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The Regulation of the Human Sphingosine 1-Phosphate Receptor, $S1P_3$

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Abstract

The $S1P_3$ receptor is a member of the cell surface G-protein-coupled receptor superfamily (GPCR). S1P-mediated activation of the $S1P_3$ receptor elicits an array of biological effects including angiogenesis, the process of new blood vessel formation, and may have an important role in atherosclerosis. After sustained exposure to agonist, many GPCRs undergo desensitisation, defined as the waning of receptor responsiveness in the face of persistent stimulation. The relative ability or inability of a GPCR to undergo classic agonist-mediated receptor phosphorylation and subsequent internalisation away from the cell surface is an important marker of GPCR desensitisation. This study has characterised, for the first time, the phosphorylation and internalisation of the human $S1P_3$ receptor.

Whole cell phosphorylation studies on hamster lung CCL-39 fibroblasts stably expressing human $S1P_3$ receptors showed that $S1P_3$ is phosphorylated in response to S1P in a time and concentration-dependent manner. In contrast activation of multiple 2nd messenger-activated kinases was without effect on $S1P_3$ phosphorylation under the same conditions. It is well known that the C-terminal region of GPCRs often holds the primary site of interaction for receptor phosphorylation and interaction with β-arrestin. Engineering constructs that truncate the receptor by removing significant and potential sites of phosphorylation allowed the identification of a region bounded by residues Leu^{332} to Val^{352} as containing the sites of receptor phosphorylation. As GPCRs are known to be phosphorylated by GRKs a panel of GRKs were investigated, of these GRK2 appeared to phosphorylate the $S1P_3$ receptor *in vitro* suggesting that GRK2 or a similar kinase is present during $S1P_3$ phosphorylation.

$S1P_3$ receptor internalisation is not detectable in CCL-39 cells as determined by biotinylation assays. Also, sucrose density gradient experiments cound not detect a wholesale shift of receptor between lipid raft and non-lipid raft compartments after S1P exposure. Although, studies of $S1P_3$ interaction with β-arrestin are currently preliminary, initial findings from this research suggest that both receptor and arrestin co-localise after agonist stimulation. This hints at the possibility that internalisation may be obstructed by something other than interaction with arrestin and provokes a receptor-complex paradigm that involves both receptor, arrestin and another protein which is cell-type specific in order to allow internalisation. Experiments in HEK293 cells demonstrated that in this system, WT and phosphorylation resistant $S1P_3$ receptors cound both internalise over identical time courses following agonist exposure. This suggests that $S1P_3$ sensitivity to internalisation is regulated specifically depending on celltype and does not require receptor phosphorylation. This has implications in

embryogenesis and also angiogenesis where cell growth and proliferation are vital to development of organs and vessels.

The impact of unique regulatory elements on functional desensitisation $S1P_3$ was determined by measuring Ca^{2+} mobilisation, a classical G-protein-mediated response. The kinetics of WT and phosphorylation-resistant $S1P_3$ receptor desensitisation were indistinguishable. This indicates that phosphorylation is not important in observing desensitisation of $S1P_3$-G-protein coupling. Rather, given the lack of any effect of disrupting phosphorylation on receptor internalisation and desensitisation, this study poses the suggestion that phosphorylation of the C terminal domain regulates another aspect of $S1P_3$ function.

An interaction of an as yet unknown protein and the SH3 interaction motif within the C-terminal tail of the $S1P_3$ receptor may occur. While yeast two hybrid results remain elusive, GST-fusion studies which were implemented to ascertain the functionality of the RXXPXXP motif revealed that Fyn kinase can interact with the SH3 domain binding motif of $S1P_3$. It is likely that a kinase similar to, or indeed Fyn, prevents internalisation in some cells by its absence which allows finely tuned regulation of the $S1P_3$ receptor to confer downstream signalling of multiple pathways.

Together, the data suggest that the $S1P_3$ receptor is uniquely regulated by agonist-stimulated phosphorylation within an 18 amino acid stretch within its C-terminal domain. Given that deletion of phosphorylation fails to alter sensitivity to internalisation or rapid desensitisation kinetics, this domain may play an alternative novel role in $S1P_3$ function. This is supported by the presence of a consensus class I SH3 domain interaction motif and the ability of the C-terminal domain to specifically bind the SH3 domain from Fyn, but not those from Src, fodrin or PI-3-kinase. In addition, because the interaction was not modulated by prior phosphorylation of the receptor, it is suggested that specific SH3 domain-containing proteins may be constitutively associated with the receptor in certain cell types. The potential for such an interaction to block the ability of agonist-bound receptor to engage with clathrin-coated pits might explain the resistance of $S1P_3$ to undergo internalisation in some cell types, exemplified by CCL39 cells, but not in others, e.g. HEK293 cells. The development of S1P-responsive chimeric $S1P_1$-$S1P_3$ receptors in which the phospho-acceptor regions have been swapped can now provide one approach with which to test the potential functional importance of RXXPXXP-SH3 domain interaction within the context of a full-length GPCR in intact cells.

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Most of all to my Family,
My Selfless, Unconditional Mamma, My Welshness Pops, My Sis Cavell,
Indy, Monty and Mondi Biondi.
Love Understands.

Abbreviations

AC	Adenylyl cyclase
Akt	Protein kinase B
AP	Adaptor protein
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
AMP	Adenosine 5' monophosphate
cAMP	Cyclic adenosine 5' monophosphate
ATP	Adenosine 5' triphosphate
ADP	Adenosine 5' diphosphate
β_2AR	β_2 Adrenergic receptor
βARK	β_2 Adrenergic receptor kinase
BCA	Bicinchonic acid
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
$[Ca^{2+}]_i$	Intracellular calcium concentration
CCL39	Chinese Hamster Lung Fibroblast cells
CKIα	Casein kinase I α
CKII	Casein kinase II
E-Cadherin	Epithelial cadherin
P-Cadherin	Placental cadherin
VE-Cadherin	Vascular endothelial cadherin
CCL-39	Hamster fibroblast cell
CHO	Chinese Hamster Ovary
CO_2	Carbon dioxide
CTx	Cholera toxin
Da	Dalton
DAG	Diacylglycerol
DHS	DL-*threo*-dihydrosphingosine
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid

DOC	Deoxycholate
DTT	Dithiothreitol
EC_{50}	Concentration of the drug required to induce a half-maximal response
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
ECV	Endosomal carrier vesicles
EDG	Endothelial differentiation gene
EDTA	Diaminoethanetetra-acetic acid disodium salt
ER	Endoplasmic reticulum
ERK	Extracellular-regulated protein kinase
ET	Endothelin
FAK	Focal Adhesion Kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GAP	GTPase-activating protein
GDP	Guanosine 5 diphosphate
GDI	GDP dissociation inhibitor
GEP	Guanine nucleotide exchange protein
GFP	Green Fluorescent Protein
GF109203X	Bisoindolylmaleimide I
GPCR	G-Protein-coupled receptor
GRK	G-Protein-coupled receptor kinase
GTP	Guanosine 5'triphosphate
HBSS	HEPES buffered saline solution
HCl	Hydrochloric acid
HEK	Human embryonic kidney
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanosulfonic acid
HIF-1	Hypoxia inducible factor-1
hmw	High molecular weight
HRP	Horseradish peroxidase
HSP	Heat shock protein
HUVEC	Humam umbilical vein endothelial cell

IgG	Immunoglobulin G
IP_3	Inositol-1,4,5-triphosphate
JNK	c-Jun N-terminal kinase
K_d	Concentration of ligand that will bind to half the receptors at equilibrium
K_i	Affinity of the receptor for the competing drug
LB	*Lurta-Bertani* medium
lmw	Low molecular weight
LP	Lysophospholipid
LPA	Lysophosphatidic acid
MAP Kinase	Mitogen-activated protein kinase
MBS	MES Buffered Saline
MEF	Mouse embryonic fibroblast
MES	2-Morpholinoethanesulfonic acid
$MgCl_2$	Magnesium chloride
MMP	Matrix metalloproteinase
NBCS	New born calf serum
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	Nitric oxide synthase
dNTP	Deoxynucleoside triphosphate
NP-40	Nonidet P-40
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PH	Pleckstrin homology
PI_3K	Phosphatidylinositol 3-kinase
PKA	cAMP-dependent protein kinase
PKB	Protein kinase B
PKC	Protein kinase C
PBS	Phosphate buffered saline
PLA_2	Phospholipase A_2

PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonylfluoride
RGS	Regulator of G-protein signalling
RIPA	Radioimmunoprecipitation buffer
PTx	Pertussis toxin
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	Standard error
SH	Src homology
Src	Src tyrosine kinase
Shc	Src homology 2 domain containing protein
Sos	Son of sevenless
S1P	D-*erythro*-Sphingosine-1-phosphate
SR-BI	Scavenger receptor, class B, type I
$T_{1/2}$	Time required to see 50% effect
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	Transforming growth factor
TM	Transmembrane domain
TRH	Thyrotropin-releasing hormone
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VSMC	Vascular smooth muscle cell
WT	Wild type

Standard one and three letter amino acid codes have been used throughout.

CHAPTER 1

Introduction

1.1 Signal Transduction in Cells

For maintenance and development, all cells must communicate with each other to pass vital information through the organism to which they belong [Marchese *et al.*, 2003]. In all cells the majority of signal transduction is initiated at the plasma membrane, which defines the cell's surface [Singer and Nicolson, 1972]. The cell surface has been likened to a communications network [Marinissen and Gutkind, 2001].

The regulation of the sphingosine 1-phosphate 3 receptor ($S1P_3$) [Rutherford *et al.*, 2005] has been studied to identify aspects of $S1P_3$ signalling that may determine which biological responses are stimulated downstream of $S1P_3$ receptor-ligand interaction. This thesis will begin with a synopsis of the general characteristics of G-protein-coupled receptors (GPCRs), mechanisms of action, to put the $S1P_3$ receptor in context with similar receptors as well as recent discoveries in $S1P_3$ receptor research.

1.1.1 G-Protein-Coupled Receptors

Transmembrane signal transduction in response to drugs, hormones and neurotransmitters is largely mediated by G protein-coupled receptors [Lefkowitz, 2004]. Estimates of the number of GPCRs in the human genome vary widely. A recent estimate using the Interpro protein family database [http://www.ebi.ac.uk/interpro/index.html] yielded 1405 results for a search on human GPCRs. One author suggests there are around 800 human GPCRs [Fredriksson *et al.*, 2003]. This represents the largest known family of integral membrane proteins and is responsible for the transduction of a diverse array of extracellular signals, including light, Ca^{2+}, odorants, amino acids, nucleotides, peptides, fatty acid derivatives and various polypeptide ligands [Gether and Kobilka, 1998; Gether, 2000; Howard *et al.*, 2001]. Contemporary pharmacological research manipulates GPCRs as they are abundant in variety in normal and patho-physiological processes. Over 50% of GPCRs are being researched by pharmaceutical companies as potential drug targets [Stadel *et al.*, 1997; Howard *et al.*, 2001], with 52% of all medicines available today acting on GPCRs [Oliveira *et al.*, 2004].

1.1.2 Structural Features of GPCRs

Several common structural elements are shared by GPCRs. The most common elements are the seven distinct regions of hydrophobic residues, which traverse the cellular membrane. These transmembrane (TM) regions, each comprise approximately 20-27 amino-acids. By extrapolation from the structure of rhodopsin, TM regions are predicted to form α-helical domains of unequal length and can extend beyond the lipid bilayer [Unger *et al.*, 1997], (Figure 1.1).

GPCRs have an amino (N)-terminal region located exterior to the cell and an intracellular carboxyl (C)-terminal tail. These are connected by three extracellular and three intracellular loops comprised largely of hydrophilic residues [Ulloa-Aguirre *et al.*, 1999; Gether, 2000; Howard *et al.*, 2001]. The sequences of the loops and tails of different GPCRs tend to be far more divergent than TM domains.

The size of the N-terminal region also varies and can contain between ten and five hundred residues, whilst the loop sections can consist of as few as five amino acids to greater than two hundred residues and the C-terminal tail between twelve and four hundred residues. This suggests that a great amount of ligand binding specificity is dependent on the precise conformation of a receptor in terms of its amino acid charges and location.

1.1.3 GPCR Classification

Over 800 GPCR-encoding sequences have been identified in the human genome [Fredriksson *et al.*, 2003]. GPCRs can be categorised as chemosensory, "csGPCRs", i.e. those that respond to exogenous stimuli such as pheromones and odours, and "endoGPCRs", which are activated by endogenous molecules. There are as many as 367 endoGPCRs in the human genome and most of these have identified ligands [Vassilatis and Hohmann, 2003].

The term orphan receptor, "oGPCR", is given to a GPCR with an as yet unidentified ligand. As oGPCRs represent potential novel targets for therapy there is great interest in de-orphanisation [Stadel *et al.*, 1997; Brezillon *et al.*, 2003] Roth *et al.*, 2003]. This is because as many as 50% of marketed drugs target GPCRs yet only 10% of GPCRs are known drug targets [Roth *et al.*, 2003] and more than 75% of identified GPCRs are oGPCRs [Karnik *et al.*, 2003].

Observations taken from phylogenetic analyses, classify each GPCR as a member of one of four main classes:

Class A comprises the rhodopsin-like receptors and contains the most endoGPCRs (284), which are activated by peptides (e.g. bradykinin, chemokines, orexin) and small molecules including lipids (e.g. S1P, LPA and cannabinoid receptors) and neurotransmitters (e.g. acetylcholine, dopamine and serotonin receptors), with the remaining receptors of the family being orphans. The sub-families of family A rhodopsin-like receptors are the largest and the most studied.

Class B consists of fifty secretin/glycagon like receptors, of these, sixteen are activated by peptides and thirty-four are orphan receptors.

Class C GPCRs are similar to metabotropic glutamate receptors and number seventeen in total, of which six are orphans.

Class F/S GPCRs are the smallest family with eleven members and no orphans.

There is also a NO family group which includes five genes that cannot be assigned to any of the above four class [Vassilatis and Hohmann, 2003].

Recently, to distinguish human GPCRs the initial class system has been revised by the GRAFS classification [Fredriksson *et al.*, 2003]. Unlike the class A-E classification it does not include receptors from several species, only the human genome. The GRAFS classification is divided as below:

Family G for Glutamate-like receptors with 15 members which can be likened to class C.

Family R (represents class A) for Rhodopsin-like receptors, which is further divided into 4 main groups, α–δ, with 13 branches.

1) The α–group consists of 5 main branches; prostanoid (15 members), amine (39 members), opsin (9 members), melatonin (3 members) and melanocortin-endoglin-cannabinoid-adenosin receptors (22 members).

2) The β–group has no main branches but includes 36 receptors that bind peptides e.g., neuropeptide Y.

3) The γ–group has 3 branches; somatostatin-opiod-galanin receptors (15 members), melanin-concentrating hormone receptors (2 members) and chemokine receptors (42 members).

4) The δ–group consists of 4 branches; the MAS-related receptors (8 members), the glycoprotein-receptors (8 members), the purine receptors (42 members) and the olfactory receptors (460 members).

Family A for Adhesion-like receptors (24 members) with either three or four branches.

Family F for Frizzle-like receptors (24 members).

Family S consisting of Secretin-like receptors (15 members) with 4 subgroups that each bind large peptides [Karnik *et al.*, 2003].

1.1.4 Structure of GPCRs

For this thesis, details of the GPCR subfamily of S1P receptors will be discussed. S1P receptors are members of GPCR class A which contains a number of conserved motifs, although overall homology between family members is quite low. There are several proline residues present in TM domains IV, V, VI and VII, and these provide the α-helices with kinks, to act as a pivot that is important to allow each receptor to bind efficiently to its ligand. TMs I, IV and VII contain only one hydrophobic residue and are therefore more hydrophilic than TMs II, III, V and VI, which contain several ionic and/or neutral residues. Hydrogen bonds and salt bridges between residues of the same TM and other TMs are vital for maintaining a tightly packed TM core [Pebay-Peyroula *et al.*, 1997].

Located at the intracellular end of TMIII is the conserved D/ERY motif which appears to be important in receptor activation [Ulloa-Aguirre *et al.*, 1999; Howard *et al.*, 2001]. The sole residue which is always conserved amongst Class A members is the arginine in this motif's amino acid sequence.

Many class A receptors possess two cysteine residues, one present within the second extracellular loop, the other located at the top of TMIII. A disulphide bridge exists between these two residues and serves to subdivide this extracellular loop into two smaller loops and may play a role in constraining the TM bundle.

Class A receptors have extracellular amino-terminal tails of varying sizes, which often contain N-linked glycosylation motifs (N-X-T/S). Presently, this post-translational modification has an unestablished role, but it may be involved in the trafficking of many GPCRs to the cell surface and be implicated in the stabilisation of protein conformation, protection of proteins from proteases and modulation of protein function [George *et al.*, 1986; Davidson *et al.*, 1995; Davidson *et al.*, 1996; Ulloa-Aguirre *et al.*, 1999].

The intracellular C-terminal tail is also highly variable in length (12-359 amino acids), and typically it is rich in serine and threonine residues, the sites of phosphorylation by intracellular kinases. The C-terminal tail also contains one or more cysteine residues, these are palmitoylation sites which allow tethering of the tail to the cytoplasmic face of the plasma membrane to create a fourth intracellular loop [Wess, 1998; Ulloa-Aguirre *et al.*, 1999; Ferguson, 2001], more details will be given about this later in the chapter.

The ligand binding site of Class A GPCRs is dependent on the type of ligand. If the ligand is small like a biogenic amine, purine, eicasanoid or lipid it is believed to bind within the TM crevice; whilst larger peptide and protein ligands appear to interact with residues of the extracellular loop and tail regions of the receptor.

1.1.5 Ligand Binding and Receptor Activation

Receptor signalling and ligand binding are distinctly dissociable functions involving specific interactions of precise domains of the GPCR with the ligand. Regions of the receptor responsible for binding and activation are dependent on the GPCR sub-family as well as structure and ligand size.

Many theories have been created to isolate specific receptor characteristics, these include the ternary complex model, the extended ternary complex model and the cubic ternary complex model [Kenakin, 2004]. Affinity and efficacy are properties possessed by GPCR ligands. Affinity describes the ligands ability to bind to a receptor, whereas efficacy depicts the extent of change in receptor activation induced by the ligand. Radioligand binding is a technique that can

be used to determine the affinity of a ligand for its receptor. This allows a calculation of the apparent dissociation constant (K_d) of that ligand for that receptor. Positive efficacy occurs when the ligand induces a functional response after binding, this is the behaviour of an agonist. Relative efficacy gives a description of the maximal response induced by that agonist expressed as a percentage of that achieved by a full agonist (in this case S1P for $S1P_3R$). An agonist that exhibits a relative efficacy of less than 100% is termed a partial agonist.

Functional assays and receptor internalisation provide a way to measure the consequences of a ligand binding to a receptor and can be used to determine the potency of a ligand. Agonist potency is given as the EC_{50} value, a value of the agonist concentration required to elicit 50% of the maximal response specific for each system.

There are ligands that can prevent activation of receptors by agonists, by binding to these receptors and not initiating a stimulus. These ligands have zero efficacy and are termed antagonists. Contemporary research has reclassified many antagonists as inverse agonists, which reflects a receptors ability to display activity in the absence of agonist stimulation (usually referred to as constitutive activity). An inverse agonist possesses negative efficacy and is able to reduce receptor constitutive activity [Milligan, 1995; De Ligt *et al.*, 2000; Jensen and Spalding, 2004].

Original dogma identified GPCRs as generally existing in equilibrium between an inactive (R) and active (R*) conformation, possibly the most accepted model for describing agonist activation of GPCRs is the Ternary Complex Model, which accounts for the cooperative interactions among receptor, G-protein and agonist. This model has been reviewed and revised several times in the last decade and is currently referred to as the Extended Ternary Complex Model, (Figure 1.2).

GPCRs are allosteric proteins that adopt inactive (R) and active (R^*) conformations in equilibrium. Preferred agonist ligand binding to the R* state promotes an isomerisation step that stabilises the receptor in a relaxed state and shifts the equilibrium toward the active R* conformation, leading to the activation of intracellular heterotrimeric G proteins. The receptors can then mediate a variety of intracellular responses to regulate cellular function. There is a degree of variability amongst GPCRs concerning the equilibrium between R and R* conformations. In the basal state of most GPCRs, the majority of receptors are in the inactive (R) state [Milligan and Bond, 1997] R^* is promoted by agonists or occurs spontaneously, leading to constitutive activity of the receptor. Conversely, inverse agonists promote R and decrease constitutive activity.

True antagonists ligands with no preference for the R and R* receptor conformation are difficult to differentiate from inverse agonists, where only a small proportion of R* GPCRs can be preferentially converted to an R state by an inverse agonist. On the other hand, a substantial fraction of R* state receptors are found with other GPCRs, such as the histamine H2 receptor(cimetidine, ranitidine) and antagonists (burinaide) [Smit *et al.*, 1996].

A select group of GPCRs, including the Kaposi's sarcoma-associated herpes virus and the cnidarian *Renilla koellikeri*, aminergic-like receptor Ren1 exhibit full constitutive activity and may not require or possess an endogenous ligand [Arvanitakis *et al.*, 1997; Rosenkilde *et al.*, 2001; Bouchard *et al.*, 2003]. After the discovery of constitutive activity, the existence of another pharmacological entity, referred to as "protean" agonists (after Proteus, the Greek god who could change shape), was assumed on theoretical grounds [Kenakin, 2004]. The rationale was that the reversal from agonism to inverse agonism (i.e., protean agonism] would occur when an agonist produces an active conformation of lower efficacy than the constitutively active conformation. It was predicted from the existence of constitutive activity that the same ligand of this class could act either as an agonist or an inverse agonist at the same GPCR, depending on the level of constitutive activity.

1.2 Heterotrimeric G-Proteins

G-proteins are composed of three subunits, termed α, β, and γ [Downes and Gautam, 1999; Willard and Crouch, 2000], (Figure 1.3). Characteristically, GPCRs bind G-proteins that act as mediators of receptor stimulated effector activation.

After a receptor is activated, bound GDP, situated in the guanine nucleotide-binding site of the GTPase domain of the G_α subunit is released and exchanged for GTP, as a result of higher intracellular GTP concentrations. GTP binding facilitates the dissociation of the α-subunit from the βγ dimer, which then allows both the G_α subunit and the $G_{\beta\gamma}$ dimer to activate effectors [Hamm, 1998; Downes and Gautam, 1999]. G-protein deactivation is rate-limiting for cell response termination and occurs when intrinsic GTPase activity of G_α subunit hydrolyses the GTP to GDP and then the G_α subunit reassociates with the $G_{\beta\gamma}$ dimer [Hamm, 1998; Downes and Gautam, 1999].

1.2.1 G_α subunit

The α–subunit contains two domains; a domain involved in binding and hydrolysing GTP that is structurally identical to the large superfamily of GTPases, and a unique helical domain that buries the GTP into the core of the protein [Hamm, 1998]. The family of G-proteins have been subdivided into four categories based upon the α–subunit composition (Figure 1.4): $G_{\alpha i}$,

which was originally shown to result in adenylate cyclase inhibition; $G_{\alpha s}$, which stimulates adenylate cyclase; $G_{\alpha q}$, which activates phospholipase C (PLC) and $G\alpha_{12/13}$ implicated in the activation of small G-proteins, such as Rac and Rho, 2001 [Downes and Gautam, 1999; Ulloa-Aguirre *et al.*, 1999; Offermanns, 2001; Radhika and Dhanasekaran, 2001].

1.2.2 $G_{\beta\gamma}$ subunit

The β-subunit (35-36kDa) consists of an N-terminal helix followed by a seven membered β-propeller structure based on its seven WD-40 repeats. The γ–subunit (6-10kDa) interacts with the β-subunit through the N-terminal coiled coil and then all along the base of the β–subunit, forming a functional unit under physiological conditions that is not dissociable except upon denaturation [Hamm, 1998]. Currently, there are 6 β-subunits and 12 γ-subunits which are gene encoded.

The βγ subunits also play a significant role in signal transduction by regulating the activity of several effectors such as the adenylyl cyclase types I, II and IV, isoforms 1-3 of phospholipase Cβ (PLCβ) [Camps *et al.*, 1992], as well as the activity of the muscarinic-gated K^+ channels [Hamm, 1998; Ulloa-Aguirre *et al.*, 1999; Radhika and Dhanasekaran, 2001].

1.2.3 Structural Features of G-proteins

The simplicity of the GTP hydrolysis cycle provides a switch mechanism that controls numerous cellular functions through many classes of GTPases, for example Ras-like small GTPases regulate cell proliferation/differentiation, cytoskeleton organization and intracellular membrane trafficking [Li and Zhang, 2004].

The G-protein level also provides a location where desensitisation can occur. RGS proteins (regulators of G-protein signalling) can increase the rate of GTP hydrolysis bound to G_i and G_q α-subunits which attenuates signalling *via* G_i- and G_q- regulated signalling pathways [Dohlman and Thorner, 1997; Hepler, 1999; Ferguson, 2001].

1.2.4 Modifications of G-Proteins

G-proteins are also sensitive to a number of covalent modifications. Most G_α subunits undergo N-myristoylation and/or palmitoylation [Willard and Crouch, 2000; Chen and Manning, 2001]. In addition, G_γ subunits are subject to prenylation [Chen and Manning, 2001]. Each of these lipid modifications has been implicated in membrane targeting and to the interactions of these subunits with each other and other proteins [Hamm, 1998; Chen and Manning, 2001]. Some G_α subunit and a G_γ subunit undergo phosphorylation, important in signal amplitude and duration [Chen and Manning, 2001].

A valuable experimental tool is the selective susceptibility to endotoxins of certain G_α subunit members. For example, $G_{\alpha s}$ subunits are adenosine 5' diphosphate (ADP)-ribosylated in the presence of cholera toxin, which catalyses the transfer of ADP-ribose to an arginine residue present in the $G_{\alpha s}$ subunit. This modification stabilises the GTP bound form of the α subunit of G_s, and through inhibition of the intrinsic GTPase activity of the $G_{\alpha s}$ subunit it is in a permanently activated state. [Hamm, 1998; Willard and Crouch, 2000; Chen and Manning, 2001]. Similarly, $G_{\alpha i}$ proteins undergo ADP-ribosylation in the presence of pertussis toxin (PTx) [Hamm, 1998; Willard and Crouch, 2000; Chen and Manning, 2001]. This occurs at a cysteine residue close to the C-terminus and is thought to uncouple the G-protein from its receptor [Willard and Crouch, 2000].

1.2.5 Small G-proteins

Small G-protein activation can be triggered when $G_{12/13}$-coupled receptors are activated. Small GTP-binding proteins are monomeric G-proteins with a molecular weight of 20-40kDa [Takai *et al.*, 2001]. Each small G-protein contributes to a superfamily of more than 100 members found in eukaryote systems ranging from yeast to human and is made of five subfamilies: - Ras, Rho, Rab, Arf and Ran [Bourne *et al.*, 1990; Hall, 1990; Takai *et al.*, 1992]. Six members belong Ras family which regulates gene expression [Takai *et al.*, 1992]. Rho/Rac/Cdc42 regulate cytoskeletal reorganisation and gene expression [Takai *et al.*, 1992; Evers *et al.*, 2000; Sah *et al.*, 2000; Fukata *et al.*, 2001]. Intracellular vessicle trafficking is regulated by Rab and Arf. During G1, S and G2 phases nucleocytoplasmic transport is regulated by Ran [Takai *et al.*, 1992].

Small G-proteins share consensus amino acid sequences responsible for specific interaction with GDP and GTP, for GTPase activity for bound GTP hydrolysis to GDP and Pi and a region for downstream effector interaction [Bourne, 1991; Takai *et al.*, 1992; Ikeda *et al.*, 2004]. C terminal sequences of Ras, Rho/Rac/Cdc42 and Rab undergo posttranslational lipid modifications, which include farnesyl, geranylgeranyl, methyl and palmitoyl moieties and proteolysis [Takai *et al.*, 1992; Glomset and Farnsworth, 1994; Casey and Seabra, 1996]. Two interconvertible forms of small G-proteins exist :- GDP-bound inactive and GTP-bound active [Benard *et al.*, 1999]. After stimulation by an upstream signal, GDP dissociates from the GDP-bound form followed by GTP binding. A conformational change of the downstream effector-binding region allows interaction with downstream effectors, enabling their function to be altered. The GTP-bound form is then converted back to the inactive GDP-bound form *via* the

intrinsic GTPase activity of the small G-protein, which ends in the release of the bound downstream effectors [Benard *et al.*, 1999].

GDP/GTP exchange is governed by a rate limiting step which dissociates GDP from the GDP-bound form [Benard *et al.*, 1999]. Through regulation by an upstream signal, guanine nucleotide exchange proteins (GEFs) can increase the dissociation rate. GEFs interact with the GDP-bound form and release bound GDP to form a binary complex of small G-proteins and GEF. GEF is then replaced by GTP, which results in the formation of the active GTP-bound form [Takai *et al.*, 1992; Benard *et al.*, 1999; Takai *et al.*, 2001]. Many GEFs, such as Rab3GEF and son of sevenless (Sos), a Ras GEF, are specific for each member or subfamily of small G-proteins [Boguski and McCormick, 1993; Buday and Downward, 1993; Wada *et al.*, 1997].

Wider substrate specificity is exhibited by some GEFs, as exemplified by dbl, a GEF active on Rho/Rac/Cdc42 proteins, [Yaku *et al.*, 1994; Hart *et al.*, 1998]. Rho/Rac/Cdc42 and Rab GDP/GTP exchanges are also regulated by the GDP dissociation inhibitors (GDIs), Rho GDI and Rab GDI respectively [Araki *et al.*, 1990; Ueda *et al.*, 1990; Fukui *et al.*, 1997]. These molecules cause inhibition of the basal and GEF-stimulated dissociation of GDP from the GDP-bound form and maintain the small G-protein in the inactive GDP-bound form. A wider substrate specificity is exhibited by Rho GDI and RabGDI than that exhibited by GEFs and GTPase-activating proteins (GAPs). Rho GDI and RabGDI are active on all Rho/Rac/Cdc42 and Rabs accordingly [Takai *et al.*, 2001]. Activation of Rho/Rac/Cdc42 and Rab is susceptible to positive and negative regulators.

1.3 GPCR Desensitisation

Regulation of GPCR signal transduction must be properly controlled in order to prevent overstimulation and achieve signal termination to render the receptor responsive to subsequent stimuli. The definition of GPCR desensitisation is stated as the process whereby receptor signalling responses plateau and then diminish despite the continuous presence of agonist [Palmer, 1996; Ferguson, 2001].

The GPCR desensitisation mechanism involves many different processes (Figure 1.17). Rapid, homologous desensitisation is believed to involve uncoupling of a receptor from its associated G-proteins within a few minutes of agonist exposure and seems to involve receptor phosphorylation [Palmer *et al.*, 1995; Jockers *et al.*, 1996; Bouvier *et al.*, 1998; Appleyard *et al.*, 1999; Xiao *et al.*, 1999; Small *et al.*, 2001].

Receptor internalisation (sequestration), away from the cell surface may facilitate receptor dephosphorylation and subsequent resensitisation after agonist removal as well as reducing the number of cell surface receptors available. [Koenig and Edwardson, 1997; Mukherjee *et al.*,

1997; Cavalli *et al.*, 2001; Ferguson, 2001]. Internalisation of receptors can also be required for intracellular signalling, as exemplified by the dopamine D3 receptor [Sorkin *et al.*, 1993]. Agonist exposure after several hours may stimulate down-regulation of receptor where there is a decrease in the total number of receptors expressed [Clark, 1986; Bouvier *et al.*, 1989; Ferguson, 2001; Tsao *et al.*, 2001]. An increase in gene expression is required to compensate for the loss of receptor protein expressed [Tsao *et al.*, 2001].

1.3.1 The Role of Phosphorylation in GPCR Desensitisation

The desensitisation of GPCRs is diverse from attenuation (β_2AR) to complete inhibition (visual/olfactory systems) [Zhang *et al.*, 1997; Sakmar, 1998; Ferguson, 2001]. For many GPCRs, the capability to undergo agonist-induced receptor phosphorylation is integral to the subsequent rate and extent of receptor desensitisation [Palmer, 1996; Clark *et al.*, 1999; Ferguson, 2001]. Desensitisation of GPCRs was shown in several receptors to be regulated by phosphorylation by G-protein receptor kinases, arrestins and second messenger-dependent kinases [Yuan *et al.*, 1994; Jockers *et al.*, 1996; Tang *et al.*, 1998; Appleyard *et al.*, 1999; Oakley *et al.*, 1999; Ferguson, 2001]. The β2-adrenergic receptor is the classic example of rapid GPCR desensitisation [Moffett *et al.*, 1993; Jockers *et al.*, 1996; Menard *et al.*, 1997; Luttrell *et al.*, 1999; McLean *et al.*, 1999].

A number of GPCRs seem to share similar regulatory mechanisms, including the m2-muscarinic, rhodopsin, and thrombin receptors [Ferguson, 2001]. Here, the predominant form of the agonist-induced β_2AR desensitisation is caused by altered conformation of the agonist-occupied receptor that facilities receptor phosphorylation by G-protein receptor kinases (GRKs) [Jockers *et al.*, 1996; Menard *et al.*, 1997; Ferguson, 2001; Pierce and Lefkowitz, 2001]. After phosphorylation of the β_2AR, β-arrestin a scaffold protein binds to the phosphorylated receptor and uncouples β_2AR from the heterotrimeric G-proteins [Tohgo *et al.*, 2003]. Accompanying the desensitisation of the β_2AR, β-arrestin also functions as an adaptor for clathrin, by mediating receptor internalisation *via* clathrin-coated vesicles [Menard *et al.*, 1997; Gaidarov *et al.*, 1999b; Luttrell *et al.*, 1999; Miller and Lefkowitz, 2001; Takei and Haucke, 2001].

1.3.2 Second Messenger-Dependent Kinases

Receptor phosphorylation arises from the activation of second messenger-dependent kinases, independently of agonist occupation. Subsequently, "heterologous" receptor desensitisation occurs as the requirement of agonist occupancy is negated [Ferguson, 2001]. For the β_2AR, phosphorylation of a consensus site within its third intracellular loop by PKA causes partial uncoupling of the receptor from G_s (about 40-60%) [Yuan *et al.*, 1994], this mechanism is

usually triggered by very low occupancy of receptor (2-5nM adrenaline) as it requires minute increases in cAMP to fully activate PKA, occurring rapidly, with a $T_{1/2}$ of 1-2 min [January *et al.*, 1997]. In contrast, GRK-mediated phosphorylation has an EC_{50} approaching the K_d for agonist binding (50-200nM adrenaline) [Clark *et al.*, 1988; Hausdorff *et al.*, 1989; January *et al.*, 1997]. Hence, as agonist concentration increases, β_2AR desensitisation shifts from being almost exclusively PKA-mediated towards a progressively larger GRK-mediated mechanism.

Furthermore, GRK-mediated phosphorylation has a time course which usually occurs within seconds [Clark *et al.*, 1999; Ferguson, 2001]. The relatively slower time-course of second messenger-mediated receptor phosphorylation can be attributed to the time required for second messenger kinase activation whereas phosphorylation mediated by GRKs only requires the necessary conformation change of the receptor. Notably, β_2AR phosphorylation by PKA also switches the receptor from G_s to G_i coupling [Daaka *et al.*, 1997a].

Together with PKA, a number of other second messenger-dependent kinases are involved in desensitisation of GPCRs. PKC as an example, has been shown to phosphorylate and desensitise a number of G_i- and G_q-coupled GPCRs, which includes α_{1b}-adrenoceptor and the type 1A angiotensin II receptor [Diviani, 1997; Liang *et al.*, 1998; Tang *et al.*, 1998].

1.3.3 The G-Protein Receptor Kinase Family

GRKs 1-7 are the seven members of the G-protein receptor kinase family [Ferguson, 2001; Pierce and Lefkowitz, 2001; Penela *et al.*, 2003]. The main characteristics of each GRK are: a central common catalytic domain, an N-terminal domain that controls substrate recognition and contains a conserved RGS domain, and a C-terminal domain responsible for targeting GRKs to the plasma membrane. The GRK family can be subdivided into three groups:

1) GRK1 (rhodopsin kinase) and GRK7 (cone opsin kinase)
2) GRK2 (β-adrenergic kinase 1, βARK1) and
   GRK3(β-adrenergic kinase 2, βARK2)
3) GRK4, GRK5 and GRK6.

Farnesylation occurs at CAAX motifs within the carboxyl termini of GRK1 and GRK7. GRK1 is also shown to be regulated by phosphatidylinositol 4,5-bisphosphate binding to a region that exhibits homologous sequence to a pleckstrin homology (PH) domain contained within the carboxyl-terminal domains of GRK2 and GRK3 [Pitcher *et al.*, 1992; Touhara *et al.*, 1994]. The carboxyl-terminal domain of GRK5 contains a length of 46 basic amino acids that mediates plasma membrane-phospholipid interactions. GRK5 activity decreases in the presence of PKC

whereas GRK2 activity is increased [Chaung *et al.*, 1995; Chaung *et al.*, 1996; Winstel, 1996] Chaung *et al.*, 1996; Winstel *et al.*, 1996].

Association of calmodulin to the N-terminal of GRK5 decreases the ability of the kinase to bind the receptor and phospholipids and decreases the activity of GRK5 by promoting the autophosphorylation of serine and threonine residues which are distinct from those involved in kinase activation [Pronin and Benovic, 1997; Pronin *et al.*, 1997; Iacovelli *et al.*, 1999a; Iacovelli *et al.*, 1999b]. Regulation of plasma membrane localisation of GRK4 and GRK6 is maintained by the palmitoylation of cysteine residues within the C-termini of GRK4 and GRK6 [Stoffel *et al.*, 1994; Stoffel, 1998].

1.3.4 GRK Targeting and Regulation

Selective phosphorylation by GRKs is achieved at serine and threonine residues within the third intracellular loop (m2 mAChR and a2α AR) or the C-terminal tail (rhodopsin and β2AR) of agonist-occupied GPCRs [Ferguson, 2001]. GPCRs also isomerise to an activated conformation in the absence of agonist which affords GRKs the potential to contribute to basal GPCR phosphorylation [Pie *et al.*, 1994; Rim and Opriann, 1995]. Conformational change in GPCRs triggered by interaction with agonist exposes two domains which are physically and functionally distinct. One domain contains a sequence that is phosphorylated by GRK and the second domain acts as a GRK activator [Chen *et al.*, 1993; Iacovelli *et al.*, 1999a], as exemplified by the sites within the m2-muscarinic receptor phosphorylated by GRK2 and the domains able to activate this kinase which were found to be located in different intracellular regions of the receptor [Nakata *et al.*, 1994; Iacovelli *et al.*, 1999a].

A number of mechanisms have been discovered for targeting GRKs to their membrane-bound receptor substrates. GRK2 and GRK3 appear to be largely cytosolic enzymes. When a GPCR is stimulated by an agonist it causes the receptor to interact with a heterotrimeric G protein leading to dissociation of α and βγdimer subunits. The βγsubunit complex, which is prenylated with a geranylgeranyl group at the C terminus of the γ is membrane-bound. Free Gβγ and membrane phosphatidylinositol bisphosphate (PIP2) appear to bind to a C-terminal domain of GRK2 or GRK3, the pleckstrin homology domain [Boivin and Lecomte, 1997]. Interaction of ligands with the pleckstrin homology domain translocates or targets the kinase to the membrane-bound, agonist-occupied receptor, where it is available to interact with its substrate. Different Gβγ isoform combinations have preferential affinity for either GRK2 or GRK3, which may permit specificity in GRK-receptor interactions [Daaka *et al.*, 1997b; Shenoy and Lefkowitz, 2003a].

1.3.5 The role of GRKs in GPCR Desensitisation

There is a surfeit of evidence that suggests that GPCR desensitisation is associated with GRK phosphorylation [Lefkowitz, 1998]. Coexpression of GRKs with GPCRs in cells resulted in augmented desensitisation of receptors including the α_{1b}-adrenergic [Diviani, 1997], α2-adrenergic [Jewell-Motz and Liggett, 1996], β1-adrenergic [Freedman *et al.*, 1995], β2-adrenergic [Pippig *et al.*, 1993], angiotensin II_{1A} (AT_{1A}) [Opperman *et al.*, 1996], A3 adenosine [Palmer *et al.*, 1995], m2 muscarinic [Schlador and Nathanson, 1997], histamine H2 [Shayo *et al.*, 2001] and m3 muscarinic receptors [Willets *et al.*, 2001].

1.3.6 Other kinases which phosphorylate GPCRs

There are also other kinases apart from GRKs and/or second messenger kinases that use GPCRs as substrates for phosphorylation. Tyrosine phosphorylation of agonist-occupied μ-opioid receptors [Pak *et al.*, 1999] has been suggested to be an important signal for receptor downregulation. Tyrosine kinase inhibitors blocked bradykinin-mediated prostaglandin E_2 production for the bradykinin B_2 receptor, demonstrating that tyrosine kinase phosphorylation of the receptor is critical for its signal transduction [Jong *et al.*, 1993]. Research has identified that casein kinase 1α can phosphorylate the m3 muscarinic receptor on the third intracellular loop [Tobin, 2002], although mutant receptors where the potential casein kinase 1α phosphorylation sites were absent still underwent agonist-mediated desensitisation [Budd *et al.*, 2000]. The phosphorylation of TRH receptor on its C-terminal tail by casein kinase II [Hanyaloglu *et al.*, 2001] was considered to be important for receptor internalisation but not desensitisation.

1.3.7 The role of Arrestins in Desensitisation

Stability of receptor/arrestin complexes may be regulated by the GRK-mediated phosphorylation of clusters of serine and threonine residues within receptor C-tails [Oakley *et al.*, 1999; Miller and Lefkowitz, 2001; Lefkowitz and Whalen, 2004]. Arrestins act as adaptor proteins that preferentially bind agonist-activated and GRK-phosphorylated GPCRs, with which they form a complex that results in receptor uncoupling from G-proteins. As well as forming complexes they can target the receptor for internalisation *via* clathrin-coated vesicles and arrestins can also mediate the activation of alternative signalling pathways [Gaidarov *et al.*, 1999a; Gaidarov *et al.*, 1999b; Luttrell *et al.*, 1999; Ferguson, 2001; Miller and Lefkowitz, 2001; Pierce and Lefkowitz, 2001]. Arrestins are a family made up of two groups. One group contains visual arrestin (S-arrestin) which is found within rod outer segments and is localised primarily to the retina [Smith *et al.*, 1994] and cone arrestin (C- or X-arrestin) which is highly expressed within neuronal tissues and the spleen where they regulate signalling of many different GPCRs

[Attramadal *et al.*, 1992; Pierce and Lefkowitz, 2001]. The other group contains β-arrestin 1 and β-arrestin 2 (also called arrestin 3). Crystal structure analysis and mutagenesis studies of visual arrestin have identified 3 functional domains (a secondary receptor-binding domain, a receptor activation domain and a phosphate sensor domain) and two regulatory domains, located at the amino terminal and carboxyl-terminal [Gurevich *et al.*, 1995; Granzin *et al.*, 1998; Ferguson, 2001]. Clathrin- and β-adaptin-binding domains are located at the C-terminal region and are conserved among non visual arrestins [Krupnick, 1994; Laporte *et al.*, 2000]. Within the N-terminal domain of β-arrestin 1 and β-arrestin 2, but not the visual arrestins, there is a proline-rich region [Luttrell *et al.*, 1999].

1.3.8 Structural features of Arrestins

Visual arrestin and the β-arrestins now possess alternative splice variants. Like visual arrestin, the β-arrestins express at least two alternative spliced forms. The variant form of β-arrestin 1 has an eight amino acid insertion between residues 333 and 334 [Parruti *et al.*, 1993] and the alternate β-arrestin 2 has an eleven amino acid insert between residues 362 and 363 [Sterne-Marr *et al.*, 1993]. No differences in activity of the β-arrestin splice variants have been reported.

Arrestins preferentially bind to phosphorylated, ligand-activated receptors suggests that there is a domain(s) that makes specific contacts with GPCRs in the active state. Initial investigations to locate the activation-recognition region suggested that it was present in the N-terminal half of the protein (residues 1-191) as a truncated visual arrestin containing retained its ability to bind light-activated state of rhodopsin [Gurevich and Benovic, 1992]. Moreover, it had been previously shown that the p44 visual arrestin mutant binds with high affinity to rhodopsin [Palczewski, 1994; Palczewski *et al.*, 1994].

Mutagenesis studies have mapped the phosphorylation-recognition site to a discrete region within the N-terminus. Arrestin truncated at residue 185 bound to phosphorylated light-activated rhodopsin and phosphorylated dark rhodopsin, while arrestin truncated at residue 158 exhibited a reduction in its ability to detect the phosphorylated form of the receptor, thus focusing the location of the phosphorylation-recognition region to between residues 158 and 185 [Gurevich *et al.*, 1993].

Mutagenesis of individual residues within region 158-185 of arrestin identified several basic residues, namely Arg^{171}, Arg^{175} and Lys^{176}, which werde crucial for phosphate binding [Gurevich *et al.*, 1995]. Futhermore, Arg^{175} was suggested to function as a phosphorylation-sensitive trigger, since mutation of this residue to a neutral or acidic amino acid resulted in

constitutive binding of arrestin to non-phosphorylated light-activated rhodopsin [Gurevich *et al.*, 1995]. The N-terminal segment of the β-arrestins also retained the ability to recognise agonist-activated receptors, indicating that the activation-recognition region of all arrestins was contained within the N-terminal half [Gurevich *et al.*, 1995].

1.4 GPCR Internalisation

Internalisation of agonist-activated receptors is an important aspect of GPCR regulation. Studies have demonstrated that many GPCRs translocate from the cell surface to intracellular membrane compartments upon exposure to agonist (Figure 1.17).

Originally discovered in bullfrog erythrocytes, GPCR internalisation was seen as a loss of cell surface β2ARs corresponding to an increase in intracellular β2ARs [Chuang and Costa, 1979].

GPCRs are classified into Class A and Class B based on the distinct pattern of their interaction with β-arrestin [Luttrell and Lefkowitz, 2002] and the resulting rate of recovery from desensitization following agonist removal. Class A GPCRs, for example, β2AR, rapidly dissociate from β-arrestin upon internalisation. These receptors are trafficked to an acidified endosomal compartment, wherein the ligand is dissociated and the receptor dephosphorylated by a GPCR-specific protein phosphatase PP2A isoform, and are subsequently recycled to the PM. Class B GPCRs, for example, the angiotensin II AT1a, form stable receptor-β-arrestin complexes. These receptors accumulate in endocytic vesicles and are can be targeted for degradation or slowly recycled to the membrane *via* routes that are currently unknown [Luttrell and Lefkowitz, 2002].

1.4.1 The role of Phosphorylation in GPCR Internalisation

Evidence has accumulated that phosphorylation might be important for the internalisation of other GPCRs. A Ser/Thr-rich sequence was suggested to be a crucial factor in the sequestration of the m1, m2, and m3 muscarinic receptors [Moro *et al.*, 1993]. Mutation within the third intracellular loop of the m2 muscarinic receptor reduced the rate of internalisation [Moro *et al.*, 1993]. Further evidence to support the role of phosphorylation in internalisation was highlighted in studies with the thrombin receptor [Shapiro *et al.*, 1996]. Truncation or mutation of the Ser/Thr residues in the C-terminus of the thrombin receptor reduced both agonist-induced phosphorylation and sequestration. In addition, agonist-induced phosphorylation of the angiotensin AT1A receptor is localised to a serine/threonine-rich region of its cytoplasmic tail [Smith *et al.*, 1998].

The direct role of phosphorylation of the β_2AR in its sequestration was eventually demonstrated using a phosphorylation- and internalisation-defective mutant, β_2AR-Y^{326}A [Ferguson *et al.*, 1995]. Overexpression of GRK 2 enhanced both the phosphorylation and internalisation of the receptor mutant. In addition, the phosphorylation and internalisation of the wild-type β_2AR in HEK293 cells was reduced by overexpression of a dominant negative GRK 2 mutant [Ferguson *et al.*, 1995]. GRK 2 phosphorylation has been shown to mediate internalisation of other GPCRs including the AT_{1a} [Smith *et al.*, 1998], endothelin A [Bremnes *et al.*, 2000], D2 dopamine [Itokawa *et al.*, 1996].

1.4.2 The role of Arrestins in Clathrin-Mediated GPCR Internalisation

GPCRs endure agonist-induced endocytosis and recycling back to the plasma membrane [Ferguson, 2001; Pierce and Lefkowitz, 2001; Takei and Haucke, 2001] (Figure 1.17). Classically, the β2AR and many other GPCRs, such as the angiotensin AT_{1a}R, the endothelin ET1A receptor and the D2 dopamine receptor undergo endocytosis involving GRK- and arrestin-dependent recruitment of GPCRs to plasma-membrane clathrin coated pits and subsequent invagination and pinching off to form intracellular clathrin coated vesicles [Brodin *et al.*, 2000; Ferguson, 2001; Pierce and Lefkowitz, 2001; Takei and Haucke, 2001]. Clathrin comprises three light and three heavy chains that form a triskelion, a structure with three legs [Brodin *et al.*, 2000; Takei and Haucke, 2001]. Assembly of the triskelions into a basket-like convex framework of hexagons and pentagons forms the coated pits on the cytoplasmic surface of the plasma membrances [Schmid, 1997; Brodin *et al.*, 2000; Takei and Haucke, 2001].

An adaptor protein (AP) called AP2 is one of the main components of the coats formed during membrane endocytosis. The AP2 complex comprises of four subunits: two large 100kDa subunits (α-adaptin, which binds to clathrin, dynamin and Eps15 and β2-adaptin, essential for clathrin coat formation), one medium size 50kDa subunit (μ2, which recognises tyrosine-based internalisation signals) and a small 17kDa subunit (σ2) [Brodin *et al.*, 2000; Ferguson, 2001; Takei and Haucke, 2001].

Clathrin has a β-arrestin binding domain localised to residues 89-100 of the amino-terminal globular region in the terminal domain (TD) of the clathrin heavy chain at the distal end of each clathrin triskelion [Goodman *et al.*, 1997]. Specific arginine residues (Arg^{394} and Arg^{396}) in the β--arrestin 2 C terminus have been identified that mediate β-arrestin binding to AP-2 and, *in vitro*, these domains in β-arrestin 1 and β-arrestin 2 interact equally well with AP-2 independently of clathrin binding [Laporte *et al.*, 2000]. In addition, whereas β2AR/ β-arrestin complexes lacking the β-arrestin 2 clathrin binding motif redistributed to coated pits, receptor/ β-

arrestin complexes lacking the β2-adaptin binding site did not [Laporte *et al.*, 2000]. Interaction of beta-arrestin with β2-adaptin represents a selective endocytic trigger for several members of the GPCR family [Laporte *et al.*, 2002].

β-arrestin interactions with the AP-2 complex, rather than the clathrin, are necessary for the initial targeting of receptors to coated pits [Laporte *et al.*, 2000]. β–arrestins bind to both the clathrin heavy chain and the β2–adaptin subunit of AP2 [Brodin *et al.*, 2000; Ferguson, 2001]. The coat also contains a monomeric adaptor protein, AP180, which interacts with AP2 and may regulate vesicle size [Brodin *et al.*, 2000]. The formation of clathrin-coated pits is assisted by synaptotagmin, an AP2 binding protein that facilities vesicle recycling by promoting coated pit nucleation [Brodin *et al.*, 2000; Ferguson, 2001; Takei and Haucke, 2001] and has been recently implicated as an intermediary in M4 muscarinic receptor internalisation [Madziva *et al.*, 2005].

When clathrin-coated vesicles are pinching off is largely dependent upon the action of dynamin a large GTPase and is ATP-dependent [Brodin *et al.*, 2000; Takei and Haucke, 2001 [Zhang *et al.*, 1996]. When a dominant negative form of dynamin, K44A, which lacks GTPase activity was overexpressed it blocked both β2AR and AT1aR internalisation [Zhang *et al.*, 1996; Gagnon *et al.*, 1998; Brodin *et al.*, 2000; Ferguson, 2001; Tsao *et al.*, 2001]. Self-assembly of dynamin into a helical structure that wraps around the necks of forming vesicles facilitates their pinching off from the membrane [Brodin *et al.*, 2000; Takei and Haucke, 2001]. Amphyphysin, the accessory protein acts as a binding partner for clathrin, AP-2 and dynamin and has been shown to recruit dynamin to clathrin-coated pits [Volchuk *et al.*, 1998; Brodin *et al.*, 2000; Takei and Haucke, 2001]. Endocytosis connects the actin cytoskelton *via* amphiphysin and syndapins which interact with dynamin [Brodin *et al.*, 2000; Takei and Haucke, 2001].

Several accessory proteins are involved in clathrin-mediated endocytosis. These include: endophilin, a lysophosphatidic acid acyl transferase involved in pit maturation and vesicle fission, and epsin and Eps15, both of which are interacting partners for the α-adaptin subunit of AP2, synaptojanin, an inositol phosphatase that regulates PIP2 metabolism and the stability of clathrin-AP2 coats; [Mukherjee *et al.*, 1997; Simpson *et al.*, 1999; Brodin *et al.*, 2000; Cavalli *et al.*, 2001; Ferguson, 2001; Takei and Haucke, 2001]. Dynamin and clathrin among other endocytosis components, have been shown to be regulated especially as a result of the β-arrestin-mediated activation of ERK [Ahn *et al.*, 1999; Miller *et al.*, 2000; Ferguson, 2001; Miller and Lefkowitz, 2001; Pierce and Lefkowitz, 2001]. Recently several components of the ERK pathway have been found to form complexes with β-arrestins and are then recruited to GPCRs in an agonist-dependent manner [Luttrell *et al.*, 1999; DeFea *et al.*, 2000; Miller and Lefkowitz,

2001]. Additional characteristics of β–arrestins have been shown to exist in the molecular adapter proteins since β-arrestin was discovered to recruit the non-receptor tyrosine kinase Src to activated β2ARs, making arrestins components of the ERK pathway [Luttrell *et al.*, 1999].

Src molecules associated with β-arrestin and activated β_2ARs were found to be dephosphorylated on Tyr530 and therefore catalytically active [Luttrell *et al.*, 1999; Miller and Lefkowitz, 2001]. When activated Src is recruited to an agonist-occupied receptor it leads to phosphorylation of the adaptor protein Shc, formation of Shc-Grb2 complexes and mediates the phosphorylation of dynamin and clathrin, as well as ERK activation [Luttrell *et al.*, 1999; Miller and Lefkowitz, 2001].

1.4.3 Trafficking Through Endocytic Organelles

Subsequent to internalisation, receptors are transported to peripheral early endosomes [Mukherjee *et al.*, 1997; Cavalli *et al.*, 2001; Ferguson, 2001]. Rab5, the small GTPase is one of the key regulators of this process and cycles between GTP- and GDP-bound form, and GTP hydrolysis depends on Rabex-5, a specific GEF [Cavalli *et al.*, 2001; Ferguson, 2001; Takei and Haucke, 2001]. Rab5 often contributes to endocytic vesicle formation, the trafficking of vesicles to early endosomes and the fusion of endocytic vesicles with early endosomes [Cavalli *et al.*, 2001; Ferguson, 2001]. On arrival at early endosomes, recycling receptors such as the β_2AR and the transferrin receptors are returned to the cell surface, at least in part *via* recycling endosomes [Ferguson, 2001; Pierce and Lefkowitz, 2001].

Rab4, another small GTPase, is also involved in the recycling pathway where it regulates the budding and/or recycling of receptor-bearing recycling vesicles [Seachrist *et al.*, 2000; Cavalli *et al.*, 2001; Ferguson, 2001]. In contrast to recycling receptors, some endocytosed are receptors targeted to lysosomes for degradation [Gruenberg and Maxfield, 1995]. Transport from early to late endosomes is mediated through intermediates called multivesicular bodies (MVBs) or endosomal carrier vesicles (ECVs). In mammalian cells, ECVs/MVBs, once formed on early endosomes, move towards late endosomes on microtubules and then dock onto and fuse with late endosomes [Gruenberg and Maxfield, 1995; Cavalli *et al.*, 2001]. At present the potential for possible cross-talk between late endosomes/lysosomes and signalling pathways is yet to be researched. Recent studies identified a novel 14 kDa protein that interacts with the MAPK scaffold protein MP1 on late endosomes/lysosomes but its function remains unclear [Cavalli *et al.*, 2001]. Protein ubiquitination has also been implicated at multiple steps of the endocytic pathway from internalisation to the maturation of endosomes and lysosomal delivery [Cavalli *et al.*, 2001; Ferguson, 2001; Shenoy and Lefkowitz, 2003b]. It has been shown that a

β2AR mutant lacking lysine residues, which was not ubiquitinated, was internalised normally but encountered ineffective degradation [Shenoy *et al.*, 2001].

1.4.4 Alternative GPCRs Pathways of Internalisation

Internalisation that is mediated by clathrin represents the most common mechanism for GPCR internalisation. However, receptors can also internalise *via* pathways that are independent of both clathrin and β-arrestin [Mukherjee *et al.*, 1997; Anderson, 1998; Cavalli *et al.*, 2001; Ferguson, 2001]. One possible route of entry involves cell surface microdomains containing cholesterol and glycosphingolipids (rafts), (Figure 1.16) which are believed to play an important role in the internalisation of the IL-2 receptor [Mukherjee *et al.*, 1997; Anderson, 1998; Cavalli *et al.*, 2001; Ferguson, 2001]. Caveolae are flask-shaped invaginations smaller than clathrin-coated pitswhich are formed by lipid rafts associated with caveolin [Anderson, 1998]. Caveolae have been reported in a myriad of cell types, including smooth muscle cells, fibroblasts, adipocytes, endothelial cells and many epithelial cells [Mineo *et al.*, 1996; Parton, 1996; Anderson, 1998; Oh *et al.*, 1998; Kogo, 2000]. Much less is known about the molecular mechanism involved in internalisation *via* caveolae, both the agonist-occupied β_2AR and the bradykinin β2 receptors were shown to be localised in caveolae as determined by electron microscopy studies [De Weerd and Leeb-Lundberg, 1997; Haasemann *et al.*, 1998; Okamoto *et al.*, 2000b; Ferguson, 2001]. Angiotensin type 1 and m2 muscarinic acetylcholine receptors also undergo agonist-dependent sequestration in this microdomain, as observed by the recovery of receptor proteins in caveolin-rich fractions [Feron *et al.*, 1997]. Cell type is crucial for many receptors in determining the favoured pathway of receptor internalisation. The β_2AR internalises in some cell types *via* clathrin-coated pits but internalises *via* caveolae in other cell types, such as A431 cells [Raposo *et al.*, 1989; Kallal and Benovic, 2000; Ferguson, 2001]. Also the endothelin ET1A receptor has been reported to internalise *via* both pathways in a cell-type dependent manner [Okamoto *et al.*, 2000b]. The route of internalisation can also determine the intracellular trafficking of the receptor [Okamoto *et al.*, 2000b].

1.4.5 Receptor Determinants for Endocytosis

Multiple receptor domains appear to contribute to the internalisation properties of GPCRs. For many GPCRs the second and third intracellular loop domains are functionally important in GPCR internalisation. For the m2 muscarinic receptor, the determinants for internalisation are found within a serine/threonine rich domain of the receptor's third intracellular loop [Moro *et al.*, 1993]. Presumably, these residues are the sites of GRK phosphorylation that are critical for inducing receptor endocytosis [Tsuga *et al.*, 1994]. As well as the third intracellular loop, the

conserved DRYXXV/IXXPL sequence of the second intracellular loop domain is also involved in the internalisation of some GPCRs including the m1 muscarinic and GnRH receptors.

Researchers have scrutinized the role of GPCR C-terminal tails and putative GRK phosphorylation sites in regulating agonist-stimulated GPCR internalisation. Despite internalisation of the β_2AR being β-arrestin-dependent, neither the truncation of the β_2AR carboxyl tail nor the mutation of potential GRK phosphorylation sites was found to inhibit β_2AR internalisation [Hausdorff *et al.*, 1989]. Alternatively, truncation of the C-tail or mutation of putative GRK sites of the $AT_{1A}R$ blocked its internalisation [Zhang *et al.*, 1996; Smith *et al.*, 1998].

1.4.6 The role of Internalisation in receptor Desensitisation and Resensitisation

Originally, GPCR internalisation was thought to be the principal mediator of receptor desensitisation due to the physical separation of receptor and effectors [Sibley and Lefkowitz, 1985]. However, additional studies demonstrated that receptor endocytosis is not as rapid as receptor desensitisation and the majority of sequestered receptors are phosphorylated meaning they do not need to be desensitised. Furthermore, hypertonic sucrose and concanavalin A treatments that inhibit GPCR internalisation were shown not to affect β_2-AR desensitisation [Pippig *et al.*, 1995]. Many studies have reported on analysis of truncated C-tail receptors and phosphorylation-deficient mutants, that desensitisation and internalisation are distinct processes. This is observed for receptors including the H2 histamine receptors [Fukushima *et al.*, 1997].

Recent studies have highlighted the importance of internalisation in the recovery from desensitisation (a process also known as resensitisation), even if GPCR internalisation may not play a critical role in agonist-induced desensitisation. The mechanisms of GPCR resensitisation are considered to involve the internalisation of agonist-activated receptors into endosomal compartments which contain a GPCR-specific phosphatase. Endosomal acidification promotes the association of the receptor with the GPCR phosphatase and dephosphorylation of the receptor. Dephosphorylated GPCRs are subsequently recycled back to the cell surface where they can be activated by agonist once more [Wenk and De Camilli, 2004].

The role of internalisation in resensitisation was first observed in studies of the β_2AR. It was subsequently proposed that dephosphorylation of internalised receptors in the endosomes followed by recycling back to the cell surface was responsible for restoring β_2AR function [Pippig *et al.*, 1995]. The critical importance of both phosphatase activity and receptor recycling in β_2AR resensitisation was demonstrated by the ability of calyculin A, an inhibitor of protein phosphatases, and monesin, an inhibitor of intracellular trafficking, to block receptor

resensitisation [Pippig *et al.*, 1995]. Sequestration has been reported to be critical for the resensitisation of many other GPCRs including the endothelin A receptors [Bremnes *et al.*, 2000].

1.4.7 Receptor Down-Regulation

Down-regulation often occurs after prolonged agonist treatment and is manifested as a decrease in receptor density. Short term desensitization results from a rapid (in minutes) and reversible uncoupling of the receptor-G protein complex, followed by sequestration and/or internalization of receptors from the cell surface (Figure 1.17). Receptors are not degraded as removal of agonist rapidly restores receptor function. Conversely, down-regulation, displays a much longer time-course (hours to days) and is characterized by a decrease in receptor density as determined by radioligand binding. Removal of agonist will only slowly reverse down-regulation, because in most cases, new receptor synthesis is required. Downregulation occurs as a consequence of both increased lysosomal degradation of pre-existing receptors and reduced mRNA and protein synthesis. The mechanism of receptor down-regulation is not well understood, but may include an accelerated rate of removal of receptors, a decrease in the rate of appearance of receptors, or both [Heck and Bylund, 1998]. Blocking β_2AR endocytosis with chemical treatments or by expressing a dominant negative mutant of dynamin could not prevent receptor downregulation indicating that this process may occur at the plasma membrane [Jockers *et al.*, 1999].

Other studies have shown that sequestration is involved with β_2AR downregulation. Immunocytochemical techniques were used to label epitope-tagged β_2ARs, agonist treatment induced redistribution of the receptors in punctate accumulations within the cells. While the majority of internalised receptors were recycled back to the plasma membrane, a small fraction of the internalised receptors were sorted in endosomes for degradation in lysosomes [von Zastrow and Kobilka, 1992]. The dynamin-K44A mutant profoundly inhibited agonist-induced internalisation and downregulation of the β_2AR in HEK293 cells, indicating that receptor internalisation was critical for downregulation in these cells. A dominant-negative mutant of β-arrestin, β-arrestin-(319-418), also inhibited both agonist-induced receptor internalisation and downregulation illustrating that downregulation of the β_2AR is partly due to trafficking of the receptor *via* clathrin coated pits [Gagnon *et al.*, 1998].

1.5 Sphingolipids

Sphingolipids are ubiquitous molecules, with more than three hundred species that exist in a diverse array of organisms and virtually all cell types [Levade *et al.*, 2001]. Members of the sphingolipid class of mediators include ceramide, sphingosine and sphingosine 1-phosphate. Sphingolipid generation is followed by regulation of ion fluxes and activation of multiple signalling pathways. These processes lead to smooth muscle cell proliferation, endothelial cell differentiation or apoptotic cell death, cell contraction, retraction or migration. Recent observations implicating sphingolipids in physiological processes, such as vasculogenesis, and frequently in pathological conditions including atherosclerosis and its complications have highlighted the special importance of sphingolipids in cardiovascular signalling [Levade *et al.*, 2001].

Sphingolipids can be recognised by their long-chain sphingolipid backbone, generally sphingosine (Figure 1.5). Structurally, ceramide consists of a long-chain sphingoid base with an amide-linked fatty acid component of 16-24 carbons in length. There are two potential pathways for intracellular ceramide formation: *de novo* synthesis *via* the condensation of serine and palmitoyl-CoA, followed by conversion to dihydroceramide and finally to ceramide and/or the breakdown of sphingomyelin through sphingomyelinase. Ceramide performs the role of a building block for most sphingolipids [Hannun, 1994]. Different substitutions on ceramide at the 1-hydroxyl position define each sphingolipid class. The synthesis, turnover and functions of sphingolipids are shown in Figure 1.6.

1.5.1 Sphingosine 1-phoshate (S1P)-activated pathways

In 1884, sphingosine was named after the mythological Greek Sphinx due to its enigmatic nature [Thudichum, 1884]. Sphingosine has been under investigation since as early as 1970 when dihydrosphingosine-1-phosphate was postulated as an intermediate in the metabolism of C18-dihydrosphingosine to palmitaldehyde and ethanolamine phosphate [Hirschberg *et al.*, 1970]. The first evidence of sphingosine research in reference to S1P appeared when it was discovered that sphingosine stimulated cellular proliferation *via* a protein kinase C-independent pathway [Zhang *et al.*, 1990]. This lead to the discovery of the importance of S1P in cell growth regulation where it was shown to increase DNA synthesis in quiescent Swiss 3T3 fibroblasts and to induce transient increases in intracellular free calcium [Zhang *et al.*, 1991]. Research has since identified many aspects of the bioactive, pleiotropic, phospholipid S1P, including its ability to act as both an extracellular signalling molecule as well as an intracellular second messenger [Olivera and Spiegel, 1993; Cuvillier *et al.*, 1996; Lee, 1998a, b; Spiegel and Milstien, 2002], (Figure 1.6).

Successive studies have shown that S1P is a potent mitogen in diverse cell types and elicits various biological effects like the mobilisation of intracellular calcium, the regulation of cytoskeleton organisation and cell growth, differentiation, survival and motility [Im *et al.*, 1997; An *et al.*, 1999; Hong *et al.*, 1999; Pyne and Pyne, 2000b, a; Spiegel and Milstien, 2003; Maceyka *et al.*, 2005]. S1P acts as an extracellular mediator by binding to a distinct sub-family of plasma membrane GPCRs.

1.5.2 Structural features of S1P

The membranes of most mammalian cells contain the integral sphingolipid component S1P, a phosphorylated derivative of sphingosine [Spiegel and Milstien, 2000a]. The stucture of S1P comprises a long hydrocarbon chain on a three carbon backbone which contains a phosphate group (Figure 1.5). S1P is biosynthesised either *de novo* through pathways of intermediate lipid metabolism or *via* stimulus-coupled liberation of the respective precursor from glycerophospholipids and sphingolipids and subsequent enzymatic conversions [Smith and Merrill, 1995; Goetzl and An, 1998; Vesper *et al.*, 1999; Pyne and Pyne, 2000b, a]. *De novo* S1P synthesis initiates with condensation of a fatty acid-CoA and serine to form 3-ketosphinganine, which upon reduction is converted to a dihydroceramide in the ER [Goetzl and An, 1998]. The dihydroceramide is then sequentially converted to ceramide, sphingosine and eventually S1P [Goetzl and An, 1998; Vesper *et al.*, 1999]. That very little free ceramide and no free sphingosine results from this *de novo* pathway provides evidence for the much greater contributions of sphingomyelin turnover to the secreted sphingosine and S1P [Goetzl and An, 1998], (Figure 1.6).

The dynamic metabolism of sphingolipids results in the formation of a number of bioactive metabolites including ceramide, sphingosine, and S1P [Spiegel and Merrill Jr, 1996; Pyne and Pyne, 2000b, a; Saba and Hla, 2004]. Degradation of sphingomyelin in the membranes of lysosomes and endosomes and in the plasma membrane occurs in response to growth factors, pro-inflammatory cytokines and arachidonic acid and also following cellular stress [Spiegel, 1999; Pyne and Pyne, 2000b, a; Spiegel and Milstien, 2000a]. Once sphingomyelinase is activated, sphingomyelin is hydrolysed to ceramide, which is thought to be involved in cell growth arrest, differentiation and apoptosis [Hannun, 1996; Kolesnick, 1998]. The conversion of ceramide to sphingosine is catalysed by ceramidase. It was also shown that sphingosine inhibits protein kinase C (PKC) and induces apoptosis [Spiegel and Milstien, 2000b, a].

Sphingosine can be phosphorylated by sphingosine kinase to produce S1P. this implicates S1P in cell growth and the inhibition of ceramide-mediated apoptosis [Spiegel, 1999; Spiegel and Milstien, 2000b, a]. S1P catabolism is catalysed either by pyridoxal phosphorylation-

independent lyase located in the ER, which degrades S1P to phosphoethanolamine and palmitaldehyde, or by a phosphatase which converts S1P back to sphingosine [Goetzl and An, 1998]. Dynamically, balance between all sphingolipid metabolites helps determine cell fate. This is commonly recognised as the "sphingolipid rheostat" model, Figures 1.5 and 1.6, [Spiegel, 1999; Pyne and Pyne, 2000a].

The majority of extracellular S1P in the blood is derived from platelets activated either *via* stress stimuli, phorbol esters or thrombin [Igarashi and Yatomi, 1998]. Originally, it was believed that subsequent to production of intracellular S1P, S1P can be released into the extracellular space where it is present as albumin-bound S1P [Igarashi and Yatomi, 1998; Hla *et al.*, 2001]. It is now appreciated that it is also carried by, and is an important biologically active component of, HDL, LDL etc. It is now appreciated that it is also carried by, and is an important biologically active component of, HDL, LDL etc. S1P released from activated platelets into the plasma is concentrated in the lipoprotein fraction with high density lipoprotein (HDL_3) being the main carrier of S1P followed by low density lipoproteins (LDL) and very low density lipoproteins (VLDL) [Sachinidis *et al.*, 1999; Spiegel and Milstien, 2002]. Additionally, extracellular S1P can be derived from other cell types such as mast cells and monocytes [Spiegel and Merrill Jr, 1996; Hannun *et al.*, 2001]. Platelets, unlike other somatic cells, lack S1P lyase [Yatomi *et al.*, 1995], and the absence of this enzyme is likely to be responsible for S1P accumulation. The precise S1P releasing mechanisms remain to be elucidated. A recent study has also suggested that as well as newly released S1P, the extracellular S1P content could also be derived from by the extracellular metabolism of sphingomyelin since the biosynthetic enzymes, namely sphingomyelinase, ceramidase and sphingosine kinase have been shown to be secreted by cells [Tabas, 1999; Romiti *et al.*, 2000; Hla *et al.*, 2001; Maceyka *et al.*, 2005].

A study has also demonstrated that the cystic fibrosis transmembrane regulator (CFTR), a member of the ATP binding cassette family of proteins, is involved in the uptake of extracellular S1P and other related phosphorylated lipids [Boujaoude *et al.*, 2001]. This uptake would influence the balance between extracellular and intracellular S1P concentrations and hence, affect the ability of S1P to modulate biological activity *via* its interaction with cell surface GPCRs.

1.5.3 S1P Receptors

Over the course of S1P receptor investigations, controversy arose over the significance of intracellular and extracellular actions of S1P. While the intracellular targets of S1P in mammalian cell have yet to be elucidated, intracellular S1P has been implicated in the mobilisation of intracellular calcium independently of IP_3, activation of ERK, inhibition of

stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) and suppression of apoptosis [Spiegel and Milstien, 2000b]. However, the discovery in 1998 that S1P acts on cell surface G-protein-coupled receptors showed that extracellular S1P can mediate a number of biological effects, including some previously attributed to intracellular S1P [Lee, 1998b; Payne *et al.*, 2002].

The extracellular effects of S1P are due to its binding to specific members of the so-called "Endothelial Differentiation Gene" (EDG) family of GPCRs, which have been renamed to account for the division between its members into S1P and lysophosphatidic acid (LPA) receptors [Chun *et al.*, 2002], see Table 1.1. The EDG receptors are a subfamily of GPCRs consisting of 8 members, EDG1-8, now referred to as $S1P_{1-5}$ and LPA_{1-3}. $S1P_{1-5}$ are highly selective for S1P and to a lesser extent LPA [Pyne and Pyne, 2000b, a; Hla *et al.*, 2001]. Although they share low affinity for S1P, LPA_{1-3} have greatest affinity for LPA, a bioactive phospholipid with similar biological effects and structure to S1P [Contos *et al.*, 2000; Fukushima *et al.*, 2001].

The EDG family can be subdivided, according to amino acid sequence similarity, into three groups:

(1) $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ (around 50% identical);

(2) LPA_1, LPA_2 and LPA_3 (around 55% identical);

(3) $S1P_4$ which is 35-42% identical to the other EDG receptors [Lynch and Im, 1999].

From phylogenetic analysis there are also other receptors that share some homology with the SIP/LPA family [Hla *et al.*, 2001; Joost and Methner, 2002; Rosen and Liao, 2003; Parrill *et al.*, 2004] as shown in Figure 1.7 and discussed in Section 1.5.9.

The LPA group is about 35% identical to the $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ group. In addition, the LPA group each contain an intron in the region of the gene encoding TM6 which is not present in the S1P group [Contos and Chun, 1998]. The S1P/LPA proteins also share partial homology with the cannabinoid receptor subfamily (<30%), indicative that S1P/LPA proteins are lipid-selective receptors and also suggestive of a possible common ancestral gene [Lynch and Im, 1999].

S1P receptor involvement in Ca^{2+} mobilisation has been recorded as far back as 1991, where S1P was shown to induce transient increases in intracellular free calcium [Zhang *et al.*, 1991]. Regulation of calcium levels by phospholipase C is depicted in Figure 1.14. S1P can mobilize calcium from internal sources either *via* an unidentified inositol 1,4,5-trisphosphate (IP_3)-independent receptor on the ER or by activation of S1P receptor that stimulate phospholipase C. Stimulation of SK also results in decreased sphingosine levels that normally

block the store-operated calcium release-activated calcium current leading to refilling of the stores. [Spiegel and Milstien, 2002] (Figure 1.14).

The S1P/LPA proteins are integral membrane proteins that are glycosylated and are predicted to have seven transmembrane-spanning domains. However, each S1P or LPA receptor possesses distinguishing structural elements that have yet to be fully related to any aspects of ligand binding or signalling As an example, the substitution of alanine for proline in the usual seventh transmembrane NPXXY sequence of LPA_2, which is conserved in the other S1P/LPA receptors and most GPCRs [Goetzl and An, 1998].

Furthermore, it has been observed that basic amino acids within $S1P_1$, Arg^{120} and Arg^{292}, form ion pairs with the phosphate of S1P [Parrill *et al.*, 2000]. Also, the S1P receptors, $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$ all share an anionic residue corresponding to the Glu^{121} residue defined in $S1P_1$ to interact with the ammonium S1P moiety [Parrill *et al.*, 2000]. In comparison, the LPA-specific receptors, LPA_1, LPA_2 and LPA_3 possess a neutral glutamine residue at the same position which may interact with the neutral hydroxyl group in LPA [Parrill *et al.*, 2000].

The $S1P_3$ C-terminal is unique amongst S1P receptors as it contains a putative class I SH3 interaction motif domain (RASPIQP), which is important in tyrosine kinase signalling, as shown by investigations of the N-terminal of phosphodiesterase 4D4, [Beard *et al.*, 1999] and this will be discussed in detail in Section 1.5.15. The last three amino acids of the $S1P_2$ C-terminal (TVV) correspond to a consensus PDZ domain interaction motif.

Recent findings have suggested a new role for S1P as a second messenger for platelet-derived growth factor (PDGF). The controversial mechanism put forward is that of signalling through $S1P_1$-PDGF receptor cross-talk. Currently, there are two models proposed for this mechanism. One model is of sequential activation of the PDGF receptor succeeded by extracellular S1P production and activation of $S1P_1$ [Hobson *et al.*, 2001]. The integrative model suggests that the interaction of $S1P_1$ with PDGF is physical, and shows that MAPK activation by PDGF requires $S1P_1$ but not SK1 activity [Alderton *et al.*, 2001b; Waters *et al.*, 2003]. Each model is unsupported by the findings of the other but both do involve cross-talk between $S1P_1$ and PDGF. To add to this, fresh input has been delivered to add further support to cross-talk in S1P receptors as $S1P_1$ has been found to interact with VEGF to induce membrane ruffling [Endo *et al.*, 2002]. Also, $S1P_3$ has been discovered to mediate cross-talk between S1P and PDGF, and Akt phosphorylation by Akt is potentiated by $S1P_3$ [Baudhuin *et al.*, 2004] (Figure 1.15). Additionally, S1P and SK1 have been found to be important components of the transforming growth factor β (TGFβ) signalling pathway involved in up-regulation of the tissue inhibitor of

metalloproteinase-1 (TIMP-1) gene [Yamanaka *et al.*, 2004]. This transactivation of tyrosine kinase receptors by GPCRs shows a new level of regulation for S1P receptors and suggests new directions for studies of important pathologies.

It has been suggested that one route to cross-signalling is receptor dimerization, which has been reported for S1P receptors in two studies [Salim *et al.*, 2002; Van Brocklyn *et al.*, 2002]. Many effects requiring multiple S1P receptors may require dimerization, and possibly this assists in the formation of complexes with other receptor subtypes.

Respective signalling models for all five S1P receptors are shown in Figure 1.8 and will be discussed individually. General expression patterns for each receptor are shown in Table 1.2.

1.5.4 The $S1P_1$ Receptor

The $S1P_1$ receptor was the initial receptor to be cloned from the S1P family. It was originally identified as an early immediate gene product induced in phorbol ester-differentiated human umbilical vein endothelial cells (HUVECs) [Hla and Maciag, 1990]. $S1P_1$ expression is observed in most mammalian tissues with the highest expression found in skeletal structures undergoing ossification, in endothelial cells and is highly expressed in the white matter, hippocampus and the Purkinje cell layer of the cerebellum [Spiegel and Milstien, 2000a; Fukushima *et al.*, 2001; Chae *et al.*, 2004]. The $S1P_1$ receptor was the first EDG receptor identified with specificity for S1P, providing the impetus for the concept of S1P as an extracellular mediator [Lee, 1998b; Okamoto *et al.*, 1998; Zondag *et al.*, 1998]. S1P was also reported to act as a low-affinity receptor for LPA to induce $S1P_1$ phosphorylation [Lee, 1998a], although, a separate study using membranes of Sf9 cells co-expressing $S1P_1$ and G_{i2} failed to elicit any biological effects [Windh, 1999]. Other studies have not observed competition of [^{32}P]S1P binding by LPA [Van Brocklyn *et al.*, 1999]. Additionally, LPA did not function as an agonist for the murine analog of $S1P_1$, lp_{B1}, when transfected into RH7777 cells [Zhang *et al.*, 1999].

$S1P_1$ signalling is involved in cell migration, the formation of new blood vessels and vascular maturation [Pyne and Pyne, 2000b, a; Spiegel and Milstien, 2000a; Hla *et al.*, 2001]. A study using $S1P_1$-expressing Sf9 cells has demonstrated that $S1P_1$ activation by S1P results in the activation of a variety of G-protein family members, including G_{i1}, G_{i2}, G_{i3}, G_o and G_z (Figure 1.8) but not G_s, G_q, G_{12} or G_{13} [Windh, 1999]. $S1P_1$ signalling *via* a $G_{i/o}$-coupled mechanism has been demonstrated in a number of cell types, such as transfected CHO, HEL, Cos-7 and Sf9 cells, and often results in extracellular signal-regulated kinase (ERK) activation and the inhibition of adenylyl cyclase (AC) activity [Okamoto *et al.*, 1998; Zondag *et al.*, 1998; Pyne and Pyne, 2000b]. $S1P_1$ activation also activates phosphoinositide 3-kinase (PI3K) *via* the

heterotrimeric G_i protein, leading to the activation of the serine/threonine kinase Akt and phosphorylation of the Akt substrates, such as, endothelial nitric oxide synthase (eNOS), shown to be involved in endothelial cell chemotaxis [Igarashi and Michel, 2000; Igarashi, 2001; Lee *et al.*, 2001; Morales-Ruiz *et al.*, 2001]. It has been shown that activation of sphingosine kinases and consequently the S1P receptors, $S1P_1$ and $S1P_2$, by FcεRI triggering plays a crucial role in mast cell function and might be involved in the movement of mast cells to sites of inflammation [Jolly *et al.*, 2004].

$S1P_1$ activation also regulates the activation state of small GTPases of the Rho family, specifically Rac and Rho, which are downstream of the heterotrimeric G-proteins and are involved in the regulation of cytoskeletal rearrangements [Hobson *et al.*, 2001; Lee *et al.*, 2001; Paik *et al.*, 2001]. It was shown that the $S1P_1$-induced G_i- and PI3K-dependent activation of Akt leads to the phosphorylation of $S1P_1$ at Thr236 located within the third intracellular loop [Lee *et al.*, 2001]. This activates Rac, *via* an unknown mechanism and the subsequent signalling pathways required for cortical actin assembly, lamellipodia formation and chemotaxis [Lee *et al.*, 2001]. In addition, HEK293 cells transfected with $S1P_1$ have also been shown to stimulate PTx-insensitive, $G_{12/13}$-mediated Rho pathways that regulate morphogenesis, such as adherens junction assembly and induction of placental (P)-cadherin and epithelial (E)-cadherin expression [Lee, 1998b; Lee *et al.*, 1999; Liu *et al.*, 2000]. In contrast, it has recently been observed that $S1P_3$ and $S1P_2$, but not $S1P_1$, mediate an increase in the amount of GTP-bound Rho in CHO cells [Takuwa *et al.*, 2001]. It is possible that as $S1P_1$ cannot couple to $G_{12/13}$, the $S1P_1$ receptor-dependent activation of Rho must be through a different mechanism which has yet to be defined and which may also be dependent upon cell type. A recent study showed that $S1P_1$, along with $S1P_3$, regulates signalling pathways required for HUVEC morphogenesis into capillary-like networks [Lee *et al.*, 1999]. One possible mechanism of $S1P_1$ activation of Rho could be through a cross-talk mechanism with $S1P_3$. Interestingly, $S1P_1$ has recently been shown to be involved in a cross-talk mechanism with the platelet-derived growth factor (PDGF) receptor [Rakhit *et al.*, 2000; Hobson *et al.*, 2001; Rosenfeldt *et al.*, 2001; Spiegel *et al.*, 2002; Waters *et al.*, 2002; Pyne *et al.*, 2003] (see Figure 1.14). $S1P_1$ is also involved in the control of lymphocyte egress and endothelial barrier function [Brinkmann *et al.*, 2004].

1.5.5 The $S1P_2$ Receptor

The $S1P_2$ receptor is expressed widely, but is particularly enriched in the heart and lung but less so in the brain of the adult rat and mouse [Pyne and Pyne, 2000b, a; Fukushima *et al.*, 2001; Takuwa *et al.*, 2001; Payne *et al.*, 2002; Spiegel *et al.*, 2002]. However, the $S1P_2$ receptor

is more prominent in the brain during embryonic development, suggesting a role for $S1P_2$-mediated signalling in neuronal development [MacLennan and Browe, 1994; Fukushima *et al.*, 2001]. $S1P_2$ receptors couple to the G_i, G_q, G_{12} and G_{13} heterotrimeric G-proteins [An *et al.*, 1999; Ancellin and Hla, 1999; An *et al.*, 2000; Arikawa *et al.*, 2003; Meacci *et al.*, 2003a]. Research on CHO, HEL, Jurkat T and HTC4 hepatoma cell lines demonstrated that $S1P_2$ receptors are coupled to the stimulation of phospholipase C and Ca^{2+} mobilisation *via* both PTx-sensitive and PTx-insensitive G-proteins, most likely G_i and $G_{q/11}$ respectively [Okamoto *et al.*, 1998; An *et al.*, 1999; Gonda *et al.*, 1999; Kon *et al.*, 1999]. $S1P_2$ along with $S1P_3$ also mediates ERK/MAPK activation almost exclusively *via* G_i in CHO cells [Takuwa *et al.*, 2001]. In contrast, $S1P_2$ was also observed to activate JNK and p38 MAPK in a PTx-insensitive manner [Gonda *et al.*, 1999]. A recent study demonstrated that $S1P_2$ activation resulted in an increase in AC activity in CHO cells [Kon *et al.*, 1999]. However, direct coupling of $S1P_2$ to G_s was not observed in membranes of Sf9 cells [Windh, 1999]. It remains to be determined whether $S1P_2$ is directly coupled *via* G_s to adenylate cyclase.

$S1P_2$ and $S1P_3$ also regulate the activity of small GTPases. Both $S1P_3$ and $S1P_2$ have been shown to activate Rho through a $G_{12/13}$-dependent mechanism, resulting in stress fibre formation, cell rounding, neurite and serum response element-driven transcriptional activation [Buhl *et al.*, 1995; Kozasa *et al.*, 1998; Pyne and Pyne, 2000b, a; Takuwa *et al.*, 2001]. Interestingly, a recent study using transfected CHO cells has shown that, whereas $S1P_1$ and $S1P_3$ result in a PI3K-dependent activation of Rac, $S1P_2$ inhibited Rac activation and subsequently membrane ruffling and cell migration [Okamoto *et al.*, 2000a]. The physiological significance of this observation is illustrated by the fact that $S1P_2$ is expressed in cells in which S1P is an inhibitor of cell migration, such as melanoma cells and vascular smooth muscle cells [Okamoto *et al.*, 2000a]. Current research poses the question of whether $S1P_1$ or $S1P_2$ receptors that are present on mast cells are transactivated after IgE triggering and whether this is a critical event for mast cell activation [Jolly *et al.*, 2002].

Much work has been done in investigating the regulation of PLD by $S1P_2$ in skeletal muscle derived C2C12 cells [Meacci *et al.*, 1999; Meacci *et al.*, 2002; Bencini *et al.*, 2003; Meacci *et al.*, 2003a; Meacci *et al.*, 2003b; Donati *et al.*, 2005], where a role for PKC and calcium has been found in receptor-mediated activation of phospholipase D by S1P in C2C12 cells [Meacci *et al.*, 1999; Meacci *et al.*, 2002]. Additionally, down-regulation of $S1P_2$ during myogenic differentiation results in the specific uncoupling of S1P signalling to phospholipase D [Meacci *et al.*, 2003a]. S1P has effects on excitation-contraction coupling and signal transduction in mammalian skeletal muscle, and regulates myogenic differentiation, those

providing a major role for $S1P_2$ receptor [Bencini *et al.*, 2003; Meacci *et al.*, 2003b; Donati *et al.*, 2005].

Recently, it has been determined by observing the functions of $S1P_1$, $S1P_2$ and $S1P_3$ in knockout mice that $S1P_2$ knockout mice were viable but deaf. Histological analysis revealed the absence of spiral ganglion neurons in the cochlea of $S1P_2$ knockout mice. This provided a new physiological function for the S1P receptor signaling system and provides evidence that $S1P_2$ is essential for proper neuron development in the inner ear [Kono *et al.*, 2004].

1.5.6 The $S1P_3$ Receptor

The proposed structure for $S1P_3$ can be seen in a schematic plot, showing the 7TM regions and N- and C-terminal domains, Figure 1.10. The main biological roles of $S1P_3$ are summarised in Figure 1.11 and a model of currently appreciated $S1P_3$ signalling is provided in Figure 1.8.

In 1996, Yamaguchi discovered by cloning, the novel human GPCR $S1P_3$. The receptor was discovered by PCR using primers derived from cannabinoid type 1 receptor (CB1) sequences [Yamaguchi *et al.*, 1996]. A full length clone of $S1P_3$ has been isolated through screening of a human genomic library [An *et al.*, 1997]. $S1P_3$ is located at chromosome 9, in the human variant it consists of 378 amino acids (Figure 1.10) with a predicted mass of 42kd. An integral membrane protein, the $S1P_3$ receptor shows expression in all tissues but most abundantly in the heart, placenta, lung, kidney and brain where the $S1P_2$ receptor can also be found [Pyne and Pyne, 2000b, a; Spiegel and Milstien, 2000a; Fukushima *et al.*, 2001; Takuwa *et al.*, 2001]. $S1P_3$ is highly homologous to human $S1P_1$ (51.9% overall and 69.2% in 7-TM regions), and is a relatively hydrophobic membrane protein that consists of an extracellular N-terminal domain linked to a cytoplasmic C-terminal domain by seven transmembrane α-helices, see (Figure 1.10). $S1P_3$ receptors also couple to the G_i, G_q, G_{12} and G_{13} heterotrimeric G-proteins [An *et al.*, 1998; Sato and Murata, 1998; An *et al.*, 1999; Ancellin and Hla, 1999] see (Figure 1.8). Consequently, it has been demonstrated in CHO cells, HEK cells, Jurkat T cells and HTC4 hepatoma cells that $S1P_3$ receptors are coupled to the stimulation of phospholipase C and Ca^{2+} mobilisation *via* both PTx-sensitive and PTx-insensitive G-proteins, most likely G_i and $G_{q/11}$ respectively [An *et al.*, 1999; Gonda *et al.*, 1999; Kon *et al.*, 1999; Okamoto *et al.*, 2000a]. $S1P_3$ and $S1P_2$ also mediate ERK/MAPK activation almost exclusively *via* G_i in CHO cells [Sato, 1999; Takuwa *et al.*, 2001]. $S1P_3$ and $S1P_2$ regulate the activity of small GTPases [Sugimoto *et al.*, 2003] as mentioned in Section 1.5.5.

Expression of $S1P_3$ and $S1P_2$ produces a constitutive activation of G_{12} and G_{13} [Siehler and Manning, 2002]. The function of Rho in S1P receptor signalling is not fully defined [Sugimoto *et al.*, 2003]. For $S1P_3$ and $S1P_2$ receptors, coupling to $G_{12/13}$ appears as the main route of Rho activation. Rho-dependent signalling is linked with cadherin/adherens expression, stimulation of focal adhesion kinase (FAK), and PLD. A recent study has shown that, in $S1P_3$-CHO cells, increased expression of PLD1 and PLD2 prevents actinomycin D-induced apoptosis by enhanced activation of the PI3K signalling pathways [Yamada *et al.*, 2004].

Mouse models have been generated which assess the functions of $S1P_3$ in vivo. Targeted homozygous deletion of $S1P_3$ was successfully performed and unlike $S1P_1$ there was no obvious phenotypic abnormality. The embryonic development, overall health, body weight, fertility and longevity upto 18 months of the KO mice did not significantly differ from normal mice. This suggests that the other S1P receptors, which were not significantly affected by $S1P_3$ knock out, can compensate for its deletion [Ishii *et al.*, 2001]. Another study further supports this assumption, it went on to investigate the effects of double and triple receptor knockouts and found that in combination, although triple knockouts ($S1P_{1-3}$) were most deleterious, the combination of $S1P_1$ and $S1P_2$ was significantly more fatal than $S1P_1$ together with $S1P_3$ [Kono *et al.*, 2004]

In the case of tumour angiogenesis, the enzyme membrane type 1-matrix metalloproteinase (MT1-MMP) has been shown to induce migration and morphogenic differentiation, which involves the cooperation of the enzyme with platelet-derived bioactive lipids through S1P-mediated activation of $S1P_1$ and $S1P_3$. This may provide an important molecular link between hemostasis and angiogenesis [Langlois *et al.*, 2004]. It has been shown that in $S1P_3$-CHO cells, increased expression of PLDs prevents ActD-induced apoptosis by enhanced activation of the PI3K signalling pathways [Yamada *et al.*, 2004].

One investigation of a $S1P_3$ peptide derived from 9 amino acids of the $S1P_3$ second intracellular loop has identified KRX725 as a mimic for the effects of S1P. It was found that this peptide could stimulate extensive angiogenesis in the form of vascular sprouting in rat aortic rings [Licht *et al.*, 2003]. This suggests that KRX-725 could act as a therapeutic agent for treatments of vascular disorders where angiogenesis is beneficial, for example, peripheral vascular disease, myocardial ischemia, tissue grafts and diabetic wound healing, with the benefit of being able to design KRX-725 to degrade slowly and act locally in comparison to S1P [Licht *et al.*, 2003].

Of particular relevance to this thesis, the C-terminal domain of $S1P_3$ contains a unique regulatory element which is not significantly similar to any other known GPCR. $S1P_3$ contains a

proline-rich sequence that conforms to a consensus SH3 (Src homology 3) domain binding motif. SH3 domains are compact globular structures found in many proteins that have important signalling functions which will be discussed at length in Section 1.5.15.

1.5.7 The $S1P_4$ and $S1P_5$ Receptors

The $S1P_4$ and $S1P_5$ receptors represent the most recently identified and therefore the most poorly characterised S1P receptors. $S1P_4$ exhibits the most restricted expression pattern of all the S1P receptors, being expressed primarily in lymphoid and haematopoietic tissues [Graeler *et al.*, 1998]. as well as the lung [Fukushima *et al.*, 2001; Takuwa *et al.*, 2001]. The $S1P_4$ receptor has been shown to mediate S1P-induced PLC activation, intracellular Ca^{2+} mobilisation and ERK/MAPK activation, all of which are blocked by PTx-treatment [Fukushima *et al.*, 2001]. Thus, $S1P_4$ couples to G_i and possibly $G_{12/13}$ [Siehler and Manning, 2002]. $S1P_4$ also regulates cell shape and motility *via* coupling to G_i and $G_{12/13}$ [Graeler *et al.*, 2003]. A single amino acid glutamic acid residue, Glu ^{329}, present in the TM3 of S1P receptors was shown to be important for the selective recognition of S1P, *versus* the closely related lipid LPA [Holdsworth *et al.*, 2004]. This study also provided the evidence that the $S1P_4$ receptor ligand binding pocket is shorter in length than the $S1P_1$ ligand binding pocket [Holdsworth *et al.*, 2004].

The $S1P_4$ receptor has been shown to be expressed in human monocytes and macrophages [Duong *et al.*, 2004]. Although $S1P_4$ receptors are expressed similarly to LPA receptors in human monocytic cells, stimulation of the LPA_1 receptor was found to be the receptor critical for monocyte activation through LPA, mm-LDL and serum [Fueller *et al.*, 2003]. Murine $CD4^+$ and $CD8^+$ T cells express $S1P_1$ and $S1P_4$ predominantly yet T cell receptor-mediated activation of $CD4^+$ T cells suppresses expression of the two S1P receptors and eliminates their chemotactic function in mouse splenic T cells [Graeler and Goetzl, 2002]. S1P has been identified as a novel inhibitor of T-cell proliferation, and PMA plus ionomycin or anti-CD3 plus anti-CD28 down-regulated the expression of $S1P_4$ and up-regulated the expression of $S1P_5$ in T cells 3 days after stimulation. [Jin *et al.*, 2003]. Phytosphingosine 1-phosphate has been shown to be a high affinity ligand for the $S1P_4$ receptor, with greater affinity for $S1P_4$ than even S1P [Candelore *et al.*, 2002]. The immunosuppressant FTY720 also binds and down regulates $S1P_4$ [Graeler and Goetzl, 2004].

The $S1P_5$ receptor is expressed in a variety of tissue type, it is highly expressed in the human brain, specifically in white matter, spleen [Graeler and Goetzl, 2002], corpus collosum, peripheral blood leukocytes and arteries, placenta, lung, aorta and fetal tissues [Im *et al.*, 2000; Fukushima *et al.*, 2001; Im *et al.*, 2001; Takuwa *et al.*, 2001]. In contrast to $S1P_4$, $S1P_5$ does not appear to be expressed in human monocytes and macrophages [Duong *et al.*, 2004]. $S1P_5$ is also

expressed in rat neural progenitor cells [Tham *et al.*, 2003], and rat microglia, where the level of expression depends on the activation state of the cells [Harada *et al.*, 2004], also rat astrocytes have been found to express $S1P_5$ but expression is less than the majority of the other members of the S1P/LPA family [Rao *et al.*, 2003]. $S1P_5$ has been shown to couple to $G_{i/o}$ and G_{12} but not G_s or $G_{q/11}$ [Im *et al.*, 2000; Malek *et al.*, 2001] (Figure 1.8). In a recent study using CHO cells transfected with $S1P_5$, S1P treatment resulted in a PTx-sensitive inhibition of forskolin-induced cAMP accumulation and a PTx-insensitive activation of JNK and inhibition of serum-induced activation of ERK1/2 [Malek *et al.*, 2001]. The inhibitory effect of S1P on ERK1/2 activity was abolished by treatment with orthovanadate, suggesting the involvement of a tyrosine phosphatase [Malek *et al.*, 2001]. Intrinsic inhibition of unstimulated adenylyl cyclase or ERK activity by the $S1P_5$ receptor is apparently insensitive to ligand modulation [Niedernberg *et al.*, 2003a]. Notably, it has been revealed that rat $S1P_5$ in CHO-K1 cells displays antiproliferative effects and this is not seen in the human $S1P_5$ transfected in HEK293 cells, and this may represent a a celltype or species-specific effect [Niedernberg *et al.*, 2002]. The human $S1P_5$ receptor is overexpressed in the lymphoproliferative disorder, large granular lymphocyte (LGL) leukaemia [Kothapalli, 2002].

1.5.8 The LPA Receptors

The chemical structure of the agonist lysophosphatidic acid can be seen in Figure 1.5. The current relation of LPA and S1P receptors is clearly represented in the phylogenetic tree in Figure 1.7. There are four identified LPA receptors in mammals [Anliker and Chun, 2004b]. A distinct gene encodes each receptor that activates downstream signaling pathways mediated by one or more G proteins. The first three, LPA_{1-3}, share sequence homology with one another, whereas LPA_4 is divergent in sequence. LPA_1 represents the first LP receptor identified. LPA_1 is widely expressed outside the nervous system and is expressed prominently in testis and intestine [Contos *et al.*, 2000; Pyne and Pyne, 2000b; Fukushima *et al.*, 2001; Takuwa *et al.*, 2001]. LPA_1 is also prevalent in the myelinating cells of the adult nervous system where LPA promotes the G_i-mediated PI3K/Akt-dependent survival of myelinated Schwann cells from the peripheral nervous system [Weiner and Chun, 1999]. In addition, LPA_1 is also expressed in several cancers, suggesting a pathological role for receptor-mediated LPA signalling [Furui *et al.*, 1999]. LPA_1 couples to $G_{i/o}$, which leads to cell proliferation and also couples to $G_{12/13}$ to activate Rho [Weiner and Chun, 1999; Igarashi and Michel, 2000; Fukushima *et al.*, 2001; Hla *et al.*, 2001]. LPA_1 contains 364 amino acids in a seven-transmembrane receptor structure, with an apparent molecular mass of 42 kDa. Gene expression is most marked in the ovaries but is also observed at lower levels in several other tissues.. In mice, a multi-exon gene structure was reported, with the

coding region characterized by conservation of a single intron separating two coding regions at the sixth transmembrane domain. This intronic structure is shared with LPA_2 and LPA_3 [Anliker and Chun, 2004a, b]. LPA_2 is a high-affinity LPA receptor that activates the G_q pathway [Contos *et al.*, 2000; Fukushima *et al.*, 2001]. LPA_2 is constitutively expressed in $CD4^+$ T cells and inhibits the secretion of interleukin-2 (IL-2) [Hla *et al.*, 2001]. LPA_2 couples to G_i and G_q, which mediates LPA-induced PLC activation and leads to intracellular Ca^{2-} increases and inositol phosphate production [Contos *et al.*, 2000; Fukushima *et al.*, 2001; Hla *et al.*, 2001]. LPA_2 receptor expression is strongly induced in ovarian cancer cell lines where it regulates the transcription of immediate-early genes and cellular proliferation [Goetzl *et al.*, 1999]. LPA_3 is abundantly expressed in testis, heart and frontal regions of the cerebral cortex [Contos *et al.*, 2000; Contos and Chun, 2001; Fukushima *et al.*, 2001; Toman and Spiegel, 2002]. Studies of LPA_3 function within mammalian and insect cell lines have demonstrated an LPA-dependent, PTx-insensitive increase in PLC activity and intracellular Ca^{2+} concentration, suggesting that LPA_3 is coupled primarily to G_q [Contos and Chun, 2001; Fukushima *et al.*, 2001]. LPA_3 differs from the previous two LPA receptors by not coupling to $G_{12/13}$ and showing a preference for LPA molecules with unsaturated acyl chains. Although still expressed in many adult tissues, LPA_3 shows somewhat more restricted expression. Its signalling properties are generally similar to LPA_1 and LPA_2 except for AC-related effects that vary with respect to analyzed cell lines [Anliker and Chun, 2004b]. Recently, the p2y9/GPR23 receptor has been identified as the fourth LPA receptor (LPA_4), with likely evolutionary distinct ancestors [Noguchi *et al.*, 2003]. Biological roles, null mutations, and the relationship of LPA_4 to the other LPA receptors have not been reported [Anliker and Chun, 2004b]. The biological roles of LPA receptors in different systems are summarised in Figure 1.11.

1.5.9 Novel GPCRs with homology to S1P and LPA receptors

In addition to the agonist selectivity that is shared between S1P and LPA receptors, several other receptors have been found that are activated by members of the lysophopholipid family (Figure 1.7) [Kostenis, 2004]. A recent review focused on the assignment of lipid mediators to GPCRs that include GPR3, GPR6, GPR12, GPR23, GPR40, GPR41, GPR43, GPR63, TG1019 (also known as R527), and BG37 (also known as TGR5). These GPRs were recently identified as receptors for intercellular lipid messengers such as, S1P, SPC, dioleoylphosphatidic acid (doPA), LPA, free fatty acids, eicosatetraenoic acid, and bile acids [Im, 2004]. The author advises caution to the likelihood of the authenticity of these GPCRs as LP receptors as many of the studies were not carried out methodically, therefore binding studies are either lacking or unsupported by parallel studies which deems them inconclusive [Im, 2004].

The novel LP receptors include the ovarian cancer GPCR1 (OGR1) receptor (also GPR68 receptor) that is activated by sphingosylphosphorylcholine (SPC) [Xu *et al.*, 2000], the GPR4 receptor which is a receptor that displays high affinity for SPC and low affinity for lysophosphatidylcholine (LPC), and shares ~50% homology with OGR1 [Zhu *et al.*, 2001] and homology with the cannabinoid receptors CB1 and CB2 [Felder and Glass, 1998]. LPC is a high-affinity ligand for G2A, a lymphocyte-expressed GPCR whose genetic ablation results in the development of autoimmunity. Activation of G2A by LPC has been seen to increase intracellular calcium concentration, induce receptor internalization, activate ERK, and modify migratory responses of Jurkat T lymphocytes. These findings implicated a role for LPC-G2A interaction in the etiology of inflammatory autoimmune disease and atherosclerosis [Kabarowski *et al.*, 2001]. PSP24, is an orphan receptor that has been proposed to bind LPA but not in mammaliam tissues [Kawasawa *et al.*, 2000]. The orphan GPCRs, GPR3, GPR6 and GPR12 were recently identified as S1P receptors [Uhlenbrock *et al.*, 2002; Ignatov *et al.*, 2003]. The receptor encoded by GPR45 is the mammalian orthologue of a putative LPA receptor from *Xenopus laevis*, expressed in the central nervous system and periphery [Marchese *et al.*, 1999]. S1P and doPA have been identified as low affinity agonists for the orphan receptor GPR63 [Niedernberg *et al.*, 2003b]. GPR65 (T cell death associated gene, TDAG8) shares evolutionary homology to OGR1 and GPR4 yet its agonist is proposed to be psychosine (galactosyl sphingosine) [Murakami *et al.*, 2004]. Agonist and antagonist binding for S1P and LPA receptors has been reviewed in terms of development of receptor-specific ligands. [Parrill *et al.*, 2004].

1.5.10 The involvement of $S1P_1$ and $S1P_3$ receptors in angiogenesis

Among the most important of their biological roles, $S1P_1$ and $S1P_3$ act as regulators in the process of angiogenesis. Angiogenesis can be defined as the formation of new blood vessels from pre-existing vessels. A process that constitutes an integral component in numerous physiological events, from embryonic development to wound healing [Yatomi *et al.*, 1995; Vogler *et al.*, 2003] and the menstrual cycle, each requiring new blood vessel formation to simultaneously supply oxygen and nutrients and remove waste products [Carmeliet and Jain, 2000]. Angiogenesis is also critical in several pathological conditions associated with blood vessel formation. As an example, angiogenesis in excess has been linked to solid and haematologic tumour progression [Dvorak, 1986], chronic inflammation as presented in rheumatoid arthritis and in Crohn's disease, endometriosis and diabetic retinopathy [Griffioen and Molema, 2000; Summers and Nelson, 2005].

The S1P effect on blood vessel formation was attributed to it promoting migratory activities of angioblasts and early endothelial cells required for the expansion of vascular

networks [Lee, 2000]. Findings suggest that migratory events critical to the *de novo* formation of blood vessels are under the influence of S1P, possibly synthesized *via* the action of sphingosine kinase 2 (SK2), with signaling mediated by S1P receptors that include $S1P_1$, $S1P_2$, and $S1P_3$ [Argraves *et al.*, 2004]. These three receptors play important roles in carefully regulating several overlapping pathways, as findings from mouse embryonic fibroblast cells have confirmed [Anliker and Chun, 2004a] (Figure 1.9).

S1P has been shown to act synergistically with FGF and VEGF in promoting angiogenesis [Lee *et al.*, 1999]. Recent evidence suggests that S1P-dependent activation of $S1P_1$, along with $S1P_3$, results in the Rho-activation of integrin $\alpha_v\beta_3$ and β_1-containing integrins, leading to the formation of initial focal contacts required for cell spreading and migration [Paik *et al.*, 2001]. $\alpha_v\beta_3$ has also been shown to function in a synergistic manner with VEGF in the processes of cell migration and proliferation [Soldi *et al.*, 1999]. Recently, $S1P_3$ was shown for the first time to interact with PDGF as well as showing a role for $S1P_3$ in S1P-induced Akt activation [Baudhuin *et al.*, 2004] (Figure 1.15).

1.5.11 $S1P_3$ Involvement in the Cardiovascular System

A general scheme of the roles of $S1P_3$ in biological systems, including the cardiovascular system is shown in Figure 1.11. $S1P_3$ is involved in morphogenesis is the process of newly formed neovasculature remodelling into capillary-like networks which occurs after endothelial cell proliferation [Lee *et al.*, 1999; Griffioen and Molema, 2000; Cross and Claesson-Welsh, 2001], and which involves the folding of epithelial cell sheets into tubes and other structures. Morphogenesis is typically regulated *via* connection sites for actin filaments known as adherens junctions [Akhtar and Hotchin, 2001]. Cell-matrix adherens junctions allow cells to grip the extracellular matrix, and various forms of cell-to-cell adherens junctions exist. In non-epithelial tissues, cell-to-cell adherens junctions are small punctate/streak-like attachments that connect actin filaments in the cortical cytoplasm of adjacent cells [Evers *et al.*, 2000; Griffioen and Molema, 2000]. In epithelial sheets, cell-to-cell adherens junctions are a continuous adhesion belt termed the zonula adherens, that exists for each of the interacting cells in the sheet and is located near the cell apex [Evers *et al.*, 2000]. Adhesion belts in adjacent epithelial cells are directly opposed and the interacting PMs are held together by transmembrane linker proteins that are members of the family of Ca^{2+}-dependent cell-cell adhesion molecules called cadherins [Evers *et al.*, 2000].

The family of cadherins consists of around 12 members, including vascular endothelial (VE)-cadherin, found in the vascular endothelium; E-cadherin, found in epithelial cells; N-

cadherin, found in nerve, muscle and lens cells and P-cadherin, found in placenta and epidermal cells [Sumpio *et al.*, 2002].

Cells contain a contractible bundle of actin filaments adjacent to the adhesion belt and running parallel to the PM that are attached *via* a set of intracellular attachment proteins; α-, β-, and γ-catenin, vinculin, α-actinin and plakaglobin [Griffioen and Molema, 2000; Jones *et al.*, 2001; Brinkmann *et al.*, 2004]. Cadherins and attachment proteins link actin bundles in adjacent cells resulting in an extensive transcellular network. Contraction of this network mediates morphogenesis, which can be seen in Figure 1.13.

The development of HUVECs into capillary-like networks has been observed *via* S1P treatment in HUVECs where activation of $S1P_1$ and $S1P_3$ receptors further activated Rac- and Rho-dependent adherens junction assembly and cytoskeletal rearrangement, culminating in morphogenesis. [Lee *et al.*, 1999]. Notably, the action of S1P contrasts with the action of VEGF, which is known to disrupt adherens junctions [Lee *et al.*, 1999; Hla *et al.*, 2001]. S1P stimulation of $S1P_1$ and $S1P_3$ receptors expressed in HUVECs results in the activation of $\alpha_v\beta_3$- and β_1-containing integrins [Clark and Brugge, 1995; Paik *et al.*, 2001]. In addition to regulating cell spreading and migration, antagonists of $\alpha_v\beta_3$ and β_1-containing integrins inhibited S1P-induced endothelial cell morphogenesis in a three-dimensional fibrin matrix [Paik *et al.*, 2001].

1.5.12 Maturation and preservation of vasculature

After formation of the neovasculature, endothelial cells deposit a new basement membrane and recruit surrounding vessel layers composed of mural cells, such as pericytes in small vessels and smooth muscle cells in large vessels [Griffioen and Molema, 2000; Saaristo *et al.*, 2000; Richard *et al.*, 2001]. Mural cell recruitment is largely dependent upon PDGF synthesis and secretion within endothelial cells [Griffioen and Molema, 2000; Cross and Claesson-Welsh, 2001]. Once contact is made between endothelial cell and mural cell, a latent form of transforming growth factor-β (TGF-β), which is produced by both endothelium and mural cells is activated in a plasmin-mediated process [Griffioen and Molema, 2000]. TGF-β activation induces changes in myofibroblasts and pericytes, leading to the formation of a quiescent vessel, ECM production and maintenance of growth control [Griffioen and Molema, 2000].

Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) and the receptor tyrosine kinases Tie1 and Tie2 are essential for the communication of endothelial cells with the surrounding mesenchyme [Lin, 1997; Griffioen and Molema, 2000; Jones *et al.*, 2001]. The function of Tie1 is associated with endothelial cell differentiation and establishing blood vessel integrity whilst

Tie2 is necessary for vascular network formation and is only expressed on endothelial cells [Jones *et al.*, 2001]. Ang-1 and Ang-2 are Tie2-specific ligands that activate or antagonize Tie2 signalling respectively [Davis *et al.*, 1996; Maisonpierre *et al.*, 1997; Jones *et al.*, 2001]. Endothelial cells failed to associate properly with underlying support cells in the vessels of embryos lacking Tie2 or Ang1, which demonstrated that Tie2 signalling may facilitate recruitment of, and tight association with, adjacent periendothelial cells [Suri *et al.*, 1996; Patan, 1998]. Persistent Tie2 expression and its phosphorylation in quiescent adult endothelium implies a role for Tie2 in transducing a sustained survival signal [Wong *et al.*, 1997; Jones *et al.*, 2001]. In human glioblastomas, a cell-specific up-regulation of Tie2, Ang-1 and Ang-2 during tumour progression was detected in a pattern compatible with a role in tumour-induced angiogenesis [Stratmann *et al.*, 1998; Griffioen and Molema, 2000].

$S1P_1$ knockout mice have shown that $S1P_1$ is essential for vascular maturation as $S1P_1$ gene disruption resulted in impaired vascular maturation due to the failure of mural cells to migrate to arteries and capillaries to reinforce them [Liu *et al.*, 2000; Kono *et al.*, 2004]. Although $S1P_1$-null embryos died *in utero* due to massive haemorrhage, they exhibited normal vasculogenesis and a substantially normal blood vessel network, yet were severely impaired in the recruitment of smooth muscle cells and pericytes to the vessel walls and this was attributed to their defective migration [Liu *et al.*, 2000]. Extracellular S1P can directly stimulate $S1P_1$ on vascular smooth muscle cells (VSMCs), facilitating their migration to vessel walls or, alternatively, can stimulate $S1P_1$ expressed in endothelial cells that in turn may recruit VSMCs [Liu *et al.*, 2000]. Recent studies have demonstrated that the effect of $S1P_1$ on vascular maturation can be largely attributed to the cross-talk between $S1P_1$ and PDGF receptor signalling mentioned previously [Hobson *et al.*, 2001; Rosenfeldt *et al.*, 2001]. Cell migration toward PDGF, which stimulates sphingosine kinase and increases intracellular S1P, was shown to be dependent upon $S1P_1$ expression in a number of cell types, including HEK293 cells, human aortic smooth muscle cells (ASMCs) and mouse embryonic fibroblasts (MEFs) [Hobson *et al.*, 2001]. It was therefore suggested that spatially and temporally localised generation of S1P by activation of sphingosine kinase in response to PDGF results in restricted activation of S1P that in turn activates Rac, resulting in an increase in cell motility [Hobson *et al.*, 2001]. A recent study has subsequently demonstrated that the PDGF-induced cytoskeletal rearrangements, lamellipodia extensions and cell motility are abrogated in $S1P_1$ null fibroblasts [Rosenfeldt *et al.*, 2001]. Also, PDGF-induced focal adhesion formation and activation of FAK, Src and SAPK2 were disregulated in the absence of $S1P_1$ [Rosenfeldt *et al.*, 2001]. However, $S1P_1$ was not involved in mitogenicity and survival effects induced by S1P or PDGF [Rosenfeldt *et al.*, 2001].

Hence, it was suggested that $S1P_1$ acted as an integrator linking the PDGFR to lamellipodia extension and cell migration. Cells then migrate towards PDGF, enhancing PDGF receptor signalling and therefore acting as a positive feedback mechanism [Hobson *et al.*, 2001; Rosenfeldt *et al.*, 2001].

1.5.13 Functions of $S1P_3$ in Inflammation and Immunology

Both S1P and LPA receptors appear to have important roles in immunology [Payne *et al.*, 2004; Radeff-Huang *et al.*, 2004], (Figure 1.11). Results from a recent study show differential effects of S1P on polyclonal T-cell proliferation and cytokine secretion [Jin *et al.*, 2003]. Local administration of S1P caused inflammation coupled to a large eosinophil (EO) recruitment in rat-paw tissue. The inflammatory response was accompanied by an increase in $S1P_1$, $S1P_2$ and $S1P_3$ and by an enhanced expression of CCR3, which is the main chemokine receptor known to be involved in EO function. Human EOs constitutively express $S1P_1$ and, to a lower extent, $S1P_2$ and $S1P_3$ receptors. S1P *in vitro* causes cultured human EO migration and an increase in S1P receptor mRNA and strongly up-regulates CCR3 and RANTES (Regulated on Activation, Normal T cell-Expressed and Secreted) message levels. A blocking anti-CCR3 Ab inhibited S1P-induced chemotaxis, implying that S1P acts as a specific recruiting signal for EOs not only through its own receptors but also through CCR3. These results suggest that S1P is involved in EO chemotaxis and helps to unravel the complexity underlying EO recruitment in several diseases such as asthma and some malignancies [Roviezzo *et al.*, 2004].

Asthma and chronic obstructive pulmonary disease (COPD), are chronic diseases affecting millions of people worldwide and are characterized by airway obstruction, airway inflammation, and alterations in structural cell function. The role of structural cells such as airway smooth muscle (ASM), myofibroblasts, and fibroblasts was thought to be maintenance of bronchial wall integrity, but evidence suggests that these cells may be integral components of the inflammatory response as well [Panettieri, 2004]. Asthma is a complex condition where exposure to environmental antigens induces inflammatory reactions in the airway characterized by activation of mast cells and eosinophils. Mast cells are known to be the main effector cells in eliciting IgE-mediated allergic response. These cells secrete various substances that sustain inflammation and provoke airway smooth muscle (ASM) contraction. In another study, it was shown that S1P may regulate human airway smooth muscle cell (HASM) contractility, which is important in the pathobiology of asthma [Ammit *et al.*, 2001; Rosenfeldt *et al.*, 2003]. A newly recognized addition to the range of FcεRI-mediated signaling events is the activation of SK leading to the generation S1P from sphingosine. Lung secretions of S1P significantly increase

after challenge with an allergen, thus adding S1P to the variety of mediators that are released during an allergic reaction [Ammit *et al.*, 2001]. Previous reports also agreed that FcεRI cross-linking was not only found to increase cellular levels of S1P, it also markedly enhanced its secretion from rat basophilic leukemia RBL-2H3 cells. Moreover, S1P can induce the degranulation of RBL and bone marrow derived mast cells (BMMCs) cells as determined by the release of hexosaminidase. Treatment of BMMCs with the SK inhibitors, DL-threo-dihydrosphingosine and dimethylsphingosine, reduced IgE/Ag stimulated histamine release. RT-PCR analysis demonstrated that mast cells express S1P receptors $S1P_1$ and $S1P_2$ but not $S1P_3$, $S1P_4$ or $S1P_5$ transcripts. Further studies would determine whether IgE triggering results in transactivation of $S1P_1$ or $S1P_2$ present on mast cells and whether this is a critical event for mast cell activation [Jolly *et al.*, 2002].

S1P influences heart rate, coronary artery calibre, endothelial integrity, and lymphocyte recirculation through $S1P_{1-5}$. Inhibition of lymphocyte recirculation by non-selective S1P receptor agonists produces clinical immunosuppression preventing transplant rejection and is associated with transient bradycardia [Sanna *et al.*, 2004]. Knowledge of individual receptor contribution has been limited by the embryonic lethality of the $S1P_1$ knock-out and the unavailability of selective agonists or antagonists. A potent, $S1P_1$-receptor selective agonist, SEW2871, structurally unrelated to S1P, has been discovered that activates multiple signals triggered by S1P, including guanosine 5'-3-O-(thio)triphosphate binding, calcium flux, Akt and ERK1/2 phosphorylation, and stimulation of migration of $S1P_1$- but not $S1P_3$-expressing cells *in vitro*. The agonist also altered lymphocyte trafficking *in vivo*. Use of a selective agonist together with mice lacking $S1P_3$ receptor revealed that agonism of $S1P_1$ alone is sufficient to control lymphocyte recirculation. Moreover, $S1P_1$ agonist plasma levels were causally associated with induction and maintenance of lymphopenia [Sanna *et al.*, 2004]. $S1P_3$, and not $S1P_1$, is directly implicated in sinus bradycardia. The sustained bradycardia induced by S1P receptor non-selective immunosuppressive agonists in wild-type mice is abolished in $S1P_3^{-/-}$ mice, whereas a $S1P_1$-selective agonist does not produce bradycardia. Separation of receptor subtype usage for control of lymphocyte recirculation and heart rate may allow the identification of selective immunosuppressive $S1P_1$ receptor agonists with an enhanced therapeutic window. $S1P_1$-selective agonists will have future uses in understanding cell functions *in vitro*, and vascular physiology *in vivo* [Sanna *et al.*, 2004].

The novel immunomodulator FTY720 is effective in experimental models of transplantation and autoimmunity, and is currently undergoing Phase III clinical trials for

prevention of kidney graft rejection [Budde, 2002]. FTY720 is a structural analogue of sphingosine-1-phosphate (S1P) and activates all S1P receptors except $S1P_2$ [Forrest et al., 2004]. FTY720 has been shown to induce endothelium-dependent arterial vasodilation in phenylephrine precontracted mouse aortae. Vasodilation did not occur in thoracic aortic rings from eNOS-deficient mice, implicating an effect dependent on activation of the eNOS/NO pathway. Thus, FTY720 induced NO release, Akt-dependent eNOS phosphorylation and activation in human endothelial cells [Tolle *et al.*, 2005]. For biological efficacy, FTY720 required endogenous phosphorylation, since addition of the SK antagonist N',N-dimethylsphingosine (DMS) prevented activation of eNOS *in vitro* and inhibited vasodilation in isolated arteries. The endothelial phosphorylation of FTY720 was extremely rapid with also most complete conversion after 10 minutes as determined by mass spectrometry [Tolle *et al.*, 2005]. $S1P_3$ was identified as the S1P receptor responsible for arterial vasodilation by FTY720, as the effect was completely abolished in arteries from $S1P_3$-deficient mice [Tolle *et al.*, 2005]. In summary, FTY720 is the first immunomodulator for prevention of organ graft rejection in clinical development that, in addition, positively affects the endothelium by stimulating NO production, and thus potentially displaying beneficial effects on transplant survival beyond classical T cell immunosuppression, [Tolle *et al.*, 2005].

1.5.14 $S1P_3$ involvement with HDL

Atherosclerosis is a chronic inflammatory disease, with lipoproteins, vascular endothelial cells, monocytes, macrophages, smooth muscle cells, activated T lymphocytes, and platelets all interacting through adhesion molecules, cytokines, chemokines, and prothrombotic factor [Yuhanna *et al.*, 2001]. Naturally, as $S1P_3$ has been associated with many of these components it seems likely that its involvement in inflammatory diseases is of therapeutic importance. Recently, HDL-induced endothelial cell migration and survival were thought to mediated by the lipoprotein component S1P and the lipid receptors $S1P_1$ and $S1P_3$ [Kimura *et al.*, 2003].

HDLs are complex molecules known to induce a multitude of intracellular signals for which different components of HDL have been made responsible. The lysophospholipid concentrations effective in inducing vasodilation are likely to be provided *in vivo* by plasma HDL. 1mg of HDL contains 287 ± 17 pmol S1P and 290 ± 20 pmol SPC, which represents 0.58nM of these bioactive phospholipids together. HDL-induced endothelial cell migration and survival may be mediated by the lipoprotein component S1P and the lipid receptors $S1P_1$ and $S1P_3$ [Kimura *et al.*, 2003]. It was shown that HDL stimulates release of nitric oxide (NO) in human endothelial cells and induces vasodilation in isolated aortae *via* intracellular Ca^{2+} mobilization and Akt-mediated eNOS phosphorylation [Nofer *et al.*, 2004]. The vasoactive

effects of HDL could be mimicked by three lysophospholipids present in HDL: SPC, S1P, and lysosulfatide (LSF). All three elevated intracellular Ca^{2+} concentration and activated Akt and eNOS, which resulted in NO release and vasodilation [Nofer *et al.*, 2004]. $S1P_3$ deficiency abolished the vasodilatory effects of SPC, S1P, and LSF and reduced HDL effects by approximately 60%. In endothelial cells from $S1P_3$-deficient mice, Akt phosphorylation and Ca^{2+} rises in response to HDL and LPs were severely reduced. *In vivo*, intra-arterial administration of HDL or LPs lowered mean arterial blood pressure in rats. Thus, HDL has been identified as a carrier of bioactive LPs that regulate vascular tone *via* $S1P_3$-mediated NO release. This mechanism may contribute to the vasoactive effect of HDL and represent a novel aspect of its antiatherogenic function [Nofer *et al.*, 2004]. *In vivo* the $S1P_3$ receptor would encounter the whole HDL particle with its total load of LPs, resulting in a much higher biologically active concentration at the single receptor level compared with LPs in solution, and it is likely that several of the LPs contained in HDL would work together to achieve vasodilation [Nofer *et al.*, 2004].

Angiogenesis and promotion of endothelial cell survival have been shown to depend on the activation of Akt. Recently, S1P was demonstrated to induce Akt activation, eNOS phosphorylation, and NO production in endothelial cells [Lee *et al.*, 2001]. Both S1P and SPC interact with several LP receptors such as $S1P_1$, $S1P_3$, and $S1P_5$, which are all expressed in endothelial cells. The effects of HDL and HDL-associated LP on Akt activation, eNOS activation, and NO release in HUVECs were inhibited by PTX, suggesting the involvement of a PTX-sensitive G_i protein *in vitro* [Nofer *et al.*, 2004]. In isolated aortic segments, the vasodilatory effects of SPC, LSF, and S1P were completely abrogated in aortae from $S1P_3$-deficient animals, suggesting that this particular LP receptor completely mediates the vasodilatory effect of all three compounds. As the increase in $[Ca^{2+}]_i$ and Akt phosphorylation were either completely abolished (S1P and LSF) or substantially reduced (SPC), both major mechanisms of eNOS activation appear to be mediated by $S1P_3$. This is in agreement with two other studies that have implicated $S1P_3$ in Akt activation and NO production. The failure of S1P to increase $[Ca^{2+}]_i$ and activate PI-specific phospholipase C, another target of HDL-triggered intracellular signalling, has been reported previously for $S1P_3$-deficient MEFs [Nofer *et al.*, 2004]. Thus, it is suggested that the specific element of the vasodilatory effect of HDL that was lost in $S1P_3$-deficient animals (~50%) is attributable to its SPC, S1P, and LSF content acting via the $S1P_3$ receptor. Importantly, the vasoconstrictive effect was independent of $S1P_3$, as it was not different between aortae from WT and $S1P_3$ knockout animals. The further augmentation of vasoconstriction by S1P in precontracted aortae from $S1P_3^{-/-}$ or $eNOS^{-/-}$ mice suggested that S1P

has a dual action: it contracts arteries under basal conditions (but not *via* $S1P_3$) and mediates vasodilation (*via* $S1P_3$) in the case of increased arterial tone, with mediation by at least two different pathways: one impacting on endothelial cells and another on VSMCs. These effects may differ among different vascular beds [Nofer *et al.*, 2004].

In contrast to the complete loss of eNOS activation by lysophospholipids in $S1P_3$-deficient animals, the vasodilatory response to HDL, although substantially inhibited (by ~60%), was not completely abrogated. Other HDL-mediated effects, besides resulting in eNOS and/or Akt activation, may account for the remaining part of HDL-induced eNOS dependent vasodilation. It has been shown that HDL-induced eNOS activation is critically dependent on the binding of HDL to the scavenger receptor, class B, type I (SR-BI), and that Src, Akt, and MAPK signaling *via* SR-BI contribute to eNOS activation [Yuhanna *et al.*, 2001] (Figure 1.13). However, it was reported that the major SR-BI protein ligand of HDL, apoAI, failed to activate eNOS, while an antibody against the cytoplasmic C-terminus of SR-BI inhibited the ability of SR-BI to stimulate eNOS [Nofer *et al.*, 2004]. LPs are amphipathic molecules, and their ability to diffuse freely between HDL and the cell surface may be limited. Therefore, the interaction of HDL with SR-BI could provide the appropriate proximity for SPC, S1P, and LSF to effectively stimulate $S1P_3$. However, it is important to note that the data neither exclude direct activation of $S1P_3$ *via* LPs within the HDL complex nor indirect mechanisms of S1P production/ release, which could also activate $S1P_3$ [Nofer *et al.*, 2004].

In contrast to studies suggesting a role for Akt in eNOS activation by HDL, it was recently described that HDL bound to SR-BI stimulated eNOS *via* an increase in intracellular ceramide without an increase of $[Ca^{2+}]_i$ or Akt activation [Li *et al.*, 2005]. Interestingly, recent findings suggest that plasma membrane estrogen receptors activate eNOS *via* Akt and that they are biologically coupled to eNOS through $G_{\alpha i}$ [Shaul and Mineo, 2004]. This has led to the suggestion that cross-talk exists between estrogen receptors and a GPCR that signals *via* $G_{\alpha i}$ [Nofer *et al.*, 2004]. An excellent candidate emerging from recent data would be the $S1P_3$ receptor. The observation that $S1P_3$ acts as a functional HDL receptor and that LPs mediate an important part of its vasodilatory effect raises exciting questions about the role of LP receptors in the antiatherogenic activity exerted by HDL. Several potentially antiatherogenic effects have been ascribed to LP receptor signaling *in vitro* such as preservation of endothelial cell integrity, induction of endothelial cell migration, and eNOS activation [van der Giet and Tolle, 2004]. Information on LP receptor regulation and function in human atherosclerosis and in animal models of the disease is sparse, nor are there many studies on the LP content of HDL in patients with atherosclerosis or coronary artery disease, these studies may help elucidate the role of LPs

receptors in HDL-mediated atheroprotection and reveal new methods to prevent and treat clinical atherosclerosis [Nofer *et al.*, 2004]. Unravelling the mechanisms of lipid mediator binding to and release from HDL will hopefully provide novel targets for antiatherogenic therapeutics. Vasodilatory potency of HDL may vary considerably among the population depending on age, sex and concomitant disease. As currently available drugs are not efficient in raising plasma HDL levels, modulating the vasoactive properties of HDL might be a new way to combat atherosclerosis [van der Giet and Tolle, 2004].

HDL is an independent risk factor in cardiovascular disease, and raising HDL levels alone results in a significant risk reduction of major cardiovascular events in patients with coronary disease whose primary lipid abnormality was a low HDL cholesterol level. However, the mechanisms by which HDL exerts its powerful protective effects are still not clear. Among its numerous potential antiatherogenic effects, HDL cholesterol levels are directly associated with flow-mediated vasodilation in clinical patients *in vivo* [O'Connell and Genest, 2001]. Recently, intravenous administration of reconstituted HDL was shown to acutely restore abnormal endothelial function in the brachial artery of hypercholesterolemic patients. Evidence is provided that intravenous administration of HDL acutely stimulates myocardial perfusion *in vivo* in the murine heart via eNOS activation, and the HDL component sphingosine 1-phosphate (S1P) and its receptor $S1P_3$ have been identified as functional opponents of this effect [Levkau *et al.*, 2004]. It is extremely complex to compare the effects of S1P *in vitro* and *in vivo*, especially because S1P is recognized to induce both vasoconstriction and vasodilation in different settings. To detect the vasodilative effect of S1P, a study raised mean arterial blood pressure initially by infusion of endothelin. In isolated arteries, S1P also had opposing effects dependent on the initial arterial tone, whereas S1P had a vasodilative effect on arteries precontracted with phenylephrine, its effect on native, noncontracted arteries was exactly the opposite. This biological behavior of S1P resembles the action of vasodilators such as diadenosine polyphosphates and suggests that it has a dual function. S1P contracts arteries under basal conditions, whereas it dilates arteries with increased arterial tone. Although S1P is a physiological component of HDL and can substantially reduce myocardial perfusion it does not appear from the results of this particular investigation to diminish the stimulatory effect of HDL on perfusion because no further increase in perfusion by HDL in $S1P_3^{-/-}$ mice was observed [Levkau *et al.*, 2004].

1.5.15 SH3 domain interactions in receptor signalling

Interaction domains such as the SH2, SH3 and PDZ binding domains control a broad range of cellular functions, including signal transduction, cytoskeletal architecture, protein

trafficking, gene expression, chromatin organization, organelle biogenesis, cell polarity and regulated proteolysis [Bladt *et al.*, 2003], as has already been exemplified in Section 1.5.14.

The Src cytoplasmic tyrosine kinase in its natural state is a peripheral membrane protein that contains an SH3 domain, an SH2 domain, a tyrosine kinase domain and a short C-terminal tail. In its autoinhibited state, c-Src becomes phosphorylated by c-Src kinase at a tyrosine residue within the tail. From this ensues an intramolecular interaction with the SH2 domain, which moves the SH3 domain to associate with the SH2-kinase linker region. Shaped like this, the activity of the kinase domain is repressed, though the inhibition can be lifted by breaking the contacts *via* dephosphorylation of the C-terminal tail, or by binding of a high-affinity ligand to either the SH2 or SH3 domain. Once activated, the SH3 and SH2 domains are liberated to bind cytoplasmic targets with appropriate motifs, and the kinase domain is activated to phosphorylate such binding partners [Mayer, 2001].

As an example of the interplay between various domains the Grb2 adaptor protein, which possesses a central SH2 flanked by two SH3 domains, can be seen to associate with several motifs. The SH2 domain binds phosphorylated motifs with the sequence pTyr-X-Asn, found in activated tyrosine kinases and docking proteins. The N-terminal SH3 domain binds proline-rich motifs in the C-terminal tail of Sos, a GEF for Ras GTPase. The C-terminal SH3 domain binds a scaffolding protein, Gab1, which in turn activates the PI3-kinase, generating PI-3,4,5-P_3 at the plasma membrane. PIP3 provides a docking site for the PH domains of the serine/threonine kinases PKB/Akt and PDK1, which collaborate to activate signalling pathways involved in cell survival and cell cycle control [Bladt *et al.*, 2003].

It has been demonstrated that of the five known isoenzymes generated by the phosphodiesterase 4D gene (PDE4D), the brain specific PDE4D4 form can interact with certain SH3 domains expressed as GST fusion proteins. A screen of SH3 fusion proteins revealed selective interaction with certain Src family tyrosyl kinases SH3 domains. It has been suggested that this may indicate a tendency of this isoenzyme to interact with specific SH3 domain-containing proteins [Beard *et al.*, 1999]. Due to the relative importance of these interaction domains in signal transduction it is useful to identify potential sites of interest within the $S1P_3$ receptor, and examine how they exert their role in S1P signalling. As mentioned previously, the $S1P_3$ receptor has an SH3 binding motif domain present within its C-terminal (Figure 1.10). The RASPIQP fragment was chosen for investigation of its protein-protein interactions using both the yeast 2 hybrid system and fusion protein methodologies. This will be discussed in further detail in Chapter 5.

1.6 Yeast Double Hybrid System Analysis

Usual tools available for the analysis of protein-protein interactions in multicellular organisms have been restricted to biochemical methods employing physical means of detection. Most familiar is the use of acrylamide gels, but also affinity chromatography, affinity blotting, immunprecipitation and cross-linking are available. Protein-protein interactions are intrinsic to nearly every cellular process, all DNA related processes from replication through to translation, to secretion, control of cell cycle, stages of metabolism, formation of enzymatic complexes and the manifestation of cellular macrostructures [Bartel and Fields, 1997; Milligan and White, 2001]. Complex interactions of proteins result in the formation of cytoskeleton, nuclear scaffold and the mitotic spindle. Protein-protein interactions seem to play crucial roles in the formation of comparatively smaller structures such as nuclear pores, centrosomes and kinetochores. As well as a vast structural requirement fulfilled by protein-protein interactions, many cellular processes are also controlled and regulated by transient protein-protein interactions. Enzymes interact only transiently with their substrates. Many enzymes can modify an array of proteins that cover fundamental processes like cell growth, cell cycle, metabolic pathways and signal transduction. Extremely large complexes also mediate many of these enzymatic activities. In many signal transduction pathways, catalytic activities involved, such as protein kinases, may bind strongly to their protein susbstrates. Structural proteins required for signal transmission have been suggested to act as scaffolds, bridging several proteins involved at consecutive steps in a protein structures through which a signal is being transmitted. Alterations in these interactions contributes to diseases. There is cause then for manipulation of these important interactions as potential therapeutic strategy. The Two-Hybrid System is a molecular genetic tool which facilitates the study of protein-protein interactions. The protein under investigation is called the bait. The protein caught with the bait is the prey. This is interaction is facilitiated by using a cDNA library in a special vector so that the yeast translates the gene cloned as a cDNA as a fusion with part of a Gal4 (the *beta*-galactosidase gene) protein. The intact Gal4 protein is a transcriptional activator which has two separate functions (a DNA-binding domain and an activating domain). If the bait and prey proteins interact, then a reporter gene e.g. Gal1-lacZ, is transcriptionally activated, and a colour reaction on specific media is observed. This can be used to study the interaction between two proteins which are expected to interact and/or as a means of cloning a gene encoding a protein which interacts with a protein for which the gene is already available [Bartel and Fields, 1997].

The specificity, selectivity and duration of GPCR signaling are optimized by the scaffolding of functional multi-protein complexes associated with receptor C-terminal tails.

Although the C-terminal tails of GPCRs have been readily accepted for the fine-tuning of G protein activation, their roles as bait to find GPCR-associated proteins have only recently been established [Bockaert *et al.*, 2003]. Interestingly, many GPCR splice variants differ in their C-termini, for example, eight C-terminal splice variants have been described for the 5-HT_4 receptor [Bockaert *et al.*, 2004], and four C-terminus splice variants exist for mGluR1 [Fagni *et al.*, 2000], and prostaglandin EP3 receptors [Hatae *et al.*, 2002], variants of which bind to different intracellular scaffolding/signaling molecules that specify their transduction signals [Bockaert *et al.*, 2003].

Many studies use the yeast two hybrid system and GST fusion assays in tandem to provide supporting evidence for physiological interactions of proteins, rather than artificial interactions due to proximity.

1.6.1 GST Fusion Studies

The study of protein interactions using GST fusion proteins was first performed between the human β2-adrenoceptor and the α-subunit of its cognate G protein G_s. The fusion protein was produced by ligating together cDNAs encoding both proteins. The results from this study suggested that agonist occupation of the fusion protein resulted in the activation of AC and elevation in potency was suggested to reflect a covalent link between the receptor and the G-protein, which causes more efficient coupling between partner proteins [Milligan, 2000]. GST fusion proteins are unique tools for explorative pharmacology as they allow investigation of ligand efficacy, the contribution of post translational modifications to GPCRs and G-proteins to agonist function and the quantitative effects of point mutations in GPCRs and G proteins on the affinity of their interactions and transfer of information between the proteins [Milligan, 2000]. The benefit of GST fusions is that the stoichiometry of expression in the two proteins is always 1:1 and is not dependent on the cell system used. The fusion approach, if analysed at the appropriate interaction should ensure directly comparable agonist-binding affinity and EC_{50} for G-protein activation. This approach supports investigations of the relationship between ligand binding and receptor activation [Milligan, 2000].

A study using both the GST fusion strategy and the yeast two hybrid system identified endophilin 1/2/3 as novel binding partners for β1ARs. This study suggest that interaction between proline-rich motifs and SH3-containing proteins may represent a previously underappreciated aspect of G-protein coupled receptor signaling [Tang *et al.*, 1999]. More on GST fusion studies will be discussed in Chapter 5.

1.7 The Study of GPCR Cell Surface Distribution and Agonist-Induced Internalisation Using Green Fluorescent Protein (GFP)

Classical approaches to studying GPCR internalisation include radioligand binding, physical fractionation techniques and immunoflurescence [Kallal and Benovic, 2000]. However, the recent application of green fluorescent protein (GFP) as a tool in the study of agonist-induced GPCR internalisation has proved invaluable [Drmota *et al.*, 1998; McLean *et al.*, 1999; Milligan, 1999; Kallal and Benovic, 2000; Sarvazyan *et al.*, 2002]. The gene encoding GFP was originally isolated from the jellyfish Aequorea Victoria in 1992 [Prasher *et al.*, 1992; Inouye and Tsuji, 1994].

GFP is an autofluorescent protein of 238 amino acids that emits green light with an emission maximum of 509nm upon fluorscent excitation at 488nm derived from either standard fluorescence microscope light sources or fluorescein isothiocyanate (FITC) excitation and emission filters [Tsien, 1998]. The use of GFP in the study of receptor internalisation has been used for a variety of GPCRs, including the cholecystokinin CCK1 receptor, β2AR, thyrotropin-releasing hormone TRH1 receptor and the vasopressin V2 receptor [Tarasova *et al.*, 1997; Drmota *et al.*, 1998; Schulein *et al.*, 1998; McLean *et al.*, 1999].

There are a number of advantages gained from using GFP [Milligan, 1999; Kallal and Benovic, 2000]. Since there are no cofactors or substrates required for fluorescence, the time and expense of using primary and secondary antibodies on fixed cells can be avoided. GFP-expressing cells can also be studied on living cells in real time so that the dynamics of protein trafficking can be observed. Cells expressing GFP can also be fixed, as GFP is relatively chemically resistant. Also, since GFP is covalently attached to the protein of interest, non-specific fluorescence is avoided. One of the major disadvantages of using GFP is that the expression of GPCR-GFP chimeras in cells results in the labelling of protein biosynthetic compartments, such as the endoplasmic reticulum and Golgi, which can influence the interpretation of results [Kallal and Benovic, 2000].

Many examples of GFP-tagging of GPCRs, such as the β2AR and TRH1 receptor, have shown that the addition of GFP has no effect on the receptors' ability to bind ligand and that the ability to generate second messengers and to desensitise was unaffected [Tarasova *et al.*, 1997; Drmota *et al.*, 1998; Schulein *et al.*, 1998; McLean *et al.*, 1999]. However, GFP-tagging may result in altered properties of the receptor. Hence, careful experimental comparisons to untagged proteins should be made prior to visualisation studies using confocal microscopy.

1.8 Aims

The aim of this PhD is to characterise the phosphorylation, internalisation and desensitisation of the sphingosine 1-phosphate receptor, $S1P_3$. Due to the distinct differences that exist amongst GPCRs in relation to the processes of receptor phosphorylation and internalisation a basis for studying the regulation of $S1P_3$ can be taken from the most well characterised examples of GPCR phosphorylation and internalisation, the β_2AR. Alongside this line of investigation a Yeast Two Hybrid Technique will be employed to examine any potential protein-protein interactions that may be unique to the $S1P_3$ receptor.

Figure 1.1: Comparison of predicted structure for bovine rhodopsin with the x-ray crystal structure.

A comparison of the predicted structure for bovine rhodopsin (green) with the x-ray crystal structure (blue). The TM regions have an rms deviation in alpha carbon coordinates (CRMS) of 3.1 Å. Taken from [Vaidehi *et al.*, 2002]

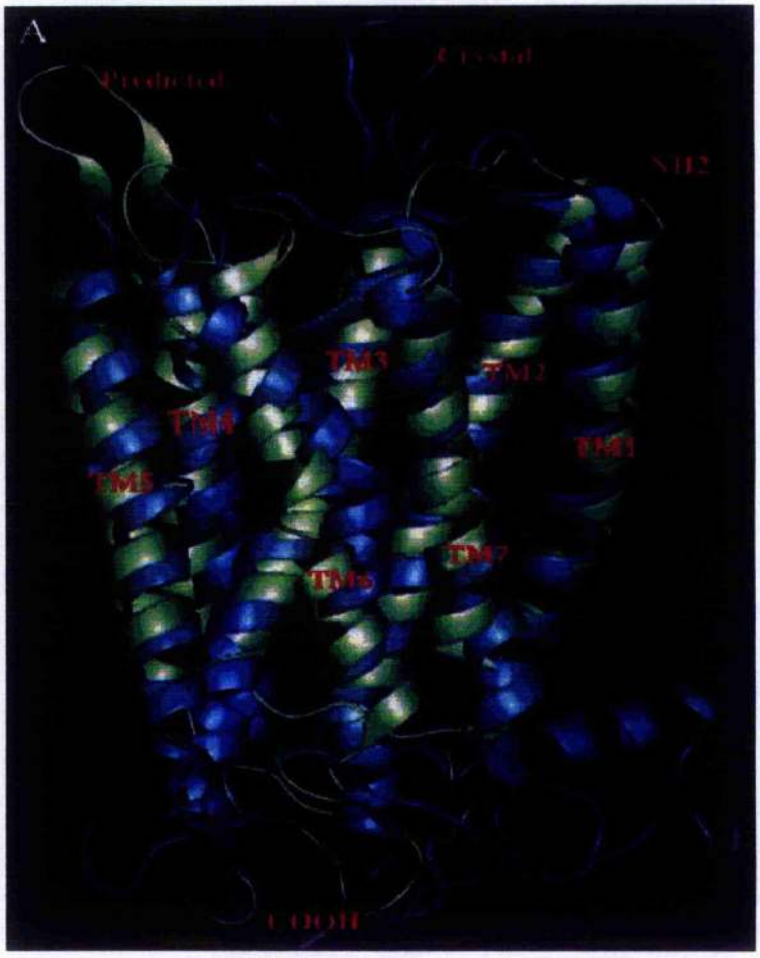
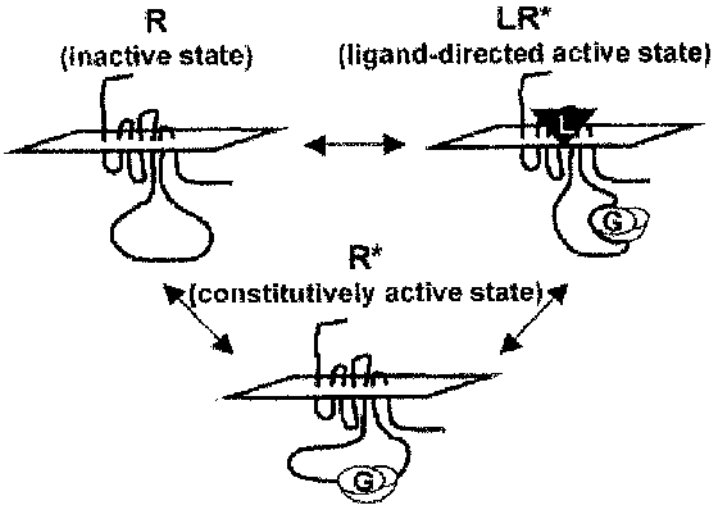

A
Predicted
Crystal
NH2
TM3
TM2
TM4
TM1
TM5
TM7
TM6
COOH

Figure 1.2: GPCRs are allosteric proteins that can adopt various conformations in equilibrium.

Contrary to the inactive state (R), the active state can interact with G proteins to initiate response. These active conformations occur either spontaneously (R^*), leading to constitutive activity of GPCRs, or through ligand binding (LR^*). The competition between LR^* and R^* for the G proteins leads to agonism, neutral antagonism, or inverse agonism. Taken from [Gbahou *et al.*, 2003].



LR* with no R*	→	Agonism
LR* with R* of lower efficacy	→	Agonism
LR* with R* of similar efficacy	→	Neutral antagonism
LR* with R* of higher efficacy	→	Inverse agonism

Figure 1.3: Structure of G-protein, showing the location of each subunit, including the βγ complex.

gg = geranylgeranyl (on γ-subunit) stable.

p = palmitate (on α-subunit) labile.

SI, SII and SIII show the switch sites.

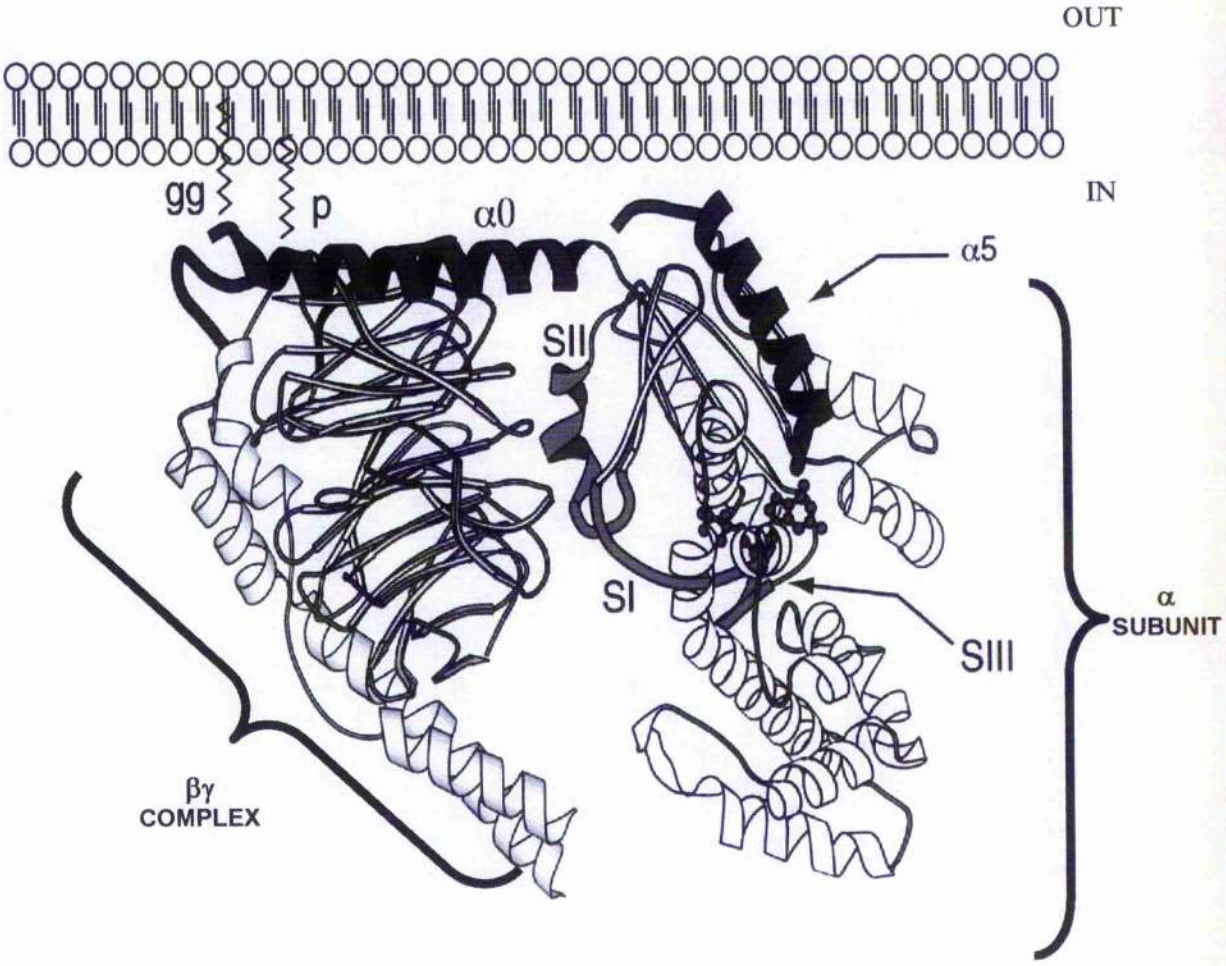

OUT
IN
gg
p
α0
α5
SII
SI
SIII
α
SUBUNIT
βγ
COMPLEX

Figure 1.4: Relationship of G-Protein subfamilies:

A phylogenetic tree showing the sequence homology of each member of the four Gα-protein subfamilies,

These include:

- $G\alpha_s$ stimulates adenylate cyclase
- $G\alpha_{olf}$ stimulates adenylate cyclase
- $G\alpha_{i1}$ undefined
- $G\alpha_{i2}$ inhibits adenylate cyclase
- $G\alpha_{i3}$ regulates K^+ channels
- $G\alpha_o$ regulates Ca^{2+} channels
- $G\alpha_{t1}$ transducin 1, activates cGMP phosphodiesterase
- $G\alpha_{t2}$ transducin 2, activates cGMP phosphodiesterase
- $G\alpha_z$ undefined
- $G\alpha_{15}$ regulates phospholipase C
- $G\alpha_{16}$ regulates phospholipase C
- $G\alpha_{14}$ regulates phospholipase C
- $G\alpha_{11}$ regulates phospholipase Cγ1
- $G\alpha_q$ regulates phospholipase C
- $G\alpha_{12}$ activates small G-proteins, such as Rac and Rho
- $G\alpha_{13}$ activates small G-proteins, such as Rac and Rho

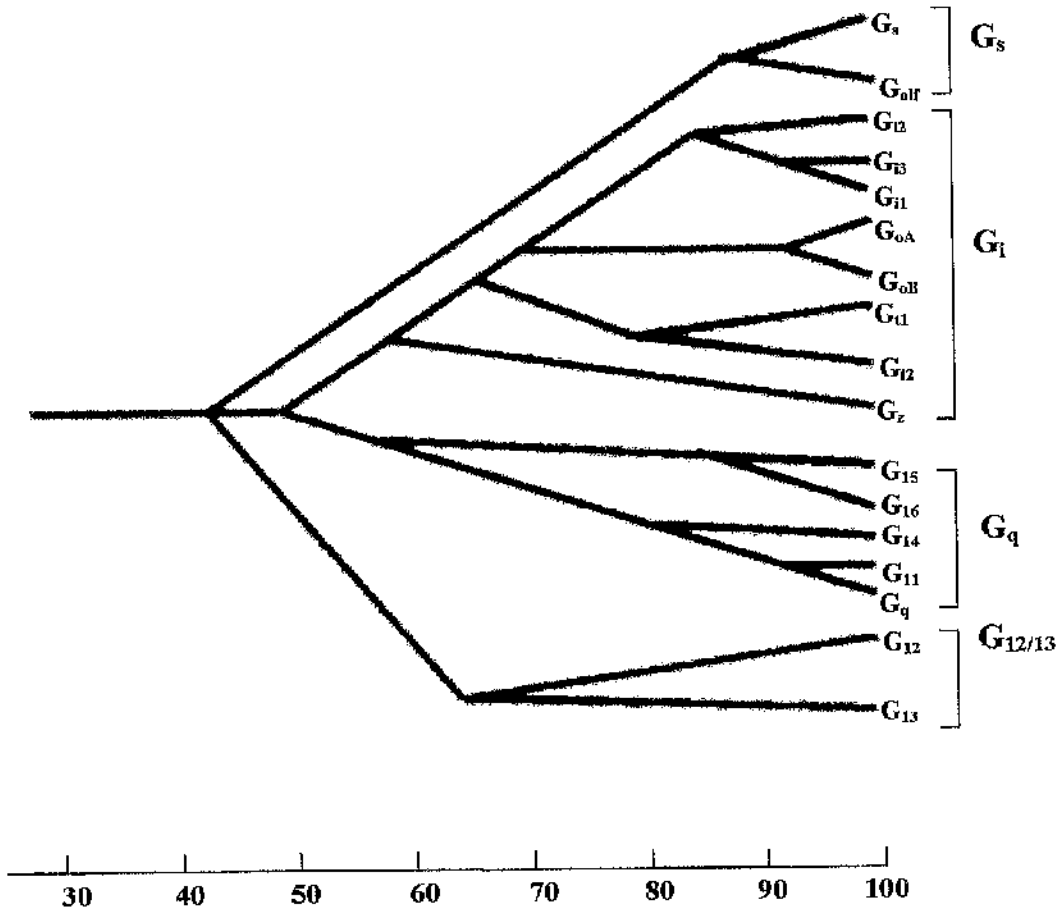

G_s
G_{olf}
G_{i2}
G_{i3}
G_{i1}
G_{oA}
G_{oB}
G_{t1}
G_{t2}
G_z
G_{15}
G_{16}
G_{14}
G_{11}
G_q
G_{12}
G_{13}
G_s
G_i
G_q
$G_{12/13}$
30
40
50
60
70
80
90
100
% Amino Acid Identity

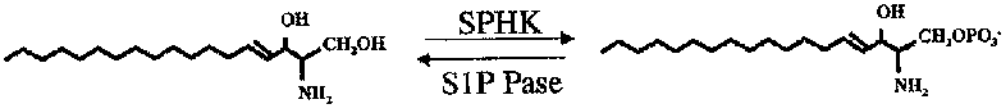
Figure 1.5: S1P and LPA Structure

A: Signaling functions of the substrate and product of the SK reaction. SKs using ATP as the phosphate donor, catalyze the phosphorylation of D-*erythro*-sphingosine to produce S1P. Several downstream targets and potential functions of both sphingosine and S1P are indicated. *PKC*, protein kinase C; *ICRAC*, calcium release-activated calcium current.

Image taken from [Spiegel and Milstien, 2002].

B: LPA; 1-acyl-2-*sn*-glycerol-3-phosphate is a naturally occurring LP that activates diverse cellular actions on many cell types. It is also an intermediate in *de novo* biosynthesis of membrane phospholipids. Although all cells contain small amounts of LPA associated with membrane biosynthesis, some cellular sources can produce significant amounts of extracellular LPA such as activated platelets, which account for the LPA found in serum [Contos *et al.*, 2000]. LPA is shown here with S1P and SPC to compare structures.

A The Sphingosine Rheostat



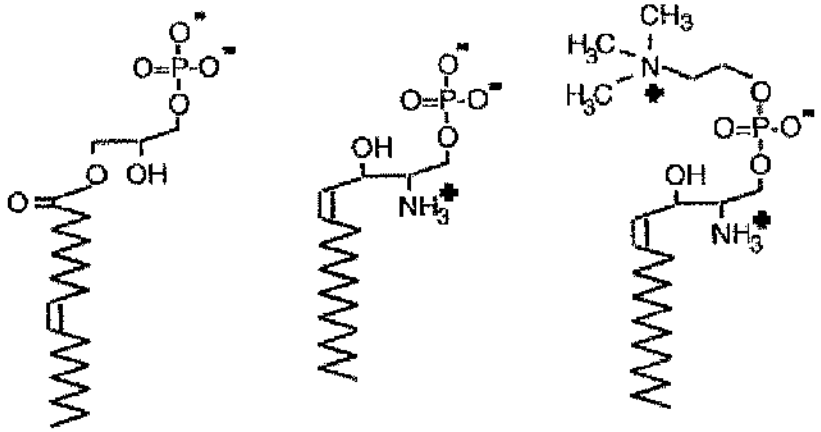
SPHINGOSINE

Growth arrest
Enhances apoptosis
Inhibits PKC
Inhibits ICRAC
Decreases thermotolerance
Regulates endocytosis
Role in cytoskeletal rearrangements

S1P

Stimulates growth
Suppresses apoptosis
Regulates motility
Regulates calcium
Increases thermotolerance
GPCR ligand
Role in cytoskeletal rearrangements

B



LPA S1P SPC

Figure 1.6: Sphingolipid synthesis, turnover and functions.

The term sphingolipid generally refers to any of an array of lipids consisting of a head group attached to the 1-OH of ceramide (Cer). Cer consist of a sphingoid base, usually referred to as a long chain base (LCB), which is *N*-acylated. *De novo* synthesis of LCBs begins with condensation of the palmitoyl CoA and serine, forming 3-ketosphinganine. This product is then reduced to sphinganine, also known as dihydrosphingosine (dihydro-Sph; 2-amino-1,3-dihydroxy-octadecane). A 14–26 carbon fatty acid chain is then added in an amide linkage with the 2-amino group, forming dihydroceramide (dihydro-Cer). A head group, such as phosphocholine or a carbohydrate, can now be added to the 1-OH, forming a SL, although most SLs of higher eukaryotes contain further modifications of the LCB. In mammalian cells, dihydro-Cer usually has a 4,5-trans double bond introduced, forming Cer. The corresponding LCBs are referred to as sphingosine (Sph, also called sphingenine) and phyto-Sph, respectively. The term ceramides refers to *N*-acylated LCB species in general, while Cer will be used to designate *N*-acyl Sph. The most common LCBs are Sph, dihydro-Sph and phyto-Sph, although there are reports of other modifications, including additional double bonds, other hydroxylations, 3-*O*-acetyl, methylations, and even 3-ketosphinganine as the LCB of some glycosphingolipids. Metabolism of SLs is a constitutive process, with removal of the head group yielding Cer. In mammalian cells, ceramidases hydrolyze the N-acyl chains, leaving the LCBs, most commonly Sph. Sph can be phosphorylated on the primary hydroxyl by SKs (SK1 or SK2), forming S1P. S1P then can be cleaved by S1P lyase. dihydro-Sph and phyto-Sph can also be phosphorylated and degraded in this manner. Although degradation of S1P by S1P lyase is irreversible, S1P can be converted back to Sph by specific S1P phosphohydrolases (SPPs), and Sph can be re-acylated to Cer by Cer synthases. Thus, dihydro-Sph can be synthesized de novo, but Sph can only arise from metabolism of SLs.

Adapted from [Maceyka *et al.*, 2005], adapted from [Le Stunff *et al.*, 2002].

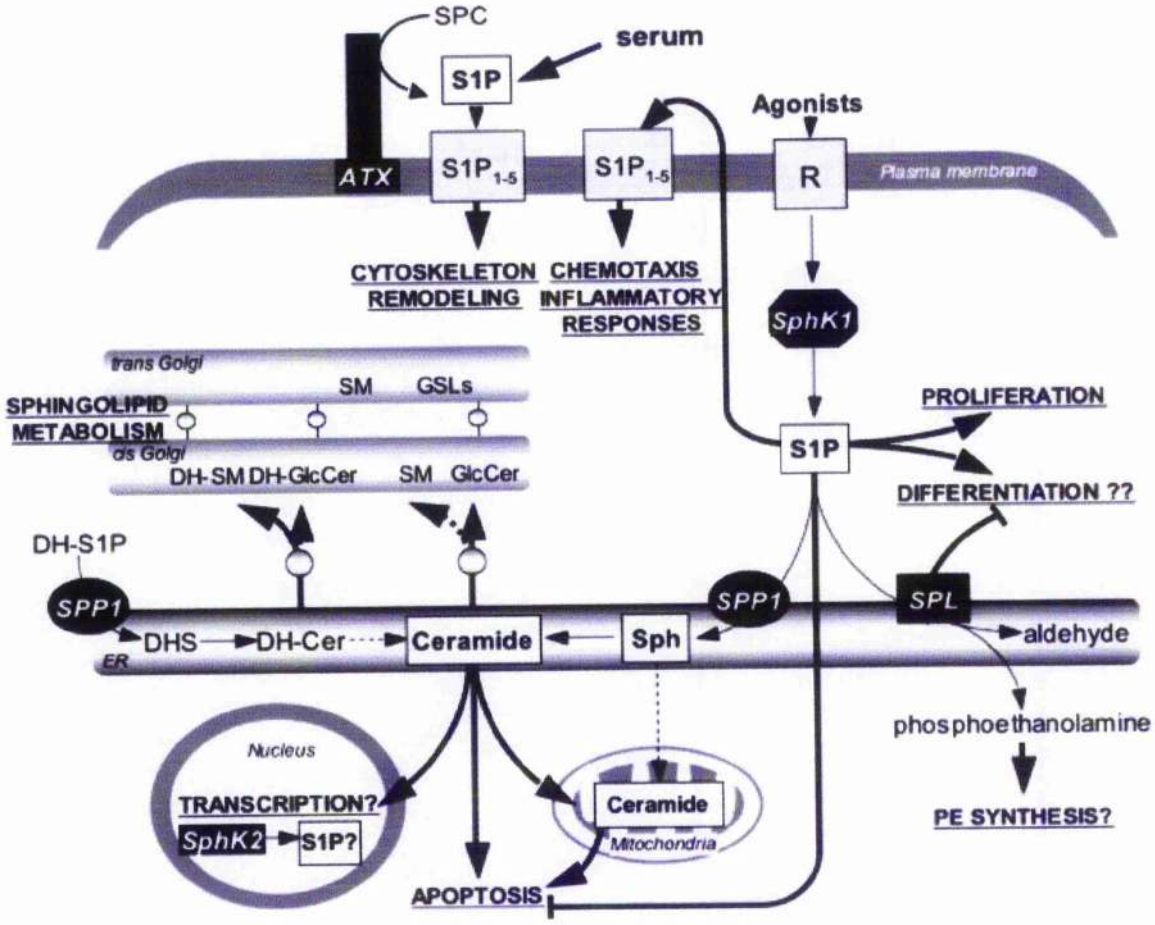

SPC
serum
S1P
Agonists
ATX
S1P1-5
S1P1-5
R
Plasma membrane
CYTOSKELETON REMODELING
CHEMOTAXIS INFLAMMATORY RESPONSES
SphK1
trans Golgi
SM
GSLs
SPHINGOLIPID METABOLISM
cis Golgi
DH-SM
DH-GlcCer
SM
GlcCer
PROLIFERATION
S1P
DIFFERENTIATION ??
DH-S1P
SPP1
SPP1
SPL
DHS
DH-Cer
Ceramide
Sph
aldehyde
ER
phosphoethanolamine
Nucleus
TRANSCRIPTION?
SphK2
S1P?
Ceramide
Mitochondria
PE SYNTHESIS?
APOPTOSIS

Figure 1.7: Phylogenetic tree of selected human G protein-coupled receptors.

The values show branch lengths that represent the evolutionary distance between each pair of sequences. The sequence divergence is equal to the sum of each value of branch length. Taken from [Kostenis, 2004].

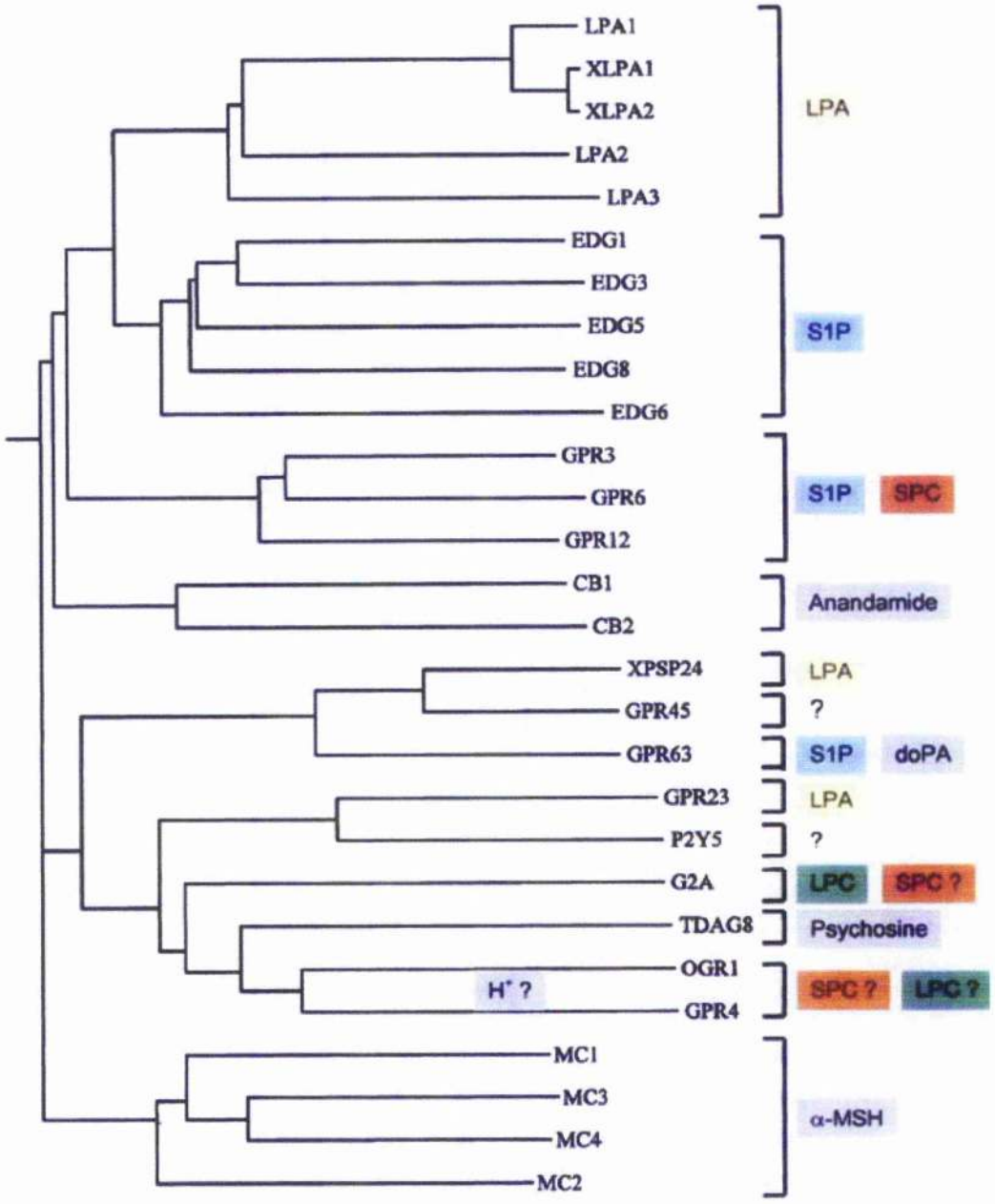

LPA1
XLPA1
XLPA2
LPA2
LPA3
LPA
EDG1
EDG3
EDG5
EDG8
EDG6
S1P
GPR3
GPR6
GPR12
S1P
SPC
CB1
CB2
Anandamide
XPSP24
LPA
GPR45
?
GPR63
S1P
doPA
GPR23
LPA
P2Y5
?
G2A
LPC
SPC ?
TDAG8
Psychosine
OGR1
H+ ?
GPR4
SPC ?
LPC ?
MC1
MC3
MC4
MC2
α-MSH

Figure 1.8: Model signalling systems for the S1P receptors

Five signalling models showing the coupling pathways of the different S1P receptors.

A: **$S1P_1$** couples only to G_i, making all its effects PTX-sensitive. The arrow from Akt to the receptor indicates phosphorylation (p) of the receptor by Akt.

B: **$S1P_3$** -

$S1P_3$ and $S1P_2$ couple to G_i, G_q, and G_{13}. The thick arrows show the predominant pathway regulating Rac. While $S1P_3$ activates Rac, $S1P_2$ inhibits it.

C: **$S1P_2$** -

D: **$S1P_4$** couples to G_i and G_{13}. It also activates adenylate cyclase (AC) through an unknown mechanism.

E: **$S1P_5$** couples to G_{12} and G_i. It inhibits ERK and activates JNK in a PTX-insensitive manner

Image taken from [Taha *et al.*, 2004].

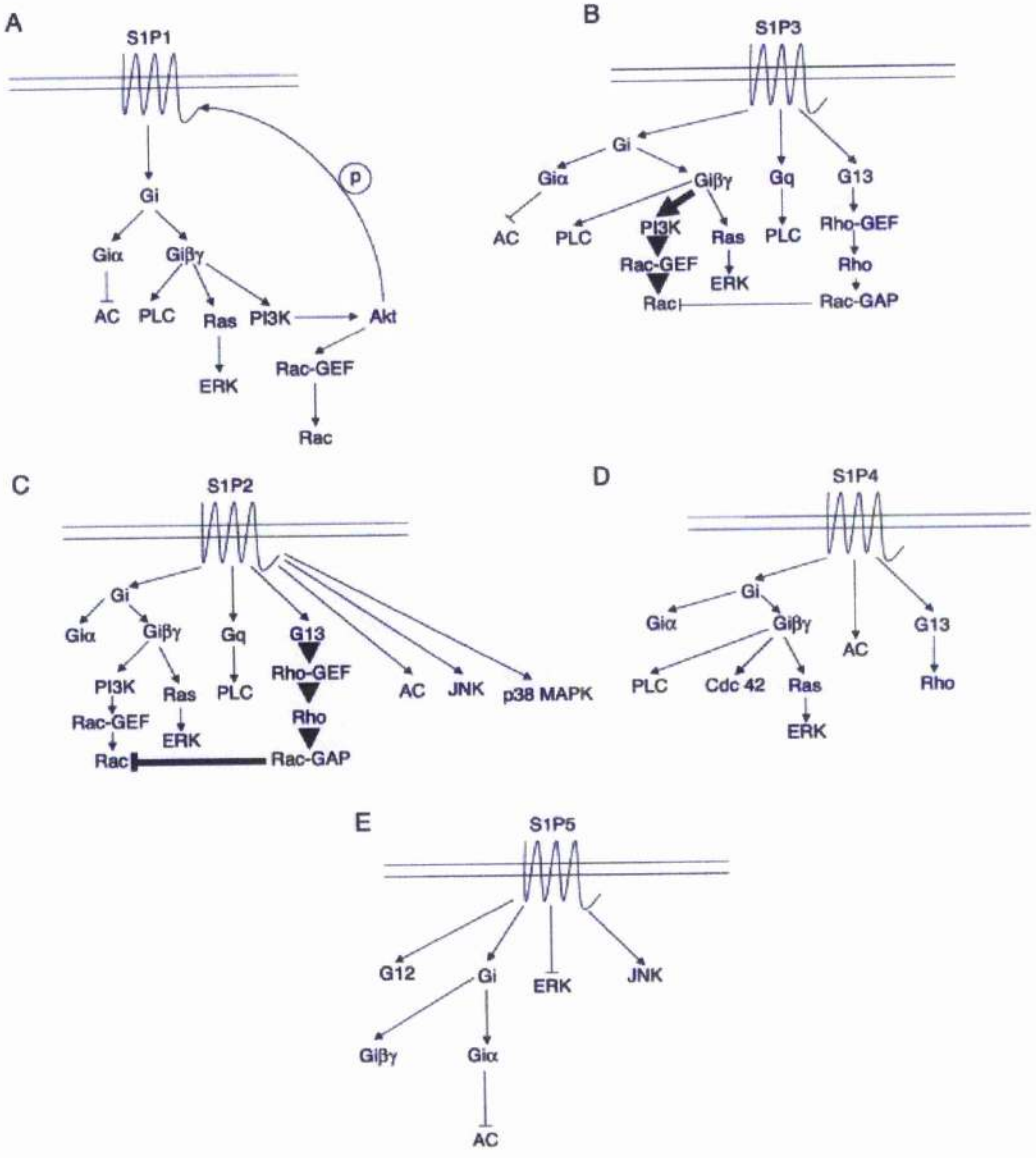
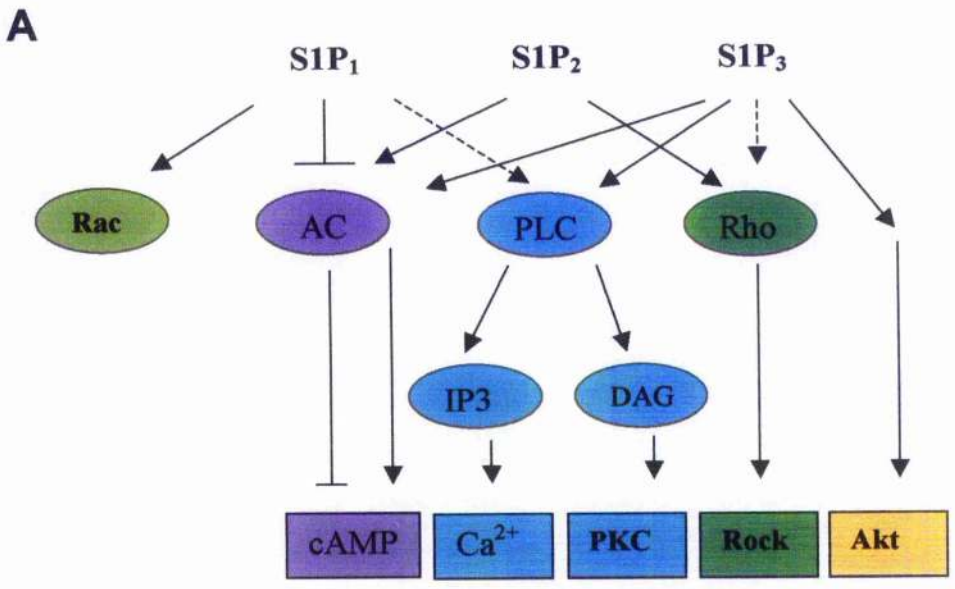

A
S1P1
P
Gi
Giα
Giβγ
AC
PLC
Ras
PI3K
Akt
ERK
Rac-GEF
Rac
B
S1P3
Gi
Giα
Giβγ
Gq
G13
AC
PLC
PI3K
Ras
PLC
Rho-GEF
Rac-GEF
ERK
Rho
Rac
Rac-GAP
C
S1P2
Gi
Giα
Giβγ
Gq
G13
PI3K
Ras
PLC
Rho-GEF
AC
JNK
p38 MAPK
Rac-GEF
ERK
Rho
Rac
Rac-GAP
D
S1P4
Gi
Giα
Giβγ
G13
AC
PLC
Cdc 42
Ras
Rho
ERK
E
S1P5
G12
Gi
ERK
JNK
Giβγ
Giα
AC

Figure 1.9: A comparison of S1P and LPA receptor signalling in mouse embryonic fibroblasts (MEFs).

A: Intracellular signalling effects of S1P through $S1P_{1-3}$ receptors. Whether $S1P_1$ mediates activation of Rac in MEFs is presently controversial. Weak activation of signalling molecules by distinct receptors are indicated by dashed arrows. AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; IP3, inositol 1,4,5-triphosphate; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; PLC, phospholipase C; Rock, Rho-associated kinase.

B: LPA_1- and LPA_2-mediated effects on signaling molecules.

Image taken from [Anliker and Chun, 2004a].


A
S1P1
S1P2
S1P3
Rac
AC
PLC
Rho
IP3
DAG
cAMP
Ca2+
PKC
Rock
Akt

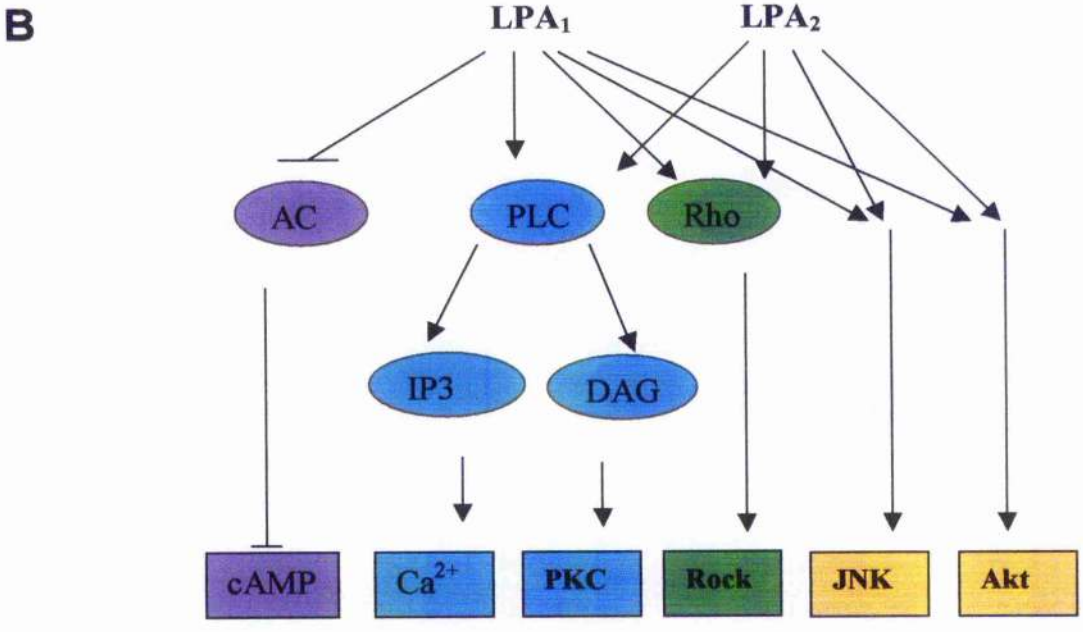

B
LPA1
LPA2
AC
PLC
Rho
IP3
DAG
cAMP
Ca2+
PKC
Rock
JNK
Akt

Figure 1.10: Schematic plot of $S1P_3$ receptor

A schematic diagram of the $S1P_3$ receptor adapted from Visuer http://www.gpcr.org/7tm/seq/diagrams_tGRAP/EDG3_HUMAN-rbdg.html showing the conformation of the receptor within the plasma membrane. Residues denoted by a white circle show regions that have been previously investigated by other researchers in the field. The missing residues are listed as follows:

A: [2] **ATALPPLQPU RGNETLREHY QYUGKLAGRL KEA** [34]

B: [139] **LTMIKM** [144]

C: [184] **PDCST** [188]

D: [220] **LVKSSSRKVA NHN** [233]

E: [269] **CRV** [271]

F: [299] **TLAS KEMRRAFFRL VCNCLVRGRG ARASPIQPAL DPSRSKSSSS NNSSHSPKVK EDLPHTAPSS CIMDKNAALQ NGIF N** [378]

A synthetic peptide MRPYDANKR derived from residues along the second intracellular loop listed in B were shown to induce a pro-angiogenic signal by $S1P_3$ [Licht *et al.*, 2003].

Residues denoted by **F** were the residues used in this research to study the effects of the C-terminal tail. RASPIQP represents the SH3 interacting domain motif.

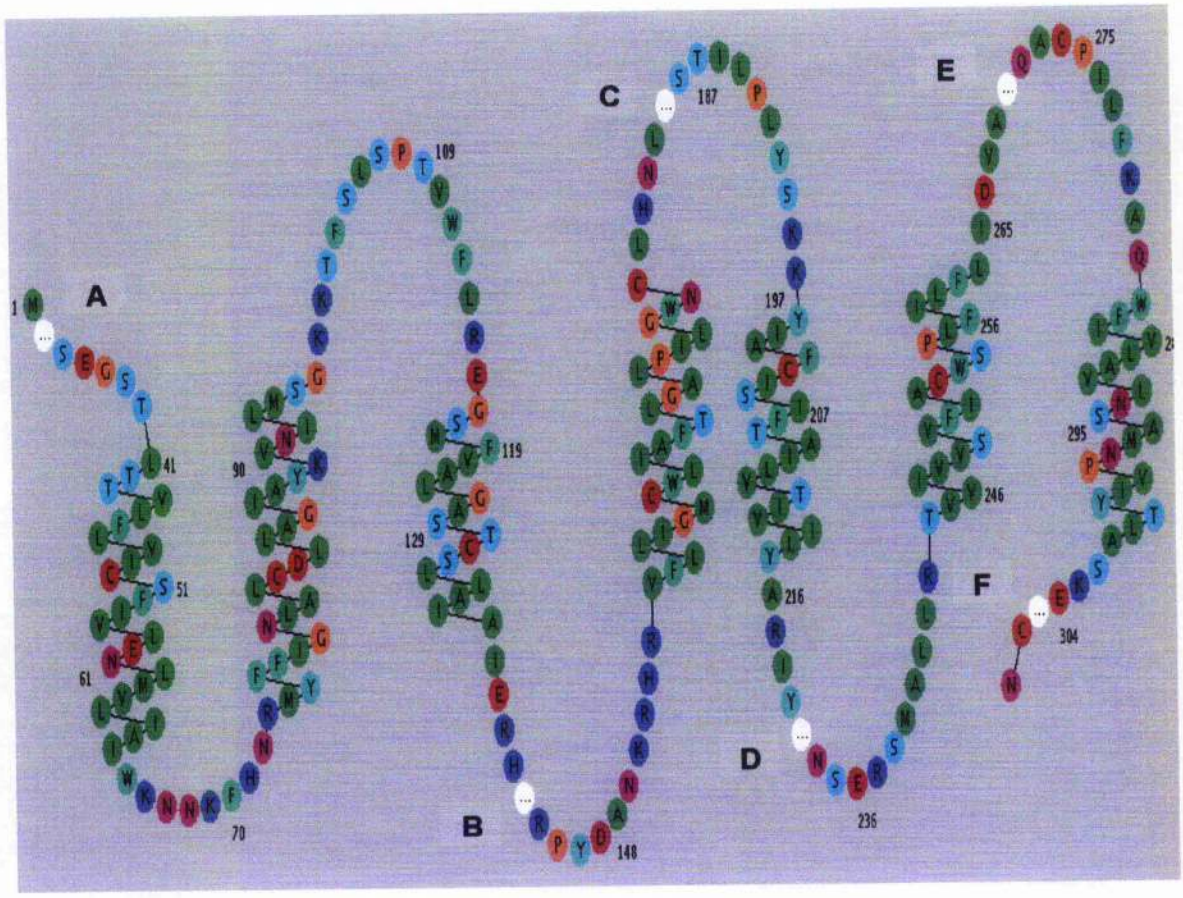

A
B
C
D
E
F
1
41
51
61
70
90
109
119
125
148
187
197
207
216
236
246
256
265
275
295
304

Figure 1.11: Biological roles of lysophospholipids in different systems

Receptor-mediated cellular responses to LPA and S1P, such as survival, proliferation, and migration, exhibit biological significance particularly within the nervous system, the cardiovascular system, the immune system, and the female reproductive system. Indicated are physiological and pathophysiological functions of LPA and S1P and the involved receptors. *IL-2*, interleukin-2; *OCCs*, ovarian cancer cells; *SCs*, Schwann cells; *VEC*, vascular endothelial cells; *VSMCs*, vascular smooth muscle cells; *HDL*, high density lipoprotein. Taken from [Anliker and Chun, 2004].

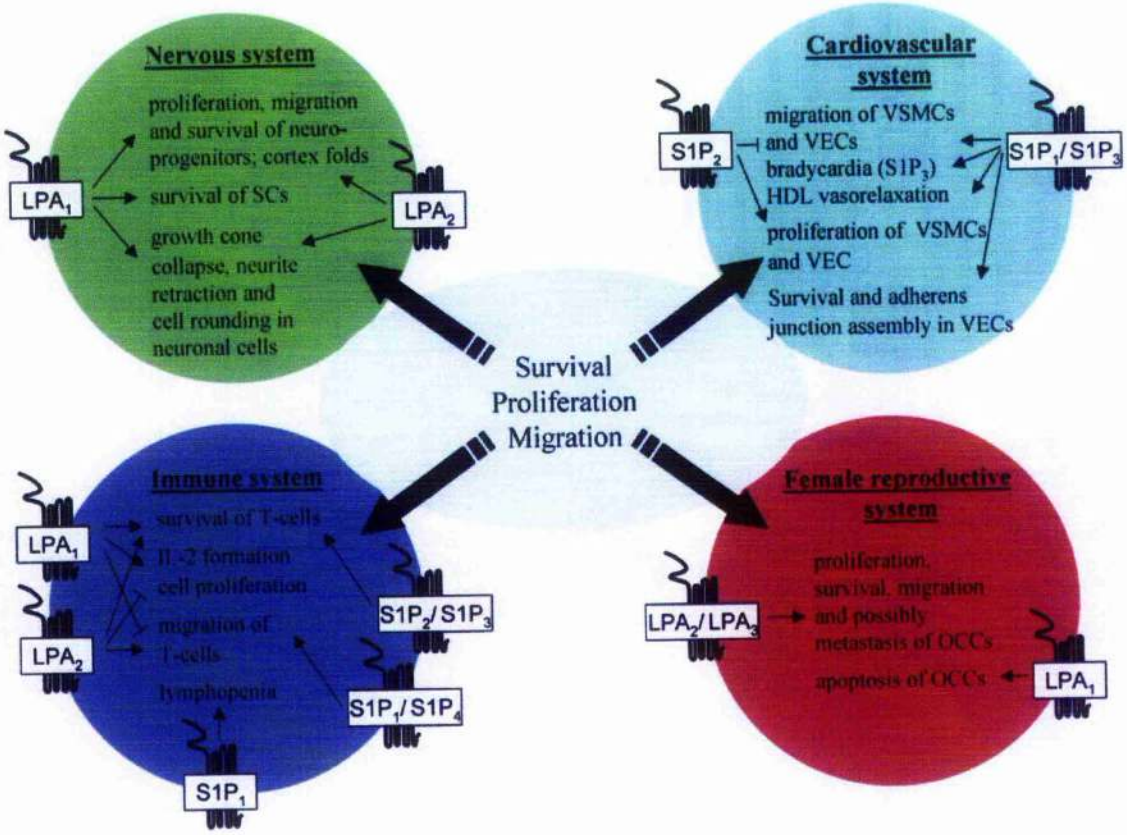

Nervous system
proliferation, migration and survival of neuro-progenitors; cortex folds
survival of SCs
growth cone collapse, neurite retraction and cell rounding in neuronal cells
LPA1
LPA2
Cardiovascular system
migration of VSMCs and VECs
bradycardia (S1P3)
HDL vasorelaxation
proliferation of VSMCs and VEC
Survival and adherens junction assembly in VECs
S1P2
S1P1/S1P3
Survival
Proliferation
Migration
Immune system
survival of T-cells
IL-2 formation
cell proliferation
migration of T-cells
lymphopenia
LPA1
LPA2
S1P2/S1P3
S1P1/S1P4
S1P1
Female reproductive system
proliferation, survival, migration and possibly metastasis of OCCs
apoptosis of OCCs
LPA2/LPA3
LPA1

Figure 1.12: HDL-induced eNOS activation *via* presentation of S1P to $S1P_3$

A model for HDL-induced eNOS activation and vasodilation by the lysophospholipid receptor $S1P_3$. PI-PLC, phosphatidylinositol-specific phospholipase C. A-I, apolipoprotein A-I; N and C, amino- and carboxyl terminus of the Scavenger Receptor Class B, Type I (SR-BI). Adapted from [Nofer *et al.*, 2004] to include FTY720 which requires endogenous phosphorylation to effect arterial vasodilation *via* $S1P_3$. FTY720 is currently in clinical trials as the first immunomodulator for prevention of organ graft rejection in clinical development that, in addition, positively affects the endothelium by stimulating NO production, and thus potentially displaying beneficial effects on transplant survival beyond classical T cell immunosuppression [Tolle *et al.*, 2005].

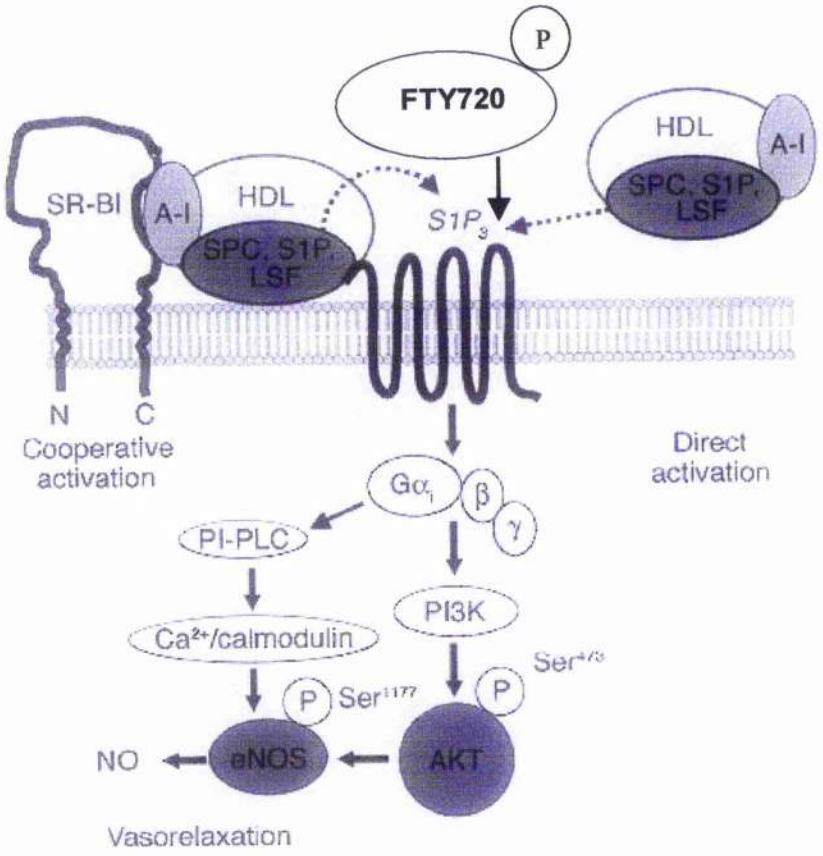

P
FTY720
HDL
A-I
SPC, S1P, LSF
SR-BI
A-I
HDL
SPC, S1P, LSF
S1P3
N
C
Cooperative activation
Direct activation
Gαi
β
γ
PI-PLC
PI3K
Ca2+/calmodulin
P
Ser1177
P
Ser473
NO
eNOS
AKT
Vasorelaxation

Figure 1.13: Preservation of vascular integrity by FTY720-P.

In endothelial cells (EC), FTY720-P induces translocation of vascular endothelial cadherin (VE-cadherin) and β-catenin to the focal contact sites between the cells, thereby promoting adherens junction assembly. The α-, β- and γ-catenins connect to the intracellular cortical actin ring of the cytoskeleton and this process stabilizes the endothelium and enhances endothelial barrier function. Similar effects are induced by S1P, suggesting that FTY720-P may not internalize but rather signal its target receptors in EC; the signaling may involve $S1P_1$ and/or $S1P_3$. Image taken from [Brinkmann *et al.*, 2004].

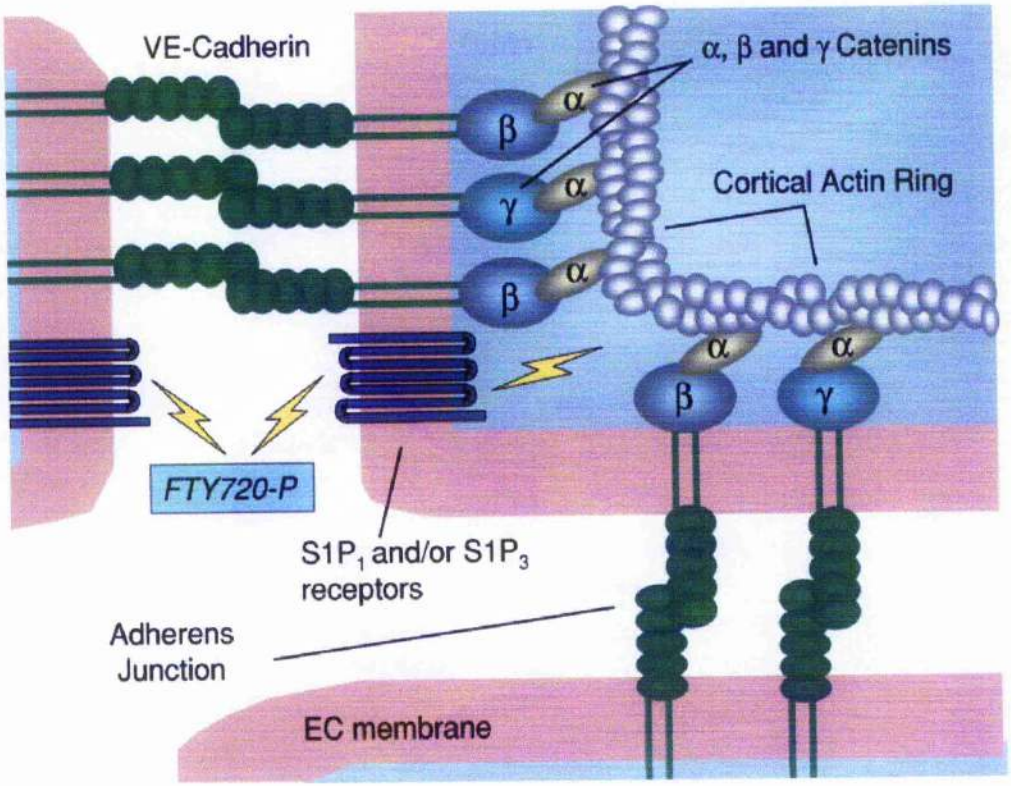

VE-Cadherin
α, β and γ Catenins
α
β
α
γ
α
β
Cortical Actin Ring
α
β
α
γ
FTY720-P
S1P1 and/or S1P3
receptors
Adherens
Junction
EC membrane

Figure 1.14: Model for receptor tyrosine kinase transactivation of S1P receptors.

The scheme depicts cross-communication between a tyrosine kinase growth factor receptor, PDGFR, and S1P receptors. Binding of PDGF to PDGFR results in activation and translocation of SK to the plasma membrane and generation of S1P is restricted.

A: S1P then activates S1P receptors leading to recruitment and/or activation of downstream signalling molecules, including Src, FAK, and Rac, which are important for cell migration.

B: Other downstream signalling, such as phospholipase C that regulates calcium levels is depicted. S1P can mobilize calcium from internal sources either *via* an unidentified inositol 1,4,5-trisphosphate (IP_3)-independent receptor on the ER or by activation of S1P receptors that stimulate phospholipase C. Stimulation of SK also results in decreased sphingosine levels that normally block the store-operated calcium release-activated calcium current leading to refilling of the stores. DAG = diacylglycerol. [Spiegel and Milstien, 2002].

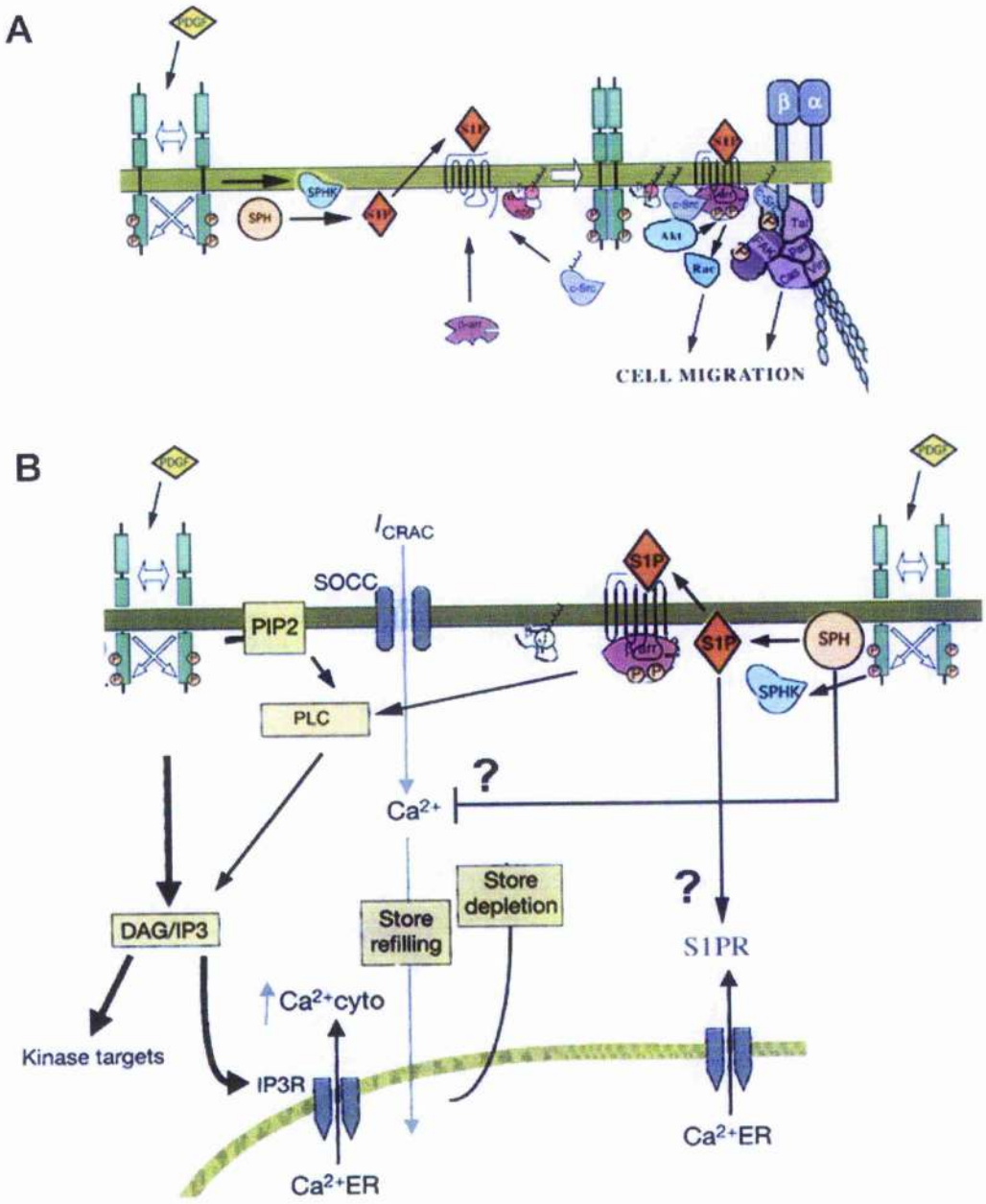

A
PDGF
SPH
SPHK
S1P
Akt
Rac
c-Src
CELL MIGRATION
B
PDGF
I_{CRAC}
SOCC
PIP2
S1P
SPH
SPHK
PLC
Ca^{2+}
?
DAG/IP3
Store refilling
Store depletion
S1PR
Ca^{2+}cyto
Kinase targets
IP3R
Ca^{2+}ER
Ca^{2+}ER

Figure 1.15: Cross talk of $S1P_1$ and PDGF and also $S1P_3$ and PDGF: Integrative and Sequential models

A schematic diagram of different types of interactions between S1P receptors and PDGFR. The left panel shows a "sequential" model which has been proposed for the $S1P_1$ receptor and PDGF [Hobson *et al.*, 2001; Rosenfeldt *et al.*, 2001]. The middle panel illustrates the proposed "integrative" model put forward, also $S1P_1$ and PDGF [Alderton *et al.*, 2001b; Waters *et al.*, 2002]. The right panel shows $S1P_3$-mediated Akt activation and crosstalk with PDGF which can be compared to the crosstalk of $S1P_1$ with PDGF.

Image taken from [Baudhuin *et al.*, 2004].

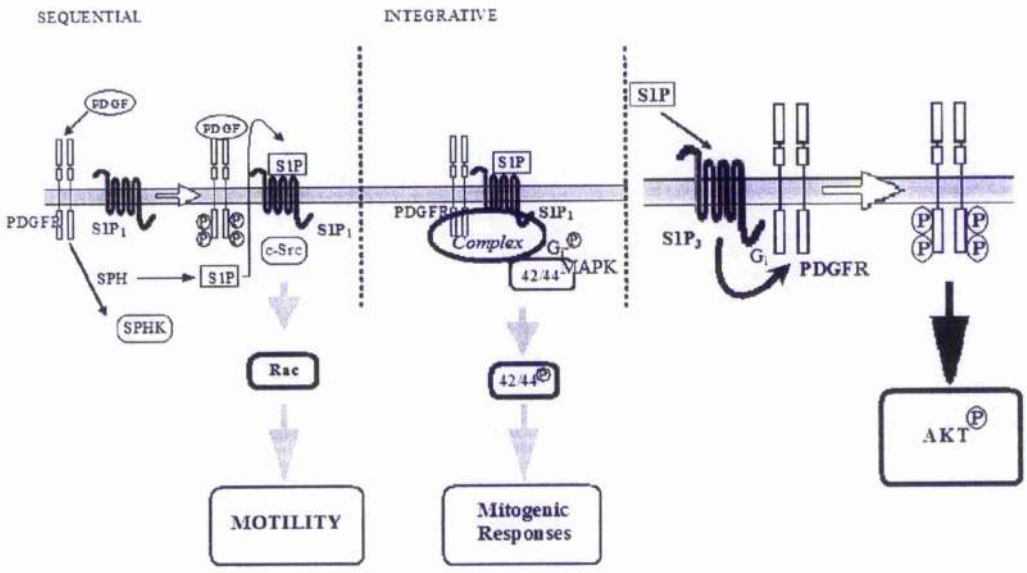

SEQUENTIAL
INTEGRATIVE
PDGF
PDGF
S1P
PDGFR
S1P1
S1P1
c-Src
S1P1
SPH
S1P
SPHK
Rac
MOTILITY
S1P
PDGFR
S1P1
Complex
Gi
MAPK
42/44
42/44
Mitogenic Responses
S1P
S1P3
Gi
PDGFR
AKT

Figure 1.16: Membrane domains classified by different lipid compositions

Membrane domains classified by different lipid compositions. Diagram of membrane depicting a caveolar and noncaveolar raft enriched in (glyco)sphingolipids and cholesterol, and flanked by semiordered lipid domains that border minimally ordered (nonraft) membrane areas. Adapted from [Hoekstra *et al.*, 2003].

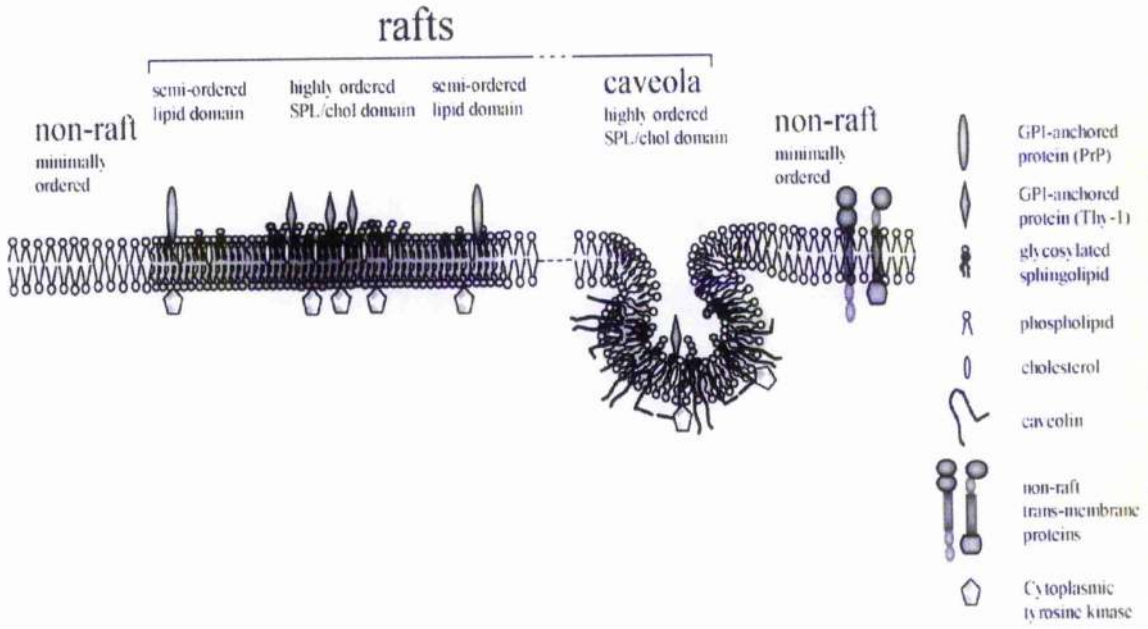

rafts
non-raft
minimally
ordered
semi-ordered
lipid domain
highly ordered
SPL/chol domain
semi-ordered
lipid domain
caveola
highly ordered
SPL/chol domain
non-raft
minimally
ordered
GPI-anchored
protein (PrP)
GPI-anchored
protein (Thy-1)
glycosylated
sphingolipid
phospholipid
cholesterol
caveolin
non-raft
trans-membrane
proteins
Cytoplasmic
tyrosine kinase

Figure 1.17: GPCR Receptor Signalling and the role of β-arrestins

The role of ß-arrestins in the desensitization, sequestration and intracellular trafficking of GPCRs.

(1) Homologous desensitization of GPCRs results from the binding of ß-arrestins (ß-arr) to agonist-occupied receptors following phosphorylation of the receptor by GRKs. ß-arrestin binding sterically precludes coupling between the receptor and heterotrimeric G proteins, leading to termination of signaling by G proteins effectors. Receptor-bound ß-arrestins also act as adapter proteins, binding to components of the clathrin endocytic machinery including clathrin, ß2-adaptin (AP-2).

(2) Receptor sequestration reflects the dynamin (Dyn)-dependent endocytosis of GPCRs via clathrin-coated pits. Once internalized, GPCRs exhibit two distinct patterns of ß-arrestin interaction. 'Class A' GPCRs, for example the ß2 adrenergic receptor, rapidly dissociate from ß-arrestin upon internalization. These receptors are trafficked to an acidified endosomal compartment, wherein the ligand is dissociated and the receptor dephosphorylated by a GPCR-specific protein phosphatase PP2A isoform, and subsequently,

(3) Recycling to the plasma membrane and down-regulation. 'Class B' receptors, for example the angiotensin II AT1a receptor, form stable receptor-ß-arrestin complexes. These receptors accumulate in endocytic vesicles and are either targeted for degradation or slowly recycled to the membrane via as yet poorly defined routes.

Adapted from [Lefkowitz, 1998].

Adapted from Biocarta.com image.

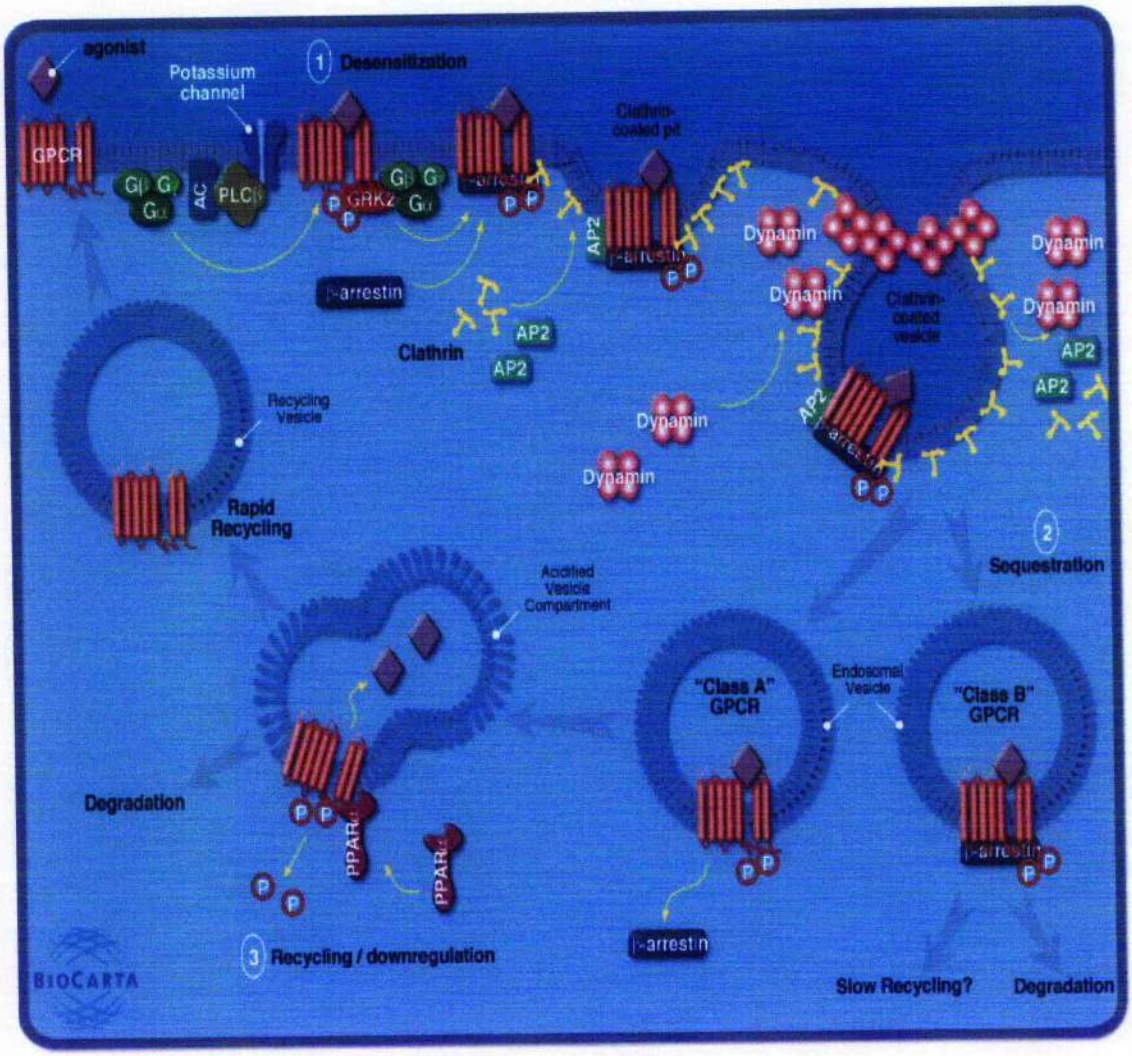

agonist
Potassium channel
1 Desensitization
GPCR
Gβ Gγ
Gα
AC
PLCβ
GRK2
P
β-arrestin
Clathrin-coated pit
AP2
Clathrin
Dynamin
Clathrin-coated vesicle
Recycling Vesicle
Rapid Recycling
2 Sequestration
Acidified Vesicle Compartment
Degradation
PPARγ
"Class A" GPCR
Endosomal Vesicle
"Class B" GPCR
3 Recycling / downregulation
Slow Recycling?
Degradation
BIOCARTA

Table 1.1: Nomenclature of S1P Receptors, Past and Present.

Internationally agreed nomenclature [Chun *et al.*, 2002].

EDG Nomenclature	S1P/LPA Nomenclature
EDG1	$S1P_1$
EDG2	LPA_1
EDG3	$S1P_3$
EDG4	LPA_2
EDG5	$S1P_2$
EDG6	$S1P_4$
EDG7	LPA_3
EDG8	$S1P_5$

Table 1.2: Characteristics of S1P receptors

S1P receptors and the G-proteins they couple with.

Receptor	Coupled G-proteins	Expression pattern
$S1P_1$/EDG-1	$G_{i/0}$	Widely expressed
$S1P_2$/EDG-5	$G_{i/0}G_{q/12/13}$	Widely expressed
$S1P_3$/EDG-3	$G_{i/0}G_{q/12/13}$	Widely expressed
$S1P_4$/EDG-6	$G_{i/0}$	Lymphoid tissues
$S1P_5$/EDG-8	$G_{i/0}G_{q/12}$	Brain, spleen

CHAPTER 2

Materials and Methods

2.1 Materials

All reagents used were of the highest grade commercially available and obtained from the following suppliers:

Amersham Pharmacia Biotech, Buckinghamshire, UK

Glutathione-Sepharose beads, Rainbow High Molecular Weight Range protein markers (14300-220000Da)

Alexis Corporation, San Diego, CA, USA

Dithiothreitol

Beckman Coulter, Buckinghamshire, UK

4ml Ultra-Clear tubes

BDH Chemicals Ltd., Poole, UK

Glass coverslips

Clontech, Palo Alto, CA, USA

Caveolin-1 mouse IgG1, 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal), Y187 library, pGBKT7 vector, pGAD GH vector, pGAD C1, AH109, Yeast Nitrogen-Base, Lysozyme

Calbiochem-Novabiochem (UK) Ltd., Nottingham, UK

Forskolin, S1P

Costar, Cambridge, MA, USA

75cm^2 tissue culture flasks, 60mm and 100mm tissue culture dishes, 6-, 12-, and 24-well tissue culture plates, cryovials

Fisher Scientific, Loughborough, Leicestershire, UK

HEPES, sodium dodecyl sulphate (SDS), EDTA, DMSO, (w/v) ethidium bromide solution, glacial acetic acid, methanol, ethanol, concentrated Hydrochloric acid, sodium fluoride, sodium phosphate

GIBCO BRL Life Technologies, Paisley, UK

Phenol:chloroform:isoamyl alcohol (w/v), LipofectAMINE, newborn calf serum, OptiMEM, phosphate-free Dulbecco's Modified Eagle's Medium (PF-DMEM)

Interactiva, Germany, Cruachem, Glasgow, UK

Oligonucleotides

Melford, Chelsworth, Ipsich, Suffolk, UK

Kanamycin

Merck, Darmstadt, Germany

Bactotryptone, agar

Molecular Probes Europe, AA Leiden, The Netherlands

Alexa^{TM} 594-conjugated goat anti-mouse IgG

New England Biolabs Inc., Beverley, MA, USA

1kb DNA Ladder, 100bp DNA ladder, restriction enzymes

NEN Life Science Products Inc., Boston

ECL reagents, ^{32}P-orthophosphate, autoradiography film

Novagen, CN Bioscience Inc., La Jolla, CA, USA

Gene Juice transfection reagent

Pierce Wariner, Rockford, IL 61105, USA

EZ-Link^{TM} Biotin-LC-hydrazide, HRP-streptavidin

Promega, Southampton, UK

T4 DNA Ligase, Promega^{TM} Wizard Plus SV miniprep kit, G-418 sulphate, restriction enzymes

Qiagen, Crawley, West Sussex

QIAquick Gel extraction kit, plasmid maxi kit

Roche Molecular Biochemicals/Boehringer-Mannheim, Mannheim, Germany

Tris, DNA molecular weight marker, restriction enzymes

Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA

Anti-GFP rabbit polyclonal IgG, Anti-IκBβ antibodies, anti-β–Arrestin-2, anti-β–Arrestin 1

Schleicher & Schuell, Inc., Keene, NH, USA

Nitrocellulose membrane

Sigma-Aldrich Comany Ltd., Poole, Dorset, UK

Triton X-100, soybean trypsin inhibitor, benzamidine, pepstatin A, IgG-free bovine serum albumin, fatty acid-free bovine serum albumin (BSA), protein A-Sepharose, protein G-Sepharose, sodium periodate, 30%/0.8% (w/v) acrylamide/bisacrylamide solution, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, rabbit anti-mouse IgG bridging antibody, thimerosal, bromophenol blue, bichinchonic acid, sodium azide, agarose, deoxycholic acid, ammonium hydroxide, isobutyric acid, ampicillin, paraformaldehyde, N,N,N',N'-tetramethylethylenediamine (TEMED), phenylmethylsulphonylfluoride (PMSF), 8-bromo-cGMP, Ham's F-12 medium, Dulbecco's Modified Eagle's Medium (DMEM), phosphate-buffered saline (PBS) (sterile), foetal bovine serum (FBS), trypsin, penicillin/streptomycin, L-glutamine, sucrose, zymolase, amino acids

Stratagene, N. Torrey Pines Rd. La Jolla, CA 92037-1073

Pfu Turbo DNA polymerase

Tocris/Semat Technical (UK) Ltd., St. Albans, Herts., UK

A23187

Upstate Ltd, Milton Keynes, UK

Anti-GRK2/3 monoclonal antibodies

Ascites containing 9E10 monoclonal antibody specific to the myc-epitope, Glu, Gln, Lys, Leu, Ile, Ser, Glu, Glu, Asp, Leu, was prepared in-house at Duke University, Durham, NC by Dr Tim Palmer.

Purified recombinant bovine GRK2 and GRK5 from Sf9 cells 1mmol phosphate/min/mg using light activated rhodopsin as a substrate, provided by Jeffrey L.Benovic, Kimmel Cancer Centre, Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia PA19107. pCMV5 expression constructs encoding bovine GRK2 and GRK5 were generated following subcloning of the open reading frames from pBC12BI/GRK2 and pBC12BI/GRK5 expression constructs donated by Prof. Benovic.

The human $S1P_3$ cDNA in pcDNA3 was the generous gift of Dr. Alan Wise (GlaxoSmithKline, Stevenage, U.K.).

Sheep α GFP antibody was the generous gift of Francis Barr.

Bacterial expression constructs encoding glutathione-*S*-transferase-Src homology 3 (SH3) domains [Beard et al., 1999] were the generous gift of Professor Miles Houslay (University of Glasgow, U.K.).

pGADT7, pGM20, pGM22 and pGM47 were the generous gift of Gerhard May ((University of Glasgow, U.K.).

2.2 Cell Culture and Transfections

2.2.1 Cell Maintenance

CCL39, HEK293 and HEKLT cells were maintained in DMEM, supplemented with 10%(v/v) FBS, penicillin (100 units/ml), streptomycin (100µg/ml) and 1mM L-glutamine in a 37°C humidified atmosphere containing 5% CO_2. Cells stably expressing $S1P_1$ or $S1P_3$ receptors were maintained in the appropriate medium supplemented with G418 in order to optimise receptor expression by maintaining selection pressure. Cells were routinely passaged 1:10. Confluent T-75 flasks were washed with PBS without calcium chloride and magnesium chloride. Cells were detached by the addition of 2ml of trypsin, followed by incubation at 37°C for 30 seconds. 8mls of medium were then added to the flasks and the cells pippetted gently to allow resuspension. Cells were either passaged into flasks to maintain the cell line or seeded for experimental analysis.

2.2.2 Transient Expression of cDNA Expression Constructs

cDNA expression constructs were transiently transfected into either HEK293, HEKLT or CCL39 cells using a Lipofectamine-mediated transfection protocol. Cells were plated into 6-well dishes at the appropriate density such that they would be 70-80% confluent the following day. Transfection mixes of 0.24ml OptiMEM, 2µg plasmid DNA and 4µl Lipofectamine were prepared in sterile microfuge tubes for each well. The tubes were then incubated at room temperature for 15-45min to allow transfection. During this incubation, each cell monolayer was washed once with 2ml/well OptiMEM prior to the addition of 0.75ml/well of OptiMEM. Following the 15-45 minute incubation, the Lipofectamine-DNA-OptiMEM mixes were added dropwise to each well and incubated for 3 hours at 37°C. The medium was then removed from each well and replaced with 3ml/well of normal growth medium. Cells were analysed 48-72 hours post-transfection.

2.2.3 Stable Expression of cDNA Expression Constructs

On day one confluent CCL39s cells in a T75 flask were split with 2ml trypsin. When detached, 6ml DMEM minus G418 was added and resuspended by pipetting. 1ml of suspension was added per 10mm dish which already contained 9ml DMEM minus G418. The following day cells were 70-80% confluent. In a sterile microfuge tube, 30µl Lipofectamine and 10µg of either pcDNA3/myc$S1P_3$ or pcDNA3/myc$S1P_3$GFP were added to 0.4ml of OptiMEM, mixed and incubated for 15-45min and then added to each dish containing 5.2ml of OptiMEM, to give a final volume of 6ml. Dishes were incubated at 37°C for 3 hours. After incubation the

Lipofectamine-DNA medium was removed and replaced with 10ml of DMEM with G418. On day 4, the cells were split for selection with 0.4mg/ml G418 into 5 dishes representing a 2:5, 1:5, 1:10, 1:20 and 1:40 cell suspension. After selection in G418, resistant colonies were isolated, expanded and screened for receptor expression by immunoblotting and, in the case of the GFP constructs, fluorescence microscopy following excitation at 488nm using an argon/krypton laser.

2.3 Molecular Biology

2.3.1 Preparation of Antibiotic Agar Plates

LB agar (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, with 1.5% (w/v) agar was prepared, autoclaved and allowed to cool before the addition of the appropriate antibiotic (either ampicillin at 50μg/ml or kanamycin at 30μg/ml final concentrations). The liquid LB was poured into 90mm Petri dishes and allowed to solidify and sweat overnight at room temperature. Plates were then stored at 4°C until required.

2.3.2 Preparation of competent XL1 Blue *E.coli*

An overnight culture of XL1 Blue *E.coli* was grown in 3ml of LB broth containing 50μg/ml tetracycline. The next day, 250ml of LB broth was inoculated with the culture and grown with aeration until the cells reached log phase. The cells were then transferred into two 250ml centrifuge tubes on ice and left for 1 hour. Log phase was defined when the optical density (OD_{600}) reached approximately 0.35-0.375. Cells were centrifuged at 4000g at 4°C for 20 minutes, the supernatant decanted and the cells resuspended and washed in ¼ starting volume of ice-cold 0.1M magnesium chloride. Following a second 20 min centrifugation, the cells were resuspended in ¼ starting volume of ice-cold 15% (v/v) glycerol with 0.1M calcium chloride. 250ml of resuspended cells were each aliquoted into sterile microfuge tubes on dry ice/methanol, to induce rapid freezing, and stored at –80°C until required.

2.3.3 Transformation of Competent *E.coli*

Approximately 30-50ng of DNA was added to a thin-walled, plastic round-bottomed 13ml Falcon tube on ice. 50μl of thawed competent *E.coli* were added and the DNA and *E.coli* mix incubated on ice for 10min. The tubes were then incubated for 5min in a 37°C water bath. 0.5ml LB/tube was then added and the tubes were incubated for 45min at 37°C. 200μl from each transformation was then plated onto an LB agar plate supplemented with the appropriate selection antibiotic and incubated overnight at 37°C.

2.3.4 Mini- and Maxi-Preparation of Plasmid DNA

Transformed colonies were picked from agar plates and used to inoculate 5-10ml LB containing appropriate selection antibiotic, and grown overnight at 37°C with shaking at

~200rpm for aeration. Plasmid DNA was prepared using the Promega™ Wizard Plus SV miniprep purification system as per the manufacturer's instructions. Larger quantities of DNA were obtained by transferring an initial overnight culture into 500ml of LB broth containing the appropriate antibiotic and grown overnight. DNA purification was achieved obtained using the Qiagen Plasmid Maxi-kit system. The concentration of DNA obtained was determined by measuring the absorbance at 260nm (A_{260}) of a 1:50 dilution of each preparation in sterile H_2O, assuming that 1 absorbance unit was equivalent to 50μg/ml of double stranded DNA. DNA purity was assessed by measurement of A_{260}/A_{280} ratios.

2.3.5 Digestion of Plasmid DNA

1-2μg of plasmid DNA was digested in a volume of 10-20ml using the buffer conditions recommended by the manufacturer with 2-4 units of the appropriate restriction enzyme. Digested DNA was analysed by agarose gel electrophoresis in which samples were prepared by the addition of a 1:3 dilution of loading dye. Electrophoresis was applied to a 1% (w/v) gel containing 2.5μg/ml ethidium bromide at 75V for 20-30min in a TAE buffer (40mM Tris-acetate, 1mM EDTA, glacial acetic acid). DNA purification from excised agarose gel chips was achieved using Quiagen QIA quick gel purification kit, as per manufacturer's instructions.

2.3.6 Ligation of DNA fragments

Ligation was carried out overnight at 4°C in a reaction volume of 10μl containing 30mM Tris-HCl, pH7.8, 10mM magnesium chloride, 10mM DDT, 1mM ATP, with 1μl T4 DNA ligase. Vector and insert DNA were present at a ratio of 1:4. Ligated DNA was then transformed into competent *E.coli* as described in section 2.3.3.

2.3.7 Construction of $S1P_3$ Receptor constructs

(i) Generation of Human Myc$S1P_3$ cDNA Expression Construct:-

Myc epitope (EQKLISEEDL) and His_6 sequences were added to the C-terminus of the human $S1P_3$ open reading frame by PCR using a pcDNA3/$S1P_3$ cDNA as the template. Standard PCR reactions contained in a volume of 100μl, 100ng template DNA, 100mM dNTPs, 50pmol sense/antisense primers, 0.002 units Pfu turbo, 10μl 10-fold concentrated amplification buffer and 5% (v/v) DMSO. The reaction was initiated by a denaturation cycle of 95°C for 5 min followed by 30-40 cycles of a 95°C (1min) denaturing step, 55°C (1min) annealing step and 72°C (1.5min) extension step. A final cycle of 95°C (1min), 55°C (1min) and 72°C (10min) was added before reactions were placed at 4°C until required. The primers used were:-

5' –CATTG**AAGCTT**CCACC*ATG*GCAACTGCCCTC-3' (sense) and

5' –CATTGT**CTAGA**GTTGCAGAAGATCCCATTCTG-3' (antisense)

The sense primer was designed to add a *Hind*III site (bold) upstream of a consensus Kozak sequence (underlined) and the $S1P_3$ initiating Met (italics). The antisense primer was designed to remove the $S1P_3$ stop codon and add a *Xba*I site (bold). This was to allow in-frame fusion of the $S1P_3$ open reading frame with the myc epitope and His6 sequences following subcloning of the *Hind*III/*Xba*I-digested PCR product into a similarly digested pcDNA3mycHis-A expression vector (Invitrogen). The completed digests were resolved on 1% (w/v) agarose gels at 75V in TAE buffer.

(ii) Generation of Myc$S1P_3$ Δ27 and Δ45 cDNA expression constructs:-

An analogous PCR strategy was used to generate epitope-tagged $S1P_3$ truncation mutants in which either 27 or 45 amino acids had been deleted from the carboxyl-terminus. The integrity of all open reading frames was confirmed by the use of DNA sequencing.

27 truncation:

5'AAA CCT **TCT AGA** GAC CTT CGG AGA GTG GCT GCT ATT GTT 3'(antisense)

45 truncation:

5'AAA CCT **TCT AGA** GTC GAG CGC AGG CTG GAT GGG 3'(antisense)

(iii) Generation of Myc$S1P_3$-GFP cDNA expression constructs:-

These were generated by PCR using the pcDNA3/myc-His-$S1P_3$ receptor as a template for the pcDNA3/myc-His-$S1P_3$-GFP construct. The primers used are shown below:-

d'TCTGGCTAACTAGAGAACC (sense)

dCAT TG**G GAT CCC** GAT GGT GAT GGT GAT GAT G (antisense)

The sense primer was designed to anneal upstream of the *Hind*III site of the $S1P_3$ receptor. The antisense primer was designed to remove the stop codon in the myc-His tag of the $S1P_3$ and replace with that of GFP following ligation of *Hind*III/*BamH*I-digested PCR product with a similarly digested pEGFPAla1 vector.

iv) *Generation of $S1P_3$ C-terminal cDNA expression constructs* :-

This construct contains the RASPIQP SH3 domain binding motif of the human $S1P_3$ receptor as a 76 amino acid peptide from Lys^{303} to Asn^{378}, (Figure 1.10), and was created for insertion into $pGBKT_7$, to be used as bait in the yeast two hybrid system. Standard PCR reactions contained in a volume of 100µl, 100ng template DNA, 100mM dNTPs, 50pmol sense/antisense primers, 0.002 units Pfu turbo, 10µl 10-fold concentrated amplification buffer and 5% (v/v) DMSO. The reaction was initiated by a denaturation cycle of 95°C for 5 min followed by 30-40 cycles of a 95°C (1min) denaturing step, 75°C (1min) annealing step and 72°C (1.5min)

extension step. A final cycle of 95°C (1min), 75°C (1min) and 72°C (10min) was added before reactions were placed at 4°C until required.

The primers for these constructs are as follows:-

5'- AAA GCT **GAA TTC** CGG CGG GCC TTC TTC CGT CTG GT-3' (sense)

5'- AAA GCT **CTG CAG** GTT GCA GAA GAT CCC ATT CTG-3' (antisense)

$S1P_3$ C-terminal constructs were created following ligation of *PstI*/*EcoR*I-digested PCR product with a similarly digested $pGBKT_7$ vector. Subcloning was applied in frame with a myc epitope in the vector.

v) *Generation of $S1P_3$ C-terminal cDNA binding partner constructs* :-

These constructs amplify the Matchmaker sequence inserts in the positive activation domain (AD)/library clones to verify the presence of an open reading frame (ORF) fused to the GAL4 AD sequence, and enable comparison of the novel sequences to those in GenBank, EMBL, or other databases. Standard PCR reactions contained in a volume of 100µl, 100ng template DNA, 100mM dNTPs, 50pmol sense/antisense primers, 0.002 units Pfu turbo, 10µl 10-fold concentrated amplification buffer and 5% (v/v) DMSO. The reaction was initiated by a denaturation cycle of 95°C for 5 min followed by 30-40 cycles of a 95°C (1min) denaturing step, 45°C (1min) annealing step and 72°C (1.5min) extension step. A final cycle of 95°C (1min), 45°C (1min) and 72°C (10min) was added before reactions were placed at 4°C until required.

5' – GAT GAA GAT ACC CCA CCA- 3'

5' – GCG GGG TTT TTC AGT ATC- 3'

2.4 Experimental Techniques

2.4.1 Preparation of S1P

1mg of commercially supplied S1P was resuspended in 0.66ml of methanol to give a final S1P concentration of 4mM. The tube was capped tightly and transferred to an 80°C oven. The S1P was then vortexed every 5min until it had gone visibly into solution. The S1P solution was then pipetted into 25µl aliquots in brown glass vials. The methanol solvent was then evaporated off using a nitrogen gas stream, and the tubes capped for storage at –80°C. Reconstitution of S1P was achieved by the addition of 0.25ml serum-free medium supplemented with 0.5mg/ml fatty acid-free BSA to give a working S1P stock concentration of 400µM. Following addition of "medium + BSA", the vial was warmed to 37°C for 30min with occasional vortexing to resuspend the S1P prior to use.

2.4.2 Preparation of Cell Extracts for Immunoblotting

Confluent monolayers in 6-well dishes were kept on ice and washed three times with ice-cold PBS. All procedures were carried out at 4°C unless indicated otherwise. Cells were solubilised by scraping into 250µl of immunoprecipitation buffer (50mM Hepes, pH 7.5, 5mM EDTA, 10mM sodium fluoride, 10mM sodium phosphate, 0.1mM phenylmethylsulfonyl fluoride, and 10µg/ml each of soybean trypsin inhibitor, leupeptin, and pepstatin A in 1% (w/v) TX100, 0.5% (w/v) deoxycholate (DOC), 0.1% (w/v) SDS. The lysate was then transferred into ice-cold microfuge tubes and solubilised by a 1 hour incubation on a rotating wheel. Lysates were clarified by centrifugation (14000g for 15 minutes) and supernatants assayed for protein content.

2.4.3 BCA Assay for protein content

Bovine serum albumin (BSA) physiological standards ranging from 0-2mg/ml were loaded as a final volume of 10µl into the wells of a 96 well plate to obtain a best-fit straight line of a plot of A_{492} versus protein concentration in a bicinchonic acid (BCA)-based protein assay (Reagent A: 5g 1% (w/v) 4,4 dicarboxy-2,2 biquinoline, disodium salt, 2% (w/v) sodium carbonate, 0.16% (w/v) sodium potassium tartrate, 0.4% (w/v) sodium hydroxide, 0.95% (w/v) sodium bicarbonate, Reagent B: 4% (w/v) copper sulphate, BCA Assay solution 200µl Reagent B to 9.8ml Reagent A). Analysis was performed using the graph package "Prism v20". The protein concentrations of 10µl samples of each sample were calculated from the equation of the best-fit straight line obtained from the BSA standards.

2.4.4 SDS-PAGE and Immunoblotting

In conjunction with prestained protein markers (6.5-175 kDa), samples were solubilised in immunoprecipitation buffer and equalised for protein content. The appropriate quantity of Laemmli sample buffer (50mM Tris (pH 6.7), 10% (v/v) glycerol, 12% (w/v) SDS, 0.0001% (w/v) bromophenol blue, 1.6mg/ml dithiothreitol) was then added to each sample to give a total volume of 30µl per sample. Samples were fractionated by SDS-PAGE using a 10cm 10% (w/v) acrylamide resolving gel (10% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 0.4M Tris (pH 8.8), 0.1%(w/v) SDS, 3% (v/v) glycerol, 0.01% (w/v) ammonium persulphate and 0.001% (v/v) TEMED) and 3% acrylamide stacking gel (3% (v/v) acrylamide, 0.1% (v/v) bisacrylamide, 0.1M Tris (pH 6.8), 0.1% (w/v) SDS, 0.01% (w/v) ammonium persulphate and 0.001% (v/v) TEMED). Electrophoresis was carried out at 150V in a running buffer containing 27.4mM Tris, 0.19M glycine and 0.1% (w/v) SDS until the bromophenol blue dye front reached the bottom of the gel. The resolved proteins were then electrophoretically transferred to nitrocellulose at 400mA for

45min in transfer buffer containing 24.7mM Tris, 0.19M glycine and 20% (v/v) methanol. Following transfer, the nitrocellulose was washed briefly in PBS and incubated for 1 hour in Blotto (5% (w/v) skimmed milk in PBS supplemented with 0.2% (v/v) Triton X-100) to block non-specific protein binding sites. Membranes were then incubated for 1 hour with the appropriate dilution of primary antibody in fresh Blotto as indicated in the appropriate figure legends. The membranes were then washed three times for 10mins with Blotto followed by two brief washes in PBS. Membranes were then incubated for 1 hour with HRP-conjugated secondary antibody in High-Detergent Blotto (10% (v/v) Blotto in PBS supplemented with 1.02% (v/v) Triton X-100 and 0.1% (w/v) SDS). The membranes were then washed three times for 10min in Blotto, followed by two washes with PBS for 10min. Membranes were then incubated for 1min at room temperature with ECL reagents (1ml solution A, 1ml solution B). Immunostained protein was then visualised on autoradiography film.

2.4.5 Electrophoretic mobility shift assay (EMSA) for NF-kB activation

Nuclear extracts were made from cells treated with vehicle or S1P by lysing cells in 0.4 ml Buffer A (10 mM Hepes, pH 7.9, 10 mM potassium chloride, 0.1 mM EDTA, 0.1 mM EGTA, 1mM dithiothreitol, 0.625% (v/v) NP40, 0.5 mM PMSF and 10μg/ml each of soybean trypsin inhibitor and benzamidine). The samples were then spun at 48,000g for 30 seconds at 4°C, and the supernatant removed. The pellet was resuspended in 50 μl of buffer B (20 mM Hepes, pH 7.9, 0.45 M sodium chloride, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol, 0.5 mM PMSF and 10μg/ml each of soybean trypsin inhibitor and benzamidine). Samples were agitated for 15 min at 4°C, centrifuged at 48,000g for 5min. and the protein content of the supernatant determined using a Bradford assay. The DNA probe was labelled as follows: 10 pmol of single-stranded oligonucleotide was labelled in a final reaction volume of 20 μl comprising 5 μCi [γ-^{32}P]ATP, 2 μl polynucleotide kinase (Promega) and 2 μl 10x-concentrated polynucleotide kinase reaction buffer. After incubation for 30 min at 37°C, 3.2 μl (equivalent to 16 pmol) of the complimentary strand and 0.5 μl 5 M sodium chloride were added and the samples incubated at 100°C for 2 min before cooling slowly to room temperature over the course of 2-3 hr. Following brief microcentrifugation 5 μg aliquots from each nuclear extract were then added to the ^{32}P-labelled double stranded DNA probe (10,000 cpm/sample) containing the consensus κB binding sequence GGGGACTTTCCC to give a final reaction volume of 25 μl containing 10 mM Hepes, pH 7.9, 0.1 mM magnesium chloride, 0.1 mM EDTA, 0.5 mM dithiothritol, 10% (v/v) glycerol, 50 mM sodium chloride and 0.625 μg/ml poly dIdC.

Following a 30 min incubation at room temperature, samples were analysed by fractionation on a non denaturing 6% (w/v) polyacrylamide gel containing 0.5x TBE buffer (45 mM Tris-borate, 1 mM EDTA) followed by autoradiography.

2.4.6 Whole Cell Receptor Phosphorylation

Cells were plated into 6-well dishes at a density of $1x10^6$ cells/well and cultured overnight. When confluent, cells were quiesced by a 16-24hr incubation in serum-free DMEM. The next day, the cells were washed twice with 3ml/well phosphate-free DMEM and incubated for 90mins at 37°C with 0.75ml of the same medium supplemented with 0.2μCi/well [^{32}P] orthophosphate. After stimulation with the indicated agonists, added as a 2X concentrated dose in 0.75ml, reactions were terminated by placing the cells on ice and washing the monolayers twice with 3ml of ice-cold PBS. All subsequent procedures were carried out at 4°C unless indicated otherwise. Cells were washed three times with PBS and solubilized by scraping into an initial 250μl of immunoprecipitation buffer. The lysate was then transferred into ice-cold microfuge tubes and the wells washed with another 250μl of immunoprecipitation buffer which was subsequently transferred to the appropriate microfuge tube. The cells were then solubilized and assayed for protein content as described in section 2.4. Equivalent amounts of soluble protein from each sample were then made up to 400μl with immunoprecipitation buffer and added to microfuge tubes containing 100μl 0.2% (w/v) IgG-free bovine serum albumin and, in the case of myc-tagged constructs, 5μl 9E10 ascites, 2μl of a rabbit antimouse IgG bridging antibody and 20μl of a 50% (v/v) suspension of protein A-Sepharose beads.

Following incubation on a rotating wheel for 1 hour, immune complexes were isolated by brief centrifugation, washed three times with 1ml immunoprecipitation buffer and eluted from the beads by the addition of 30μl Laemmli electrophoresis sample buffer (Section 2.4.4) and incubation at 37°C for 1 hour, vortexing every 15 minutes. Analysis was by SDS-PAGE using 10% (w/v) polyacrylamide resolving gels as described in Section 2.4.4 and the gels were dried under vacuum prior to autoradiography for between 16 and 40 hours at –80°C. Quantitation of phosphorylation experiments was by densitometric analysis of non-saturating autoradiographs using Totallab v2.0image quantitation software (Nonlinear Dynamics).

2.4.7 *In Vitro* Receptor Phosphorylation Assay with Purified GRKs

Confluent monolayers of transfected cells in 100mm dishes were washed with ice-cold PBS and scraped into 5ml/dish of lysis buffer (10mM HEPES, pH 7.5, 2mM EDTA, 0.25M sodium chloride supplemented with 0.1mM PMSF, 10μg/ml soybean trypsin inhibitor and 10μg/ml benzamidine). The cells were then transferred to a tight-fitting glass-on-glass Dounce

homogeniser on ice and homogenised by 20 up-and-down strokes. Following a 15min incubation on ice, the membranes were pelleted by centrifugation at 14,000g for 15min. The supernatant was then discarded and the pellet resuspended in 5ml lysis buffer as before. The membranes were then re-homogenised, left on ice and centrifuged as described previously. The pellet was then resuspended in 5ml GRK assay buffer (25mM Hepes, pH 7.5, 2.5mM EDTA and 7.5mM magnesium chloride supplemented with the above protease inhibitors) by homogenisation and re-centrifuged. The pellet was finally resuspended in 220µl GRK assay buffer. Each assay tube consisted of 40µl membrane suspension, 40µl kinase mix (GRK assay buffer supplemented with 0.25mM ATP, 0.88mM dithiothreitol, 0.15µM okadaic acid (a phosphatase inhibitor) and 10 µCi [γ-^{32}P]ATP), 10µl vehicle or 50nM purified GRK, and 10µl of vehicle or S1P, at the concentration indicated in the figure legends. After incubation at 30°C for 5 min, reactions were terminated by placing the tubes on ice and adding 0.5ml/tube stop solution (0.1M sodium phosphate, pH 7.5, 10mM EDTA). Membranes were pelleted by microcentrifugation (14,000g, 10min) and the resulting pellets solubilised in 0.3ml immunoprecipitation buffer by rotation for 60min at 4°C. After removal of insoluble material by centrifugation, detergent extracts were equalised by protein assay prior to receptor immunoprecipitation with 9E10 as described in Section 2.4.6. For Rhodopsin phosphorylation experiments, urea-treated bovine rod outer segments (ROS) were employed. In this case, each assay consisted of GRK assay buffer containing 0.5µl ROS and 2µCi [γ-^{32}P] ATP. In these experiments, reactions were terminated by the addition of 15µl Laemmli sample buffer prior to analysis of rhodopsin phosphorylation by SDS-PAGE and autoradiography.

2.4.8 Biotin Labelling-Immunoprecipitation Assay Of Cell Surface Receptor Expression

Cells were plated into 6-well dishes at a density of 1 x 10^6 cells/well and cultured overnight. Cell lines expressing $S1P_3$ constructs were then serum-starved for 16-20 hours in serum-free DMEM. The next day, the cells were washed in the appropriate medium followed by the addition of 0.75ml/well of the same medium. Incubations were initiated by the addition of 0.75ml medium supplemented with either vehicle or 2X concentrated dilution of drug as indicated in the Figure Legends. Reactions were terminated by placing the cells on ice, removing the medium and washing monolayers twice with ice-cold PBS supplemented with 0.1mM calcium chloride and 1mM magnesium chloride (PBS-CM). All subsequent procedures were performed at 4°C unless stated otherwise. The alcohol groups on the cell surface glycoproteins were oxidised to aldehydes by a 30min incubation with 0.75ml/well 10mM sodium periodate in PBS-CM. Following the removal of the periodate and washing with PBS, the

monolayers were washed twice with 3ml 0.1M sodium acetate, pH 5.5, and incubated for 30min in 0.75ml/well of the same buffer supplemented with 1mM biotin-LC-hydrazide. This reacts with the newly formed alcohol groups thereby labelling all cell-surface glycoproteins with biotin. Labelling was terminated by removal of the biotin-LC-hydrazide solution was washing the monolayers three times with 3ml PBS. Cells were then solubilised for receptor immunoprecipitation as described for whole cell phosphorylation assay (Section 2.4.4). Following fractionation of immunoprecipitated receptors by SDS-PAGE, proteins were transferred to a nitrocellulose membrane. Non-specific protein binding sites were blocked by incubation in Blotto and cell-surface biotin-labelled receptors were then identified by incubation of the membrane with 1mg/ml HRP-conjugated streptavidin for 60min at room temperature. Following three 10min washes with Blotto and two washes with PBS, reactive proteins were visualised by enhanced chemiluminescence (ECL). Agonist-induced loss of cell-surface receptor was quantitated by densitometric scanning of non-saturating blots.

2.4.9 Identification of Membrane Localisation of mycS1P$_3$ Receptors using Sucrose-Density Gradient

Confluent cells from two 100mm dishes were used for each treatment. Dishes were treated with either vehicle or 10μM S1P in DMEM for 30mins followed by 2x washes in PBS. The cells were scraped twice in 3ml PBS and diluted to 10ml (in 50ml conical flasks) followed by centrifugation at 1800g for 10min. The resulting supernatant was removed and with a blunted pipette tip the pellet was loaded in 0.8ml of 1% or 0.5% (v/v) TX100 in MES Buffered Saline (MBS) containing 25mM 2-Morpholinoethanesulfonic acid (MES), 150mM NaCl, pH 6.5). For 0.5% (v/v) TX100 in MBS, a 2.5ml stock of 20% (v/v) TX100 in 100ml MBS was ued. The samples were put into a 7ml Dounce homogeniser containing 10μg/ml of soy bean inhibitor and 10μg/ml benzamidine inhibitor and homogenised with 20 up-and-down strokes to ensure complete resuspension. Following homogenisation and centrifugation each pellet was placed on a rotating wheel at 4°C for 20mins. All the remaining steps were completed at 4°C to preserve lipid raft architecture. After rotation, samples were rehomogenised and the gradient was constructed as follows: 0.8ml of sample was combined with 0.8ml of 80% (w/v) sucrose in a centrifuge tube. This was followed by the careful addition of 1.6ml of 30% (w/v) sucrose, and lastly a layer of 1.6ml of 5% (w/v) sucrose was added. Samples were centrifuged at 200000g for approximately 18hrs. Samples were removed the following day as 400μl aliquots (this provides 11 samples and a pellet) and transferred to microfuge tubes containing 10μg/ml of soy bean and 10μg/ml of benzamidine inhibitor. (In some cases the pellet needed a further 400μl of MBS to

resuspend it, which was also dounce homogenised as required). A distinct opaque band was seen between the 5% (w/v) and 30% (w/v) sucrose, which represented lipid rafts. Special care was employed when taking these aliquots to ensure the gradient was not disturbed. Samples with a 1:4 ratio of sample:sample buffer were resolved on a 12.5% (w/v) acrylamide SDS-PAGE gel. Gels were transferred to nitrocellulose, which was cut at the 30kDa marker to make independent blots of receptor and caveolin-1 (22kDa).

2.4.10 Co-Immunoprecipitations of mycS1P$_3$ and β Arrestin-2

CCl-39/mycS1P$_3$ cells were transfected with β arrestin-2 and following serum starvation for 24hrs, treated with 10μM S1P for 2 hours. Reactions were terminated by placing the cells on ice and washing the monolayers twice with 3ml of ice-cold PBS. All subsequent procedures were carried out at 4°C unless indicated otherwise. Cells were solubilized by scraping into an initial 250μl of immunoprecipitation buffer (Section 2.4.2). The lysate was then transferred into ice-cold microfuge tubes. The wells were then washed in another 250μl of immunoprecipitation buffer which was subsequently transferred to the appropriate microfuge tube. The cells were then solubilized and analysed for protein content as described in Section 2.4.3 Equivalent amounts of soluble protein from each sample were then made up to 400μl with immunoprecipitation buffer and added to microfuge tubes containing 100μl 0.2% (w/v) IgG-free bovine serum albumin containing, 5μl anti-Arrestin-3 Ab, and 20μl of a 50% (v/v) suspension of protein G-Sepharose beads for 1 hour.

Following incubation on a rotating wheel for 1 hour, immune complexes were isolated by brief centrifugation, washed three times with 1ml immunoprecipitation buffer and eluted from the beads by the addition of 30μl Laemmli electrophoresis sample buffer and incubation at 37°C for 1 hour, vortexing every 15 minutes. Analysis was by SDS-PAGE using 10% (w/v) polyacrylamide resolving gels and then transferred to nitrocellulose membrane. Membranes were then incubated with ascites 9E10 monoclonal (primary antibody) followed by HRP-conjugated anti-mouse (secondary antibody).

2.4.11 Membrane Recruitment of β-Arrestin

Stably transfected S1P$_3$GFP CCL39 cells were used, with the contents of 1X10cm dish per condition. For each condition (time course of 0, 1, 2, 5, 8, 12 minutes) cells were treated with or without 20μM S1P. Cells were washed twice with 5mls of ice cold KHEM (Incomplete KHEM buffer: 50mM Potassium Chloride, 50mM Hepes-Potassium Hydroxide pH 7.2, 10mM EGTA, 1.92mM Magnesium Chloride. For complete KHEM buffer add 1mM DTT, and 0.1mM PMSF, 10μg/ml benzamidine, 10μg/ml soy bean trypsin). 0.5ml of complete KHEM was added

to each 10cm dish and cells were scraped and removed to a microfuge tube prior to snap freezing in liquid nitrogen or dry ice. Once thawed on ice, the cells were homogenised by ten passes through a 0.2mm gauge needle. The P1 fraction was centrifuged at 1300g for 3mins and the supernatant removed to an ultracentrifuge tube. The P2 fraction was centrifuged at 100000g for 30mins. The supernatant was removed and the S fraction withheld. The pellet was washed once in 750µl KHEM, resuspended and centrifuged at 100000g again. Finally, the pellet was resuspended in 100-200µl complete KHEM. The protein concentrations were determined by BCA assay (Section 2.4.3) and equal amounts of protein were aliquoted and sample buffer added. All samples were fractionated on a 10% SDS PAGE gel and then blotted to nitrocellulose membrane. Membranes were incubated in β-Arrestin-1 (primary antibody), followed by HRP-conjugated anti-mouse (secondary antibody) as described in Section 2.4.4. Immunostained proteins were then visualised on photographic film.

2.5 Confocal Laser-Scanning Microscopy

For fixed cell analysis of $S1P_3$-GFP-receptors, cells were grown on coverslips and treated with vehicle or agonist. Following two washes with 3ml/coverslip of PBS, the cells were fixed for 20min at room temperature in 1.5ml/coverslip of 4% (w/v) paraformaldehyde in 5% (w/v) sucrose/PBS (pH7.2). Cells were then washed twice with PBS and washed 3x 2ml/coverslip for 5min in 0.4% (v/v) new born calf serum (NBCS)/0.2% (w/v) gelatin/PBS and once with 2ml/coverslip PBS prior to mounting on microscope slides with 7µl/coverslip of 40% (v/v) glycerol in PBS.

Cells were visualised using a Zeiss Axiovert 100 laser scanning confocal microscope (Zeiss, Oberkochen, Germany) using a Zeiss Plan-Apo 63 x 1.4 NA oil immersion objective, pinhole of 20 and electronic zoom between one and four. GFP was excited using a 488nm argon/krypton laser and detected with 515-540nm band pass filter. The images were manipulated with Zeiss LSM or MetaMorph software (Universal Imaging Corporation, West Chester, PA).

2.6 Preparation of Yeast Two Hybrid System Media

A variety of selective media were employed throughout the Yeast Two Hybrid Screening Protocol. These included:-

Synthetic dextrose (SD) dropout media. Added to 1L of distilled water: 1.7g yeast nitrogen base w/o amino acids, 5g ammonium sulphate, 1.3g amino acid dropout powder, 20g dextrose, (20g agar), pH 5.8 autoclaved (allowed to cool to 50°C before pouring plates).

YNB –his-leu-trp-ade media. A selective minimal medium with quadruple knockout (QDO) of amino acids and the purine adenine. Added to 1L of distilled water: 1.7g yeast nitrogen base w/o amino acids, 5g ammonium sulphate, 0.6g Histidine-Adenine-Tryptophan-Leucine dropout powder, 20g dextrose, (20g agar), pH 5.8 autoclaved (allowed to cool to 50°C before pouring plates). Media lacking all or multiple amino-acids were made, simply adding the required amino acid(s) to make triple or double knock out media, using 0.04 mg/ml Tryptophan, 0.06mg/ml Leucine, 0.02mg/ml Adenine and 0.02mg/ml Histidine.

YPD yeast growth media (a rich medium). Added to 1L of distilled water: 10g Yeast, 20g Peptone, 20g glucose (20g agar), autoclaved (allowed to cool to 50°C before pouring plates).

2.6.1 Pre-Transformed Library Screening

Each bait colony of $S1P_3$ C-terminal/$pGBKT_7$, transformed in AH109 (which is an appropriate yeast reporter strain that serves as a mating partner for Y187), was added to 50ml of SD medium minus tryptophan and left overnight at 30°C in a rotating incubator at 250-270rpm. The following day the OD_{600} was recorded as 0.8. The cells were centrifuged at 1000g for 5min and resuspended in a residual 5ml of medium by vortexing.

A 1ml aliquot of the Matchmaker pretransformed HeLa cDNA library culture in Y187 (Lot number 7080576) was thawed to room temperature in a water bath and 10µl was saved for titering. Both the bait and library cultures were combined in a 2L flask with 45µl of YPDA medium (YPD plus Adenine medium) with kanamycin and swirled to mix. The library tube was rinsed in a further 2 X 1ml of medium and the final volume made up to 50ml. The flask was left in a rotating incubator at 30°C overnight at 30-50rpm. The following day the conjugating mix was transferred to a 100ml centrifuge bottle and centrifuged at 1000g for 10mins. The original flask was rinsed with 2 X 50ml YPDA medium with kanamycin. Both rinses were combined to resuspend the pellet and spun again for a further 10mins. The conjugating media was plated for efficiency controls using 100ml of mix in dilutions of 1:10,000, 1:1000, 1:100 and 1:10 on plates selective against leucine; against tryptophan or against both leucine and tryptophan. The remaining conjugating medium was spread 200µl per plate on 50 large (150mm) plates, equivalent to ~$2x10^{-4}$ – $2x10^{-5}$ cells per plate. 25 plates represented quadruple drop-out (QDO) media (-Adenine (A), -Histidine (H), -Leucine (L), -Tryptophan (W)), the other 50% represented triple drop-out (TDO) media (-Histidine, -Leucine, -Tryptophan). All the plates were incubated at 30°C, between 3-8 days for TDO plates and between 8-21 days for QDO.

Mating efficiency was calculated and a number of clones were screened. For colonies growing on TDO media, the surviving colonies were replica plated onto 10cm QDO dishes and

incubated at 30°C for a further 3-8 days. Adenine and Histidine positive transformant colonies were chosen for further analysis. These colonies were streaked out on to SD medium-TLAH.

2.6.2 Yeast Two Hybrid System Analysis of a Library Screening

From a master plate of yeast transformed with the target DNA binding domain-fusion protein (A master plate can be stored at 4°C for not more than 2 weeks) four 20-50ml cultures of a single large colony were inoculated in synthetic dropout (SD) medium with the appropriate selection for the target protein (e.g. -Trp). Cultures were grown for 36-48 hours, and each was transferred to a 200ml culture which was grown further overnight. During the protocol the four culture pools were kept separate. The following day each 200ml culture was transferred into 400ml of YPD medium (OD should be around 0.2 in a total volume of 600ml) and grown to an OD_{600} of about 0.7-0.75 (~ 4 hours). The resultant culture transferred into 250 ml bottles (in shifts) and centrifuged at 1000g for 5min. The supernatant was removed and the pellet resuspended in water and centrifuged at 1000g for 5min. The supernatant was removed and the 4 batches of cells were resuspended, with about 10ml of 1X TE/LiAc (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1 M Lithium acetate, made fresh) per bottle. The final volume was made up to 50 ml per tube and centrifuged at 1000g for 5min, supernatant removed and the final volume brought up to 2 ml with 1X TE/LiAc. Transformations were performed in 4 x 10 microfuge tubes containing: 200µl yeast cells, 8µg library DNA, 30µl salmon sperm DNA (10µg/ml), 7µl 10X TE/LiAc, 30µl DMSO, 1.2µl PEG/LiAc (40% (v/v) PEG-4000, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1 M Lithium actetate, freshly made). Vortexed and agitated to assure DNA is well distributed. The transformations were incubated at 30°C for 30min while gently shaken in a thermomixer, and agitiated every 10min. Followed by incubation at 42°C in a thermomixer. After incubation, cells were put on ice (1min) and centrifuged at 1500g for 1min, followed by resuspension in 300µl 1X TE. From each microfuge tube, equal amounts (133µl per plate) were spread onto 3 plates lacking the appropriate amino acids: a total of 4 x 30 large plates. Plate dilutions (1/1000, 1/10000 & 1/100000) on -Trp & -Leu plates to assay transformation efficiency, for each of the 4 competent yeast batches. Plates were then left at 30°C for 1-2 weeks.

2.6.3 Preparation of X-α-Gal Plates

Indicator plates containing the chromogenic substrate X-α-Gal (5-Bromo-4-Chloro-3-Indolyl-a-D-galactopyranoside) were used to rapidly detect protein interactions using a GAL4-based library. 1L of appropriate dropout agar medium was prepared and cooled to 55°, then 1ml

of X-α-Gal (20mg/ml) was added. The plates were poured and the medium was allowed to harden at room temperature. Cells were plated and incubated at 30°C until blue colonies formed.

2.6.4 Preparing Replica Plates

Each colony from a selection plate was spiked using a sterile toothpick and mapped to a X-α-gal assay plate. The replica plate was then left at 30°C to observe indicator activity. This assay plate was made to mirror the contents of each original transformation plate to identify the positive clones. All clones that brought back a positive blue X-gal result were mapped back to the original transformation plate and then a spike from the original colony was grown overnight in culture medium (with the appropriate selection).

2.6.5 TCA Protein Extraction from Yeast

Colonies from freshly streaked plates (<3 days) were picked and added to 5ml of Drop Out media which was then left at 30°C overnight (or two nights depending on growth rate). Cultures were centrifuged at 1000rpm and the resulting pellets were each resuspended in 1ml of distilled H_2O and transferred to microfuge tubes. To each pellet 150µl trichloroacetate (TCA) buffer (1.85M NaOH, 7.4% β-mercaptoethanol in distilled water) was added and then incubated on ice for 10min. After incubation, 150µl 55% (w/v) TCA was mixed with the buffered pellets and incubated on ice for a further 10min. Samples were centrifuged at 4°C for 15min at 1300g. The supernatant was removed and briefly centrifuged to remove any remaining supernatant. Then 100-150µl of the precipitated proteins were resuspended in High Urea Buffer (8M Urea, 5% (w/v) SDS, 200mM Tris-HCl pH6.8, 0.1mM EDTA, 0.5mg/ml bromophenol blue, 10% (v/v) β-mercaptoethanol, pH6.8) and 5µl was loaded on to a 10% SDS-PAGE gel. Protein extracts were resolved by electrophoresis on a 12.5% SDS–polyacrylamide gel and proteins were transferred to nitrocellulose membranes, which were then incubated for 2hr with anti-myc monoclonal antibody 9E10, followed by incubation with peroxidase-labelled anti-mouse antibody, expressed $S1P_3$ fusion proteins were visualised using ECL reagents.

2.7 $S1P_3$ Pull-Down Assays

PGEX-2T bacterial expression constructs encoding C-terminal fusions of glutathione-S-transferase (GST) with the SH3 domains from human fodrin, c-Src, phosphatidylinositol-3-kinase (PI3K) and Fyn, and described by Beard *et al.* (1999), were generously donated by Prof. Miles Houslay (University of Glasgow). *E coli* cultures (BL21 strain) transformed with either pGEX (for GST production) or the recombinant pGEX-SH3-containing plasmid (for GST–SH3 production), were first grown overnight at 37°C with agitation in LB containing 60 µg/ml ampicillin, diluted 1:10 in the same medium and incubated at 37°C for 1.5 hr. Fusion protein

expression was induced at 37°C by adding isopropyl β-D-thiogalactoside (IPTG, final concentration = 0.1 mM) and growth was continued at 37 °C for 4–6 h. Bacteria were then harvested by centrifugation (10,000g, 15 min) and then lysed by sonication in 20 ml PBS containing a protease inhibitor cocktail (40 mg/ml PMSF, 156 mg/ml benzamidine, 1 mg/ml apoprotinin, 1 mg/ml antipain, 1 mg/ml leupeptin, 1 mg/ml pepstatin, dissolved in DMSO). The debris was pelleted by centrifugation for 1 min at full speed in a benchtop centrifuge. 600 μl of glutathione–Sepharose 4B beads equilibrated in PBS were added and incubated end-over-end for 1 hr at room temperature. The beads were then washed three times with 200 μl of PBS over 15 min, and resuspended as a 50% (v/v) slurry. GST-SH3-immobilised beads were stored at 4°C. Protein concentration and assessment of purity was determined by SDS-PAGE and Coomasie Blue staining following elution of fusion protein from a 10µl aliquot of bead suspension *via* the addition of electrophoresis sample buffer and boiling for 5 min, fractionating samples in parallel with known amounts of BSA (0-10 µg), followed by quantification and comparison of staining intensity.

For the pull-down assays, quiescent confluent 10cm dishes of myc$S1P_3$-expressing CCL39 cells were treated with or without agonist as indicated in the Figure Legends. All subsequent procedures were performed at 4°C unless indicated otherwise. Following three washes with PBS, cells were solubilised by scraping into 1ml/dish pull-down lysis buffer (50mM sodium HEPES, pH 7.5, 100mM sodium chloride, 5mM EDTA, 10mM sodium fluoride, 10mM sodium phosphate, 1% (v/v) Triton X-100, 0.1 mM phenylmethylsulphonyl fluoride, 10µg/ml soybean trypsin inhibitor and 10µg/ml benzamidine) and rotation for 1hr. Following removal of insoluble material by centrifugation, soluble extracts were equalised for protein content and volume prior to overnight incubation with 10µg/sample of the GST-SH3 domains indicated in the Figure Legends immobilised to glutathione-Sepharose. Beads were isolated by brief centrifugation, washed three times with pull-down lysis buffer and eluted by the addition of 30µl electrophoresis sample buffer and incubation at 37°C for 60min. After fractionation by SDS-PAGE, resolved proteins were transferred to nitrocellulose for detection of myc$S1P_3$ binding by immunoblotting with 9E10.

2.8 $[Ca^{2+}]_i$ imaging and analysis

Calcium mobilisation experiments were performed on Rat1a fibroblasts transiently transfected with the $S1P_3$ expression constructs indicated in the Figure Legends. After overnight serum starvation, transfected cells on glass coverslips were loaded with the Ca^{2+}-sensitive dye Fura-2 by incubation (15-20min, 37°C) under reduced light in DMEM growth medium

containing the dye's membrane-permeable acetoxymethyl ester form (1.5μM). Loaded cells were illuminated with an ultra high point intensity 75-watt xenon arc lamp (Optosource, Cairn Research, Faversham, Kent, UK) and subsequently imaged using a Nikon Diaphot inverted microscope equipped with a Nikon 40× oil immersion Fluor objective lens (NA = 1.3) and a monochromator (Optoscan, Cairn Research), which was used to alternate the excitation wavelength between 340/380nm and to control the excitation band pass (340nm band pass=10 nm; 380nm band pass=8 nm). Fura-2 fluorescence emission at 510nm was monitored using a high resolution interline-transfer cooled digital CCD camera (Cool Snap-HQ, Roper Scientific/Photometrics, Tucson, AZ). MetaFluor imaging software (version 4.6.8, Universal Imaging Corp., Downing, PA) was used for control of the monochromator, CCD camera, and for processing of the cell image data. The desensitisation regimen involved stimulating cells every 6 min with 1 min pulses of 0.5μM S1P. Sequential images (2×2 binning) were collected every 2 sec, exposure to excitation light was 100msec/image, and all experiments were undertaken in the absence of extracellular Ca^{2+} in saline solution comprising: 130mM sodium chloride, 5mM potassium chloride, 1mM magnesium chloride, 20mM Hepes, 10mM D-glucose, 0.01mM EGTA, pH adjusted to 7.4 using sodium hydroxide. For analysis, ratio images were presented in MetaFluor intensity-modulated display mode, which associates the colour hue with the excitation ratio value and the intensity of each hue with the source image brightness. Briefly, background subtracted images acquired at 340 and 380nm excitation were first used for calculating the 340/380nm ratio of each pixel. After determination of the upper and lower thresholds, the ratio value of each pixel was associated with one of the 24 hues from blue (low $[Ca^{2+}]_i$) to red (high $[Ca^{2+}]_i$).

2.9 Statistical Analysis

All statistical analysis, unless stated otherwise was carried out using the Student t-test as described in the Graphpad software, "Prism 3.0". Elsewhere, statistical analysis using ANOVA was carried out using the "Graphpad" software as indicated in the figure legends.

CHAPTER 3

Characterisation and Analysis of Human $S1P_3$ Receptor Phosphorylation

Characterisation and Analysis of Human $S1P_3$ Receptor Phosphorylation

3.1 Introduction

Derived from sphingosine, the backbone of all sphingolipids, the evolutionarily conserved lipid mediator S1P is now recognised as a potent modulator of cell regulation [Hannun, 1996; Spiegel and Merrill Jr, 1996; Hannun *et al.*, 2001; Saba and Hla, 2004]. Many cell types, notably platelets and fibroblasts, release S1P [Olivera *et al.*, 1999; Yang *et al.*, 1999; Spiegel and Milstien, 2002]. A myriad of cellular activities initiate with S1P signalling. These include intracellular calcium mobilisation, regulation of the organisation of the cytoskeleton, cell growth, differentiation, survival and motility [Spiegel and Milstien, 2002]. This signalling is due to both intracellular and extracellular actions of S1P [Van Brockyln *et al.*, 1998; Spiegel and Milstien, 2002].

S1P exerts extracellular effects when it binds specifically to S1P receptors of the class A GPCR family [Spiegel and Milstien, 2000b, 2002]. The S1P receptors are presently 5 receptors, $S1P_1$ (previously known as EDG1) [Hla and Maciag, 1990], $S1P_2$ (EDG5) [Okazaki and Ishizaka, 1993], $S1P_3$ (EDG3) [Yamaguchi *et al.*, 1996; An *et al.*, 1997], $S1P_4$ (EDG6) [Graeler *et al.*, 1998] and $S1P_5$ (EDG8) [Im *et al.*, 2000], which each have high affinity for S1P [Chun *et al.*, 2002]. It has been demonstrated that $S1P_3$ activates the NF-κB pathway via a mechanism that relies primarily on the activation of phospholipase C by $G_{q/11}$ and perhaps some contribution from other G proteins [Siehler *et al.*, 2001]. This is presumably responsible for activating the IκB kinase (IKK) complex and thus triggering the agonist-mediated phosphorylation and degradation of IκBα, β and ε isoforms [Ghosh and Karin, 2002]. $S1P_3$ stimulates the PTx-insensitive $G_{12/13}$-mediated activation of Rho-coupled pathways [Moolenaar, 1999]. In addition, binding of S1P to the $S1P_3$ receptor subtype results in pertussis toxin (PTx)-sensitive, G_i-dependent activation of ERK2 and the inhibition of adenylyl cyclase [Im *et al.*, 1997; Ancellin and Hla, 1999]. $S1P_3$ in conjunction with $S1P_1$ regulates the signalling pathways necessary for human umbilical vein endothelial cell morphogenesis into capillary-like networks [Lee *et al.*, 1999; Wang *et al.*, 1999; Kimura *et al.*, 2000]. This also suggests that antagonists of $S1P_3$ and $S1P_1$ could be used to attenuate the enhanced angiogenesis that is seen in solid tumour growth, rheumatoid arthritis and diabetic retinopathy [Lee, 1998b]. Since $S1P_3$ signalling is prevalent in processes such as angiogenesis and vasoconstriction [Ancellin and Hla, 1999; Salomone *et al.*, 2003], the molecular mechanisms regulating $S1P_3$ signalling predispose the $S1P_3$ receptor's therapeutic potential.

As mentioned earlier, sustained agonist occupancy of many GPCRs can result in the desensitisation of receptor function, internalisation of the receptor away from the cell surface as well as GPCR coupling to alternative signalling pathways. These events are initiated by the phosphorylation of the GPCR at serine and threonine residues within the third intracellular loop and/or the C-terminal tail by both second messenger-dependent protein kinases and GRKs [Luttrell and Lefkowitz, 2002]. Upon GRK-mediated phosphorylation, the agonist-dependent conformational change of the receptor can promote the selective binding of arrestins to agonist-activated receptors [Zhang *et al.*, 1997]. Arrestin binding causes steric uncoupling of the receptor from the heterotrimeric G-proteins, triggers internalisation of GPCRs by targeting them to clathrin-coated vesicles, and on occasion can initiate different signalling pathways, for example, the arrestin-mediated increase in ERK and JNK signalling found with the β_2AR [DeFea *et al.*, 2000; Chen *et al.*, 2001; Laporte *et al.*, 2002].

Phosphorylation represents a vital step in both the rapid desensitisation of GPCR function and their subsequent mechanisms of internalisation [Bouvier *et al.*, 1998]. Thus, to begin to characterise $S1P_3$ signal regulation it is necessary to fully characterise the phosphorylation of the $S1P_3$ receptor. In this chapter, the molecular mechanisms regulating $S1P_3$ phosphorylation have been characterised in detail. Also, truncation mutants of the $S1P_3$ C-terminus were used to define specific regions conferring sensitivity to phosphorylation.

3.2 Results

The lack of a selective commercial antibody against endogenous $S1P_3$ receptors dictated the development of a $S1P_3$-expressing construct incorporating a myc epitope tag, which was incorporated into human $S1P_3$ cDNA (Figure 3.1). This allowed the identification and isolation of recombinant receptors using the anti-myc monoclonal antibody 9E10. The construct was stably expressed in CCL-39 hamster lung fibroblasts and multiple colonies were screened for stable expression of the myc$S1P_3$ receptor in CCL-39 cells. The strength of expression was determined by immunoblotting using the antibody 9E10 (Figure 3.2). Several colonies with bands denoting strong expression were expanded and colonies 8 and 23 were used for subsequent experimentation.

To confirm that the $S1P_3$ receptor is expressed at the plasma membrane a biotinylation experiment was carried out to label cell surface glycoproteins on parental CCL39 fibroblast cells and myc$S1P_1$ stably expressing CCL39 cells with the myc$S1P_3$ stably expressing CCL39 cells (Figure 3.3). $S1P_3$ could be specifically immunoprecipitated from transfected cell extracts and, like the related receptor $S1P_1$, migrated as a broad band centred at an approximate molecular mass of 50kDa.

The transcription factor nuclear factor-κB (NF-κB) responds to a large number of environmental cues and is especially relevant to inflammation and apoptotic/anti-apoptotic signalling. By using HEK293 cells, it has been shown that S1P activates NF-κB in a receptor-dependent fashion [Siehler *et al.*, 2001]. $S1P_3$ is coupled to G_i, G_q, and $G_{12/13}$, and affects activation of NF-κB, whereas $S1P_1$, which is coupled to G_i alone, does not. To assess whether stably expressed myc$S1P_3$ receptor could transmit a downstream signal, activation of the NF-κB pathway which is mediated *via* a $G_{q/11}$-dependent process [Siehler *et al.*, 2001] was assessed using the degradation of the inhibitor of NFκB kinase, IκBβ as a read-out (Figure 3.4). This demonstrated that S1P exposure induced a time-dependent degradation of IκBβ in myc$S1P_3$–expressing cells but not parental controls. Electrophoretic mobility shift assays (EMSAs) of NF-κB binding to target DNA indicated that in quiescent myc$S1P_3$-expressing cells, NF-κB activity was elevated regardless of the absence of agonist compared with parental CCL39 cells. Addition of S1P further enhanced NF-κB binding but no effect was observed in parental controls, which confirms the tagged receptors functionality.

Many GPCRs require the presence of a specific agonist to initiate G-protein coupling and receptor phosphorylation and thus trigger further regulatory events within the cell [Lefkowitz, 2004]. Current research has not examined the sensitivity of $S1P_3$ to regulatory events associated

with receptor desensitisation, such as receptor phosphorylation. To begin the characterisation of $S1P_3$ phosphorylation, a whole cell phosphorylation study using serum-starved CCL-39/myc$S1P_3$ cells was carried out in the presence of the agonist, S1P and a number of activators of second messenger-regulated kinases (Figure 3.5). These included PMA, a phorbol ester that activates conventional and novel PKC subtypes; A231567, a calcium ionophore; forskolin, a direct activator of adenylyl cyclase that increases PKA activity and 8-Br-cGMP, a non-hydrolysable analogue of cGMP that activates protein kinase G (PKG). A 5- to 12-fold increase in $S1P_3$ phosphorylation was observed following exposure to S1P (range over 8 experiments). The other activators failed to induce $S1P_3$ phosphorylation (*versus* a vehicle-treated control where S1P-induced $S1P_3$ phosphorylation was set at 100%, $p>0.05$, n=3, Figure 3.5).

After the discovery that second messenger kinases did not affect the phosphorylation of $S1P_3$ receptors it was decided to investigate the effects of S1P on $S1P_3$ phosphorylation by further characterising the S1P-induced $S1P_3$ phosphorylation seen in Figure 3.5. This was achieved using a whole cell phosphorylation study on serum-starved CCL-39/myc$S1P_3$ cells which verified that the $S1P_3$ receptor was phosphorylated upon agonist stimulation, and that this phosphorylation was only observed in cells over-expressing $S1P_3$ receptors and not in parental CCL-39 cells (Figure 3.6).

Characterisation of $S1P_3$ phosphorylation commenced performing time course analysis of $S1P_3$ phosphorylation in the presence of 10μM S1P. This showed that phosphorylation is a rapid process, with maximal $S1P_3$ phosphorylation observed after the earliest time point examined,15 seconds, this being sustained for 30 minutes (Figure 3.7). Similar whole cell phosphorylation studies were subsequently carried out in the presence of a range of S1P concentrations. This demonstrated S1P produced a concentration-dependent increase in $S1P_3$ phosphorylation (EC_{50}=0.85±0.01μM, n=3, Figure 3.8). A concentration of 5-10μM was subsequently chosen for the remaining experiments as this was shown to induce maximal $S1P_3$ phosphorylation. $S1P_3$ phosphorylation was also shown to be a reversible process, with a significant decrease in $S1P_3$ phosphorylation observed after only 30min of agonist removal, although this was somewhat slower than the time course of onset since the level of phosphorylated $S1P_3$ after agonist removal for 120min was still 55±8% ($p<0.05$ *versus* agonist-treated cells, n=3) of that observed prior to agonist removal (Figure 3.9).

Based on other examples of agonist-dependent GPCR phosphorylation, potential candidates for kinases involved in S1P-induced $S1P_3$ phosphorylation included one or more members of the GRK family. To test this hypothesis, an attempt to reconstitute S1P-dependent

$S1P_3$ phosphorylation *in vitro* was made using CCL39/$S1P_3$ cell membranes as a receptor source and purified preparations of GRK2 and GRK5 (Figure 3.11). GRK2 and GRK5 were used because they each represent a separate subfamily within the GRK family. As shown previously with $S1P_1$, it was possible to reconstitute S1P-stimulated phosphorylation of $S1P_3$ using GRK2 (Figure 3.10). This was specific for GRK2, as GRK5 did not sustain $S1P_3$ phosphorylation despite activity observed from both kinases under these assay conditions when assessed by light-dependent phosphorylation of rhodospin in urea-treated rod outer segments (Figure 3.12). Accordingly, agonist-dependent phosphorylation of $S1P_3$ in CCL39 cells *in vitro* can be mediated specifically by GRK2 but not by the related kinase GRK5.

To further assess $S1P_3$ sensitivity to GRK-mediated phosphorylation in intact cells, GRK2 and the related kinase GRK3 were used to measure the level of potentiation of agonist-mediated $S1P_3$ phosphorylation in HEK293 cells co-transfected with expression constructs of the receptor and each GRK (Figure 3.13). The switch to HEK293 cells for these investigations was a practical measure, the HEK293 cells were readily transfectable and thus allowed facile comparison of WT and mutant receptor characteristics. Both overexpression of GRK2 or GRK3 failed to potentiate $S1P_3$ phosphorylation above the level of phosphorylation seen in cells without overexpression of GRK2 or GRK3, in intact cells, despite explicit evidence of receptor and GRK expression by immunobloting. This suggests that $S1P_3$ is not a substrate for GRK2 as GRK2 is not capable of phosphorylating the $S1P_3$ receptor in both intact HEK293 cells or *in vitro* CCL39 cells. Although, this result could be due to cell-type specific characteristics between HEK293 cells and CCL39 cells, and/or the artificial indulgence of over-expressing GRKs in the cell system. Each cell line should have the opposite series of GRK experiments (intact and *in vitro*) investigated to rule out GRK2 as the mediator of $S1P_3$ phosphorylation and further investigations using siRNA knockdowns of GRK2 would alleviate the problems that arise from over-expression of receptors.

To identify whether the carboxyl-terminal domain has a role in controlling $S1P_3$ phosphorylation an inspection of the $S1P_3$ open reading frame was made. The receptor's carboxyl-terminus was identified as a prime candidate for the site of regulatory phosphorylation as it is enriched in serine and threonine residues. To evaluate the contribution of two clusters of multiple potential phosphorylation sites within this domain, two truncated $S1P_3$ mutants were generated (Figure 3.14). The ability of the truncated receptors to undergo agonist-stimulated phosphorylation *in situ* was assessed following transient receptor expression in HEK293 cells (Figure 3.15). Whilst removal of 27 residues from the carboxyl-terminus markedly elevated basal phosphorylation and significantly potentiated the effect of S1P, deletion of 45 residues

abolished $S1P_3$ phosphorylation. Immunoblotting of the same cell extracts showed that these effects could not be attributed to parallel differences in receptor expression (Figure 3.16). Together, these data suggest $S1P_3$ is phosphorylated *in situ* in an agonist-dependent manner in an eighteen amino acid sequence between Leu332 and Val352 in the receptor's carboxyl-terminal domain. They also suggest that the 27 carboxyl-terminal residues may impart a tonic inhibitory effect on receptor phosphorylation.

To ensure GRK2 is not merely mimicking the effects of a native $S1P_3$ kinase in intact cells, $S1P_3$ mutant sensitivity to phosphorylation by GRK2 *in vitro* should reflect the sensitivity observed in phosphorylation in whole cells. Removal of 27 residues from the C-terminal domain of $S1P_3$ increased GRK2-dependent phosphorylation *in vitro* when compared with the WT receptor, these results were similar to whole cell phosphorylation experiments. In contrast to the abolition of $S1P_3$ phosphorylation observed in whole cells, removal of 45 residues from the carboxyl-terminus also potentiated GRK2-mediated phosphorylation *in vitro* (Figure 3.17).

3.3 Discussion

Receptor phosphorylation is an important regulatory process in GPCR signalling. Taking the β_2AR as example, phosphorylation is considered as the critical step necessary to achieve both desensitisation and internalisation of the receptor. This chapter has characterised for the first time the phosphorylation of the S1P receptor $S1P_3$ using a myc-tagged human $S1P_3$ receptor and two C-terminal truncation mutants.

In CCL-39 hamster lung fibroblasts, there is a strong, reversible, agonist-dependent phosphorylation of $S1P_3$ (Figures 3.7, 3.8 and 3.9). Agonist-dependent $S1P_3$ phosphorylation is a rapid process, with a significant phosphorylation observed after only 15 seconds (Figure 3.7). Dose-response experiments demonstrated that S1P produced a concentration-dependent increase in $S1P_3$ phosphorylation. The EC_{50} value (0.85±0.01μM, Figure 3.7) for S1P-induced $S1P_3$ phosphorylation related to the physiological range of S1P concentration in the blood which reachs low μM concentrations upon platelet activation [Pyne and Pyne, 2000b]. Previous studies have shown that the K_D of S1P for the $S1P_3$ receptor is between 23nM and 27nM [Van Brocklyn *et al.*, 1999]. The observed K_D would suggest that an additional event subsequent to agonist binding is required, and this highlights the possibility that complexing of receptors with other proteins, perhaps arrestin recruitment for receptor trafficking [Shenoy and Lefkowitz, 2003b], or indeed dimerisation may occur to allow desensitisation and internalisation of the receptor [Milligan, 1998; Gether, 2000; Milligan, 2000; Brady and Limbird, 2002; Hur and Kim, 2002; Salim *et al.*, 2002; Jensen and Spalding, 2004] or another step is involved perhaps involving a MAPK.

S1P also induced $S1P_3$ phosphorylation *in vitro* using CCL-39/myc$S1P_3$ membranes in the presence of purified GRK2 (Figure 3.10). In contrast no $S1P_3$ phosphorylation was observed in the presence of GRK5 (Figure 3.11). This suggests that S1P-induced $S1P_3$ phosphorylation may be mediated by GRK2. Assessment continued with the investigation of sensitivity of $S1P_3$ to GRK-mediated phosphorylation. The ability of GRK2 and the related kinase GRK3 to potentiate agonist-mediated $S1P_3$ phosphorylation was tested in whole cell phosphorylation experiments after co-transfection of HEK293 cells with expression constructs for the $S1P_3$ receptor and GRK2 or GRK3. Overexpression of either GRK2 or GRK3 failed to potentiate $S1P_3$ phosphorylation in intact cell phosphorylation assays despite unequivocal evidence of receptor and GRK expression. While agonist-occupied $S1P_3$ is a substrate for phosphorylation by GRK2 *in vitro*, GRK2 cannot be responsible for the phosphorylation observed in intact cells (Figure 3.13).

To assess more precisely which region of the $S1P_3$ receptor is phosphorylated, $S1P_3$ truncation mutants removing clusters of potential Ser/Thr phophorylation sites were analysed. Both sites are situated proximal to acidic residues making them potential targets for aciditrophic kinases like GRK2 (Figure 3.15). S1P-mediated $S1P_3$ phosphorylation was abolished by the truncation of the last 45 amino acids and was significantly potentiated by the truncation of 27 amino acids (Figure 3.16).

In comparison to previously described receptors of the S1P receptor family, notably the closely related $S1P_1$ receptor, $S1P_3$ is not affected by two pathways of phosphorylation as observed with $S1P_1$. $S1P_3$ phosphorylation is not activated by an agonist-independent mechanism regulated by the activation of PKC, however, it appears that there may be some similarity in the mechanism acting *via* GRK2 kinase, as with $S1P_1$ an agonist-dependent mechanism acts *via* GRK2 *in vitro* and from this study GRK2 was also shown to reconstitute S1P-stimulated phosphorylation of $S1P_3$.

Potential problems comparing *in vitro* or whole cell phosphorylation experiments arise, when working *in vitro*, constituents of the cytoplasm are readily available to cell surface receptors located on membrane fragments. These constituents include a variety of kinases which could add to the effects observed by administered GRKs, and which may include the native $S1P_3$ kinase. Equally, when taking into account the intact cell, the model system introduces overexpression of a specific S1P receptor and chosen GRK, and this skew in enzyme equilibrium may be sufficient to alter the regulation of the receptor which is distinct from any natural occurring activity in the cell-type under investigation. To alleviate discrepancies that may occur, mutant receptor phosphorylation allows further comparison of receptors *in vitro* and in intact cells as it highlights whether phosphorylation is dependent on specific residues within the receptor, regardless of which kinase is involved. Here additional evidence is provided that GRK2 is unlikely to be the agonist-regulated $S1P_3$ kinase in intact cells. Evidence that provides confirmation that once the putative area of the receptor involved in specific kinase interaction is removed, phosphorylation is potentiated regardless of the presence or absence of GRK2 which suggests another kinase specific to $S1P_3$ kinase is involved and not GRK2.

This agonist-independent regulation of GPCR phosphorylation is seen in the M3 muscarinic acetylcholine receptor by casein kinase Iα [Budd *et al.*, 2000], casein kinase II phosphorylation of thyrotropin-releasing hormone receptor [Hanyaloglu *et al.*, 2001] and phosphorylation of Ser348 within the bradykinin B2 receptor by an unidentified kinase [Blaukat *et al.*, 2001]. Similar to GRK2 and 3, CKIα and CKII are both acidotropic kinases that typically

phosphorylate clusters of Ser and/or Thr residues in a consecutive manner [Tobin, 2002]. Casein kinases are unlikely to be involved in $S1P_3$ phosphorylation as there is a distinct lack of acidic residues in the regions upstream and downstream of $S1P_3$ phosphorylation sites.

S1P influences heart rate, endothelial integrity, and lymphocyte recirculation through the S1P receptors. Inhibition of lymphocyte recirculation by non-selective S1P receptor agonists produces clinical immunosuppression preventing transplant rejection but is associated with transient bradycardia. A potent, $S1P_1$-receptor selective agonist, SEW2871, 5-(4-phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)(1,2,4)-oxadiazole, that is structurally unrelated to S1P, was found to activate multiple signals triggered by S1P, including guanosine 5'-3-O-(thio)triphosphate binding, calcium flux, Akt and ERK1/2 phosphorylation, and stimulation of migration of $S1P_1$- but not $S1P_3$-expressing cells *in vitro*. The agonist also alters lymphocyte trafficking *in vivo*. And it was found that agonism of $S1P_1$ receptor alone is sufficient to control lymphocyte recirculation [Sanna *et al.*, 2004]. As the balance of receptor expression is found to be of specific importance in the regulation of cell behaviour it is possible that disruption of regular expression of $S1P_1$ receptors would have pathological consequences. Equally, if other S1P receptors which were co-expressed in the same tissue were not properly regulated in synergy with $S1P_1$, this too could have harmful results. This highlights the importance of the differences in sequestration of both the $S1P_1$ and $S1P_3$ receptors.

SEW2871 is a full agonist on $S1P_1$ alone on both human and murine receptors for induced GTPγS binding, calcium flux, kinase activation, and cell migration; yet it is not active on the related receptors $S1P_{2-5}$ in either species [Sanna *et al.*, 2004]. SEW2871 is a highly hydrophobic agonist that lacks any solubilizing or head groups. Despite this, it is an effective full agonist of $S1P_1$ suggesting that the headgroup interactions are not required for full agonism, and can be achieved by hydrophobic-aromatic interactions alone. $S1P_3$, and not $S1P_1$, is directly implicated in sinus bradycardia. The sustained bradycardia induced by S1P receptor non-selective immunosuppressive agonists in wild-type mice is abolished in $S1P_3$-/- mice, whereas $S1P_1$-selective agonist does not produce bradycardia. The demonstration *in vivo* that a non-selective S1P receptor agonist active on $S1P_3$ induces bradycardia in wild-type mice that is abolished in $S1P_3$-/- mice provides further support for the role of $S1P_3$ in the heart [Forrest *et al.*, 2004; Sanna *et al.*, 2004]. This evidence supports the concept of differential regulation of receptors depending on celltype as both $S1P_1$ and $S1P_3$ are expressed on cardiac endothelium and perhaps myocardium, yet deletion of $S1P_3$ alone abolishes the bradycardia induced by non-selective S1P receptor agonists, and an $S1P_1$-selective agonist does not induce bradycardia [Sanna *et al.*, 2004]. With regard to the findings of this thesis, it is possible to suggest that dysfunctional regulation of

the $S1P_3$ receptor, by irregular expression at the PM, which could be caused by improper phosphorylation, internalisation or desensitisation, is likely to be the source of atypical variations of heart rate that result in bradycardic conditions.

The unique regulation of $S1P_3$ phosphorylation may be useful in designing selective drugs. If selective drugs were designed to inhibit or promote phosphorylation, by manipulation of agonist interaction with specific S1P receptor subtypes, then downstream signalling and trafficking of receptors could be regulated *via* specific signalling pathways to enable the processes of angiogenesis, cell growth or differentiation to be accelerated or decelerated accordingly. Comparative studies have been undertaken to investigate arrestin-mediated regulation of desensitisation in μ-opiod receptors [Whistler and von Zastrow, 1998]. Studies related to S1P signalling have developed the S1P agonist FYT720, which is in trials for use as a drug in transplant rejection [Rosen and Liao, 2003], and more recently a novel immunomodulator, KRP203, similar to FTY720 is being developed for use in organ transplantation [Vincenti, 2002; Shimizu *et al.*, 2005].

Figure 3.1: Schematic Diagram Of The Myc-tagged Human $S1P_3$ Receptor

A myc epitope (pink) and six histidine residues (green) were added to the C-terminus of the human $S1P_3$ receptor using pcDNA/$S1P_3$ as a template. The sense primer incorporated a HindIII site upstream of a consensus Kozak sequence and the $S1P_3$ initiating methionine, as indicated. The diagram also shows that the antisense primer was designed to remove the $S1P_3$ stop codon and add an XbaI site. This allowed in-frame ligation of the $S1P_3$ coding region with that of the myc-His epitope tag following ligation of the HindIII/XbaI-digested PCR product with a similarly digested pcDNA3.1/myc-HisA vector.

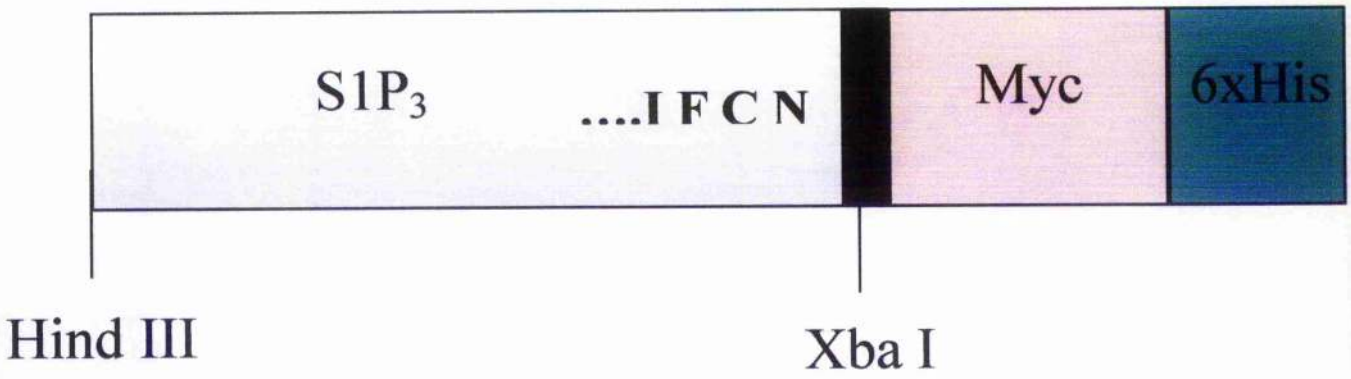

S1P3
....I F C N
Myc
6xHis
Hind III
Xba I

Figure 3.2: A screen to assess stable expression of the Myc-Tagged Human $S1P_3$ Receptor

CCL39 hamster fibroblasts stably expressing the myc epitope-tagged form of the $S1P_3$ receptor were solubilised in electrophoresis buffer and analysed by SDS-PAGE and immunoblotting using the monoclonal anti-myc antibody 9E10. Multiple colonies were screened for $S1P_3$ expression, from the example shown, of the 39 colonies screened, those with the strongest expression, colonies 1-11, 19-21, 23 and 29-31 were selected, colonies 8 and 23 were expanded for this experimental research, and the remaining strongly expressing colonies were frozen in nitrogen for experimentation at a later stage. The positive control band seen in this figure represents $S1P_1$ receptor expression.

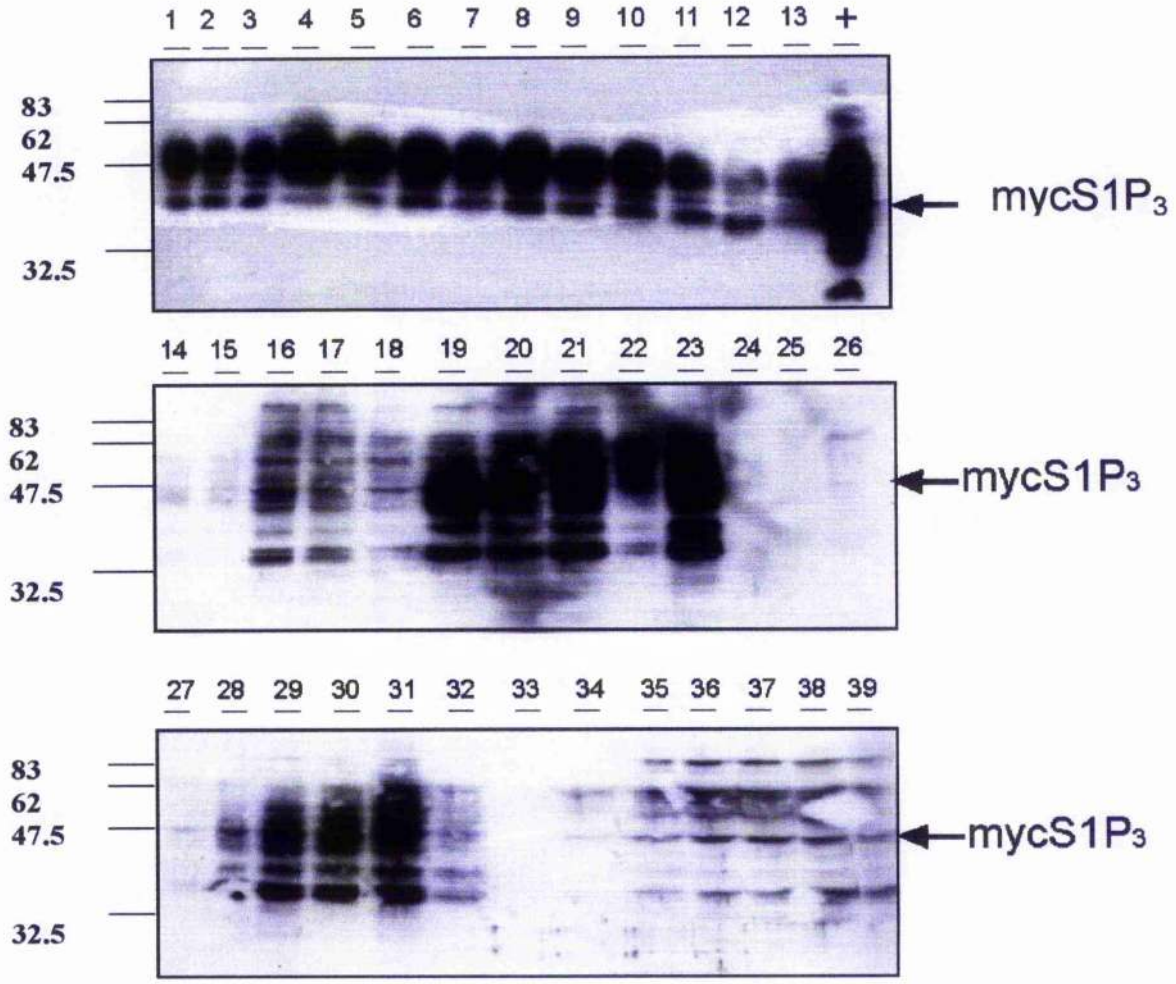

1 2 3 4 5 6 7 8 9 10 11 12 13 +
83
62
47.5
32.5
mycS1P3
14 15 16 17 18 19 20 21 22 23 24 25 26
83
62
47.5
32.5
mycS1P3
27 28 29 30 31 32 33 34 35 36 37 38 39
83
62
47.5
32.5
mycS1P3

Figure 3.3: Detection of cell surface $S1P_1$ and $S1P_3$ by biotinylation

Glycoproteins at the cell surface of parental CCL9 fibroblasts, myc$S1P_1$- and myc$S1P_3$- stably expressing cells were biotinylated as described previously in Section 2. Normalisation of soluble cell extracts was performed to adjust protein content and receptors were immunoprecipitated with anti-myc antibody 9E10. After SDS-PAGE and transfer to nitrocellulose, biotinylated proteins were observed by probing with HRP-streptavidin.

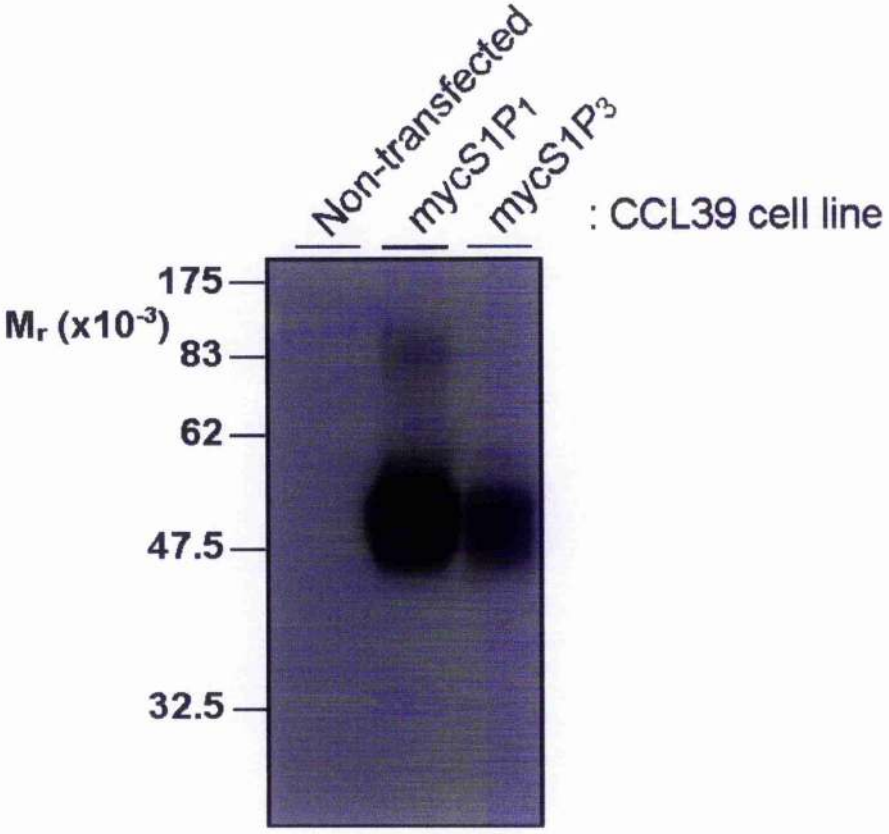

Non-transfected
mycS1P1
mycS1P3
: CCL39 cell line
175
Mr (x10-3)
83
62
47.5
32.5

Figure 3.4: Detection of IκBβ degradation to identify $S1P_3$ expression

A: Parental and myc$S1P_3$-expressing CCL39 cells were treated after quiescence with 5μM S1P for the time periods indicated prior to cell lysis and solubilisation of whole cell extracts. Protein content was then normalised and samples were fractionated by SDS-PAGE for immunoblotting with an anti-IκBβ antibody.

B: Parental and myc$S1P_3$-expressing CCL39 cells were treated after quiescence with 5μM S1P for the time periods indicated prior to cell lysis and nuclear extract preparation for EMSA analysis of NF-κB activation.

Molecular weight markers not appropriate (and thus not loaded) as it is a non-denaturing gel. Both bands represent p50/p65 NF-κB heterodimers, as determined by supershift analysis using specific antibodies (Sands and Palmer, personal communication). However, presumably the slower migrating band must also include an additional unknown protein.

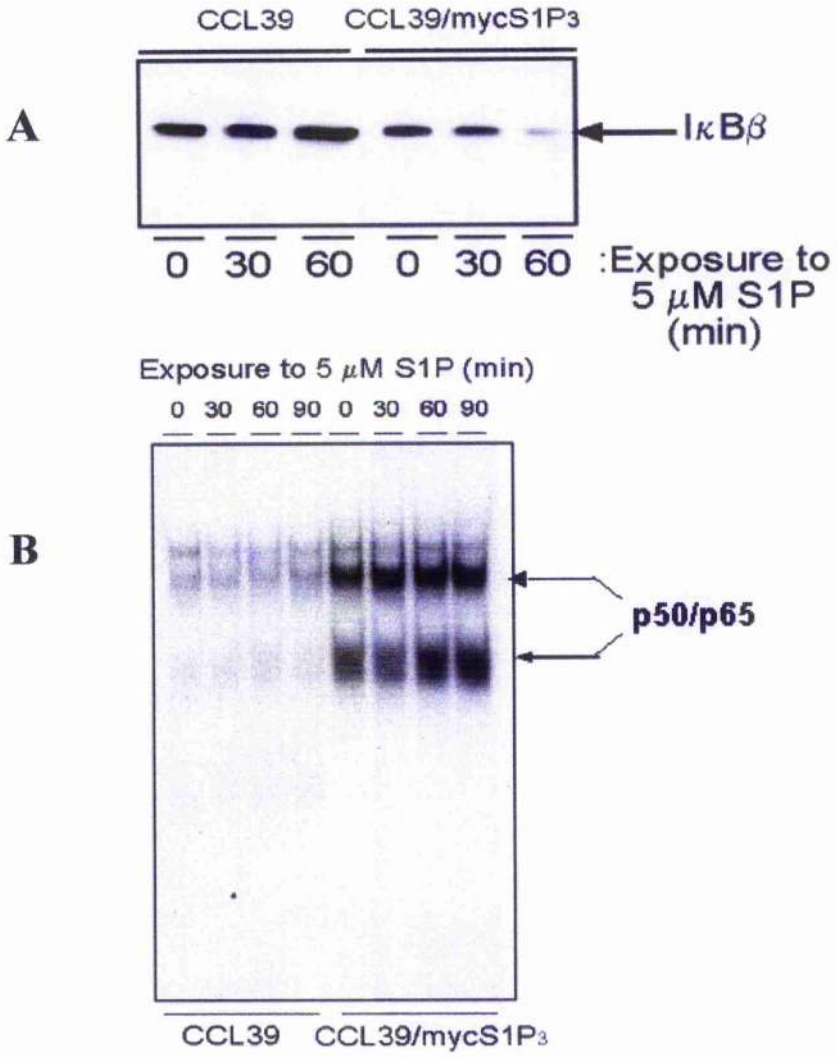

A
CCL39
CCL39/mycS1P3
IκBβ
0 30 60 0 30 60 :Exposure to 5 μM S1P (min)
B
Exposure to 5 μM S1P (min)
0 30 60 90 0 30 60 90
p50/p65
CCL39
CCL39/mycS1P3

Figure 3.5: The Effect of Second Messenger-Activated Kinase activation on phosphorylation of $S1P_3$

CCL-39/myc$S1P_3$ cells were serum-starved and treated for 10min at 37°C in the absence (vehicle) or presence of 10μM of the agonist, S1P and a range of second messenger activators; 1μM phorbol 12-myristate 13-acetate (PMA), an activator of PKC; 10μM of A23187, a calcium ionophore; 10μM Forskolin, an activator of adenylyl cyclase and 100μM 8-bromo-cGMP, an activator of cGMP. The cells were then solubilised for analysis of $S1P_3$ phosphorylation by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Typical data is shown from one of three experiments.

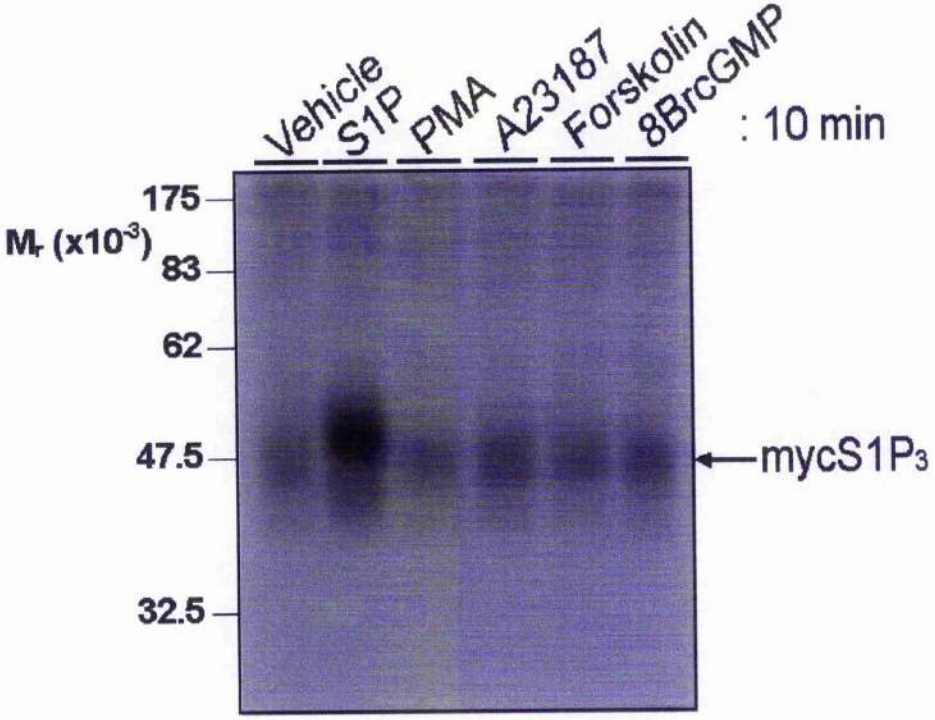

Vehicle
S1P
PMA
A23187
Forskolin
8BrcGMP
: 10 min
175
M_r ($x10^{-3}$)
83
62
47.5
32.5
mycS1P$_3$

Figure 3.6: Whole Cell Phosphorylation of the $S1P_3$ Receptor Stable Cell Line

^{32}P-labelled serum starved parental CCL-39 cells or stably transfected CCL-39/myc$S1P_3$ cells were treated with either vehicle or 10μM S1P at 37°C for 30min. The cells were then solubilised for analysis of $S1P_3$ phosphorylation by immunoprecipitation followed by SDS-PAGE and autoradiography.

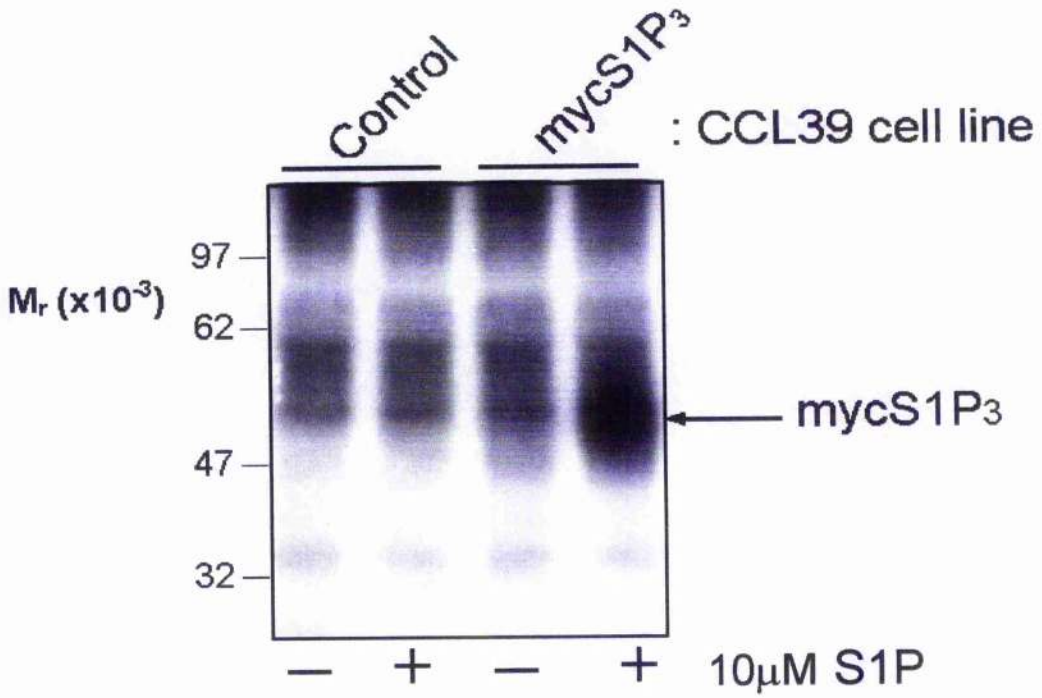
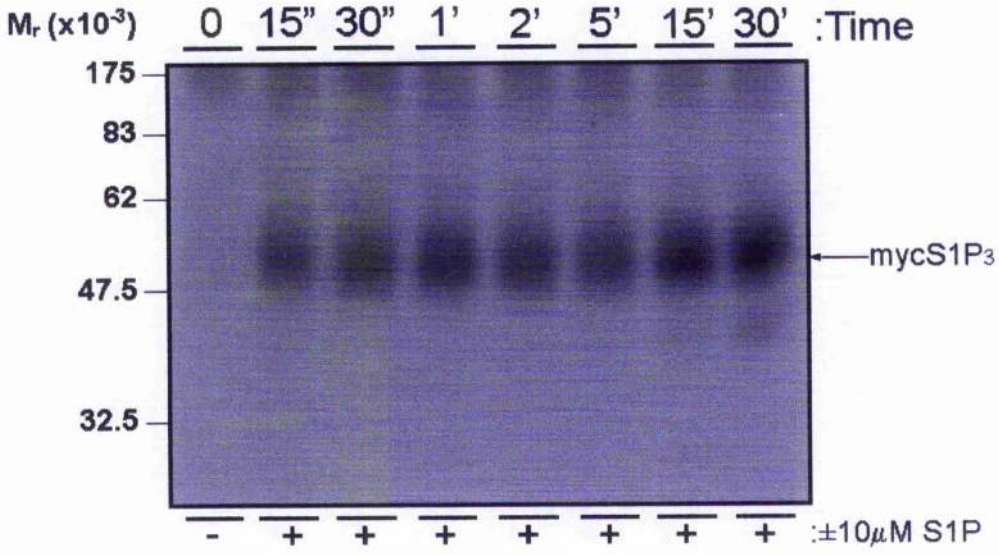

Control
mycS1P3
: CCL39 cell line
Mr (x10-3)
97
62
47
32
mycS1P3
—
+
—
+
10μM S1P

Figure 3.7: Characterisation of $S1P_3$ phosphorylation over time in intact cells

^{32}P-labelled serum starved stably transfected CCL-39/myc$S1P_3$ cells were treated with either vehicle or 10μM S1P at 37°C for the times indicated. The cells were then solubilised for analysis of $S1P_3$ phosphorylation by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Maximal S1P-induced $S1P_3$ phosphorylation was set at 100% and other results were expressed relative to this level. Data is from n=4 expts.


Mr (x10-3)
0 15" 30" 1' 2' 5' 15' 30' :Time
175
83
62
47.5
32.5
mycS1P3
- + + + + + + + :±10μM S1P

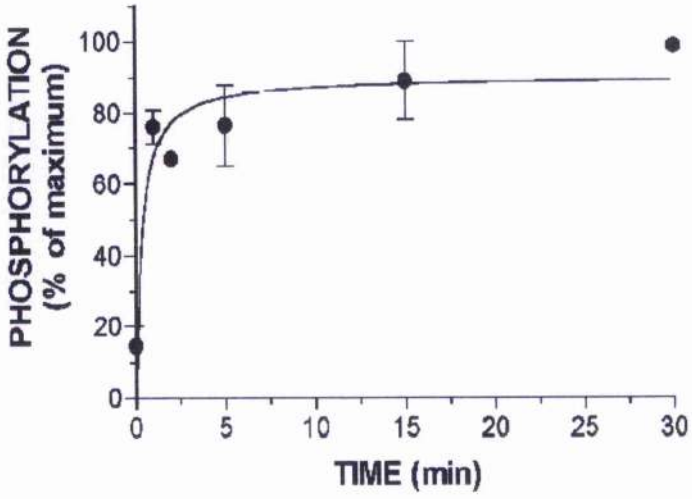

PHOSPHORYLATION
(% of maximum)
100
80
60
40
20
0
0 5 10 15 20 25 30
TIME (min)

Figure 3.8: Concentration-Dependence of $S1P_3$ Phosphorylation to Increasing Concentrations of S1P

^{32}P-labelled serum starved stably transfected CCL-39/myc$S1P_3$ cells were treated with either vehicle or increasing concentrations of S1P at 37°C for 30 minutes. The cells were then solubilised for analysis of $S1P_3$ phosphorylation by immunoprecipitation followed by SDS-PAGE and autoradiography. Maximal S1P-induced $S1P_3$ phosphorylation was set at 100% and other results were expressed relative to this level. These data represent the mean ± SEM of three similar experiments.

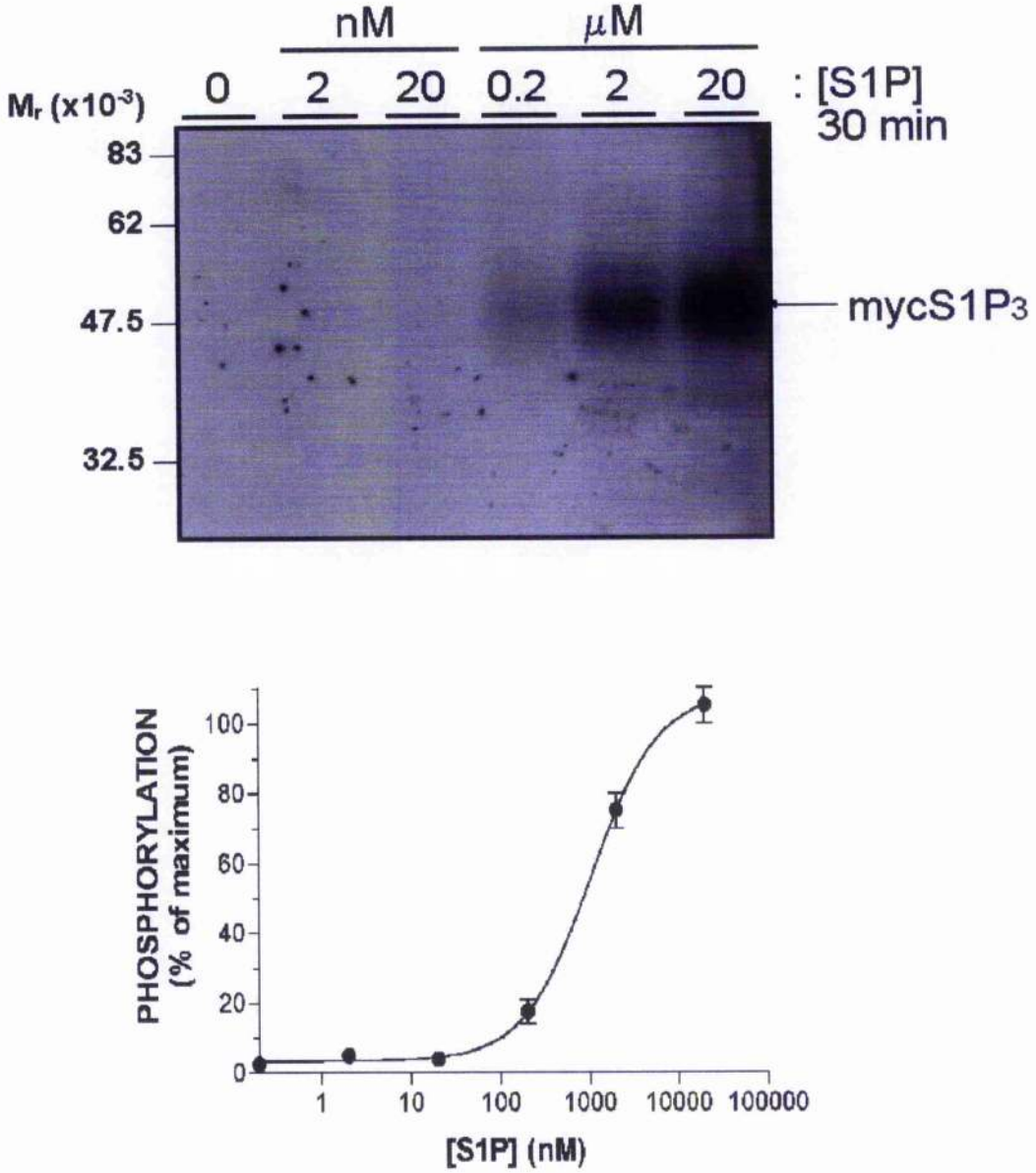
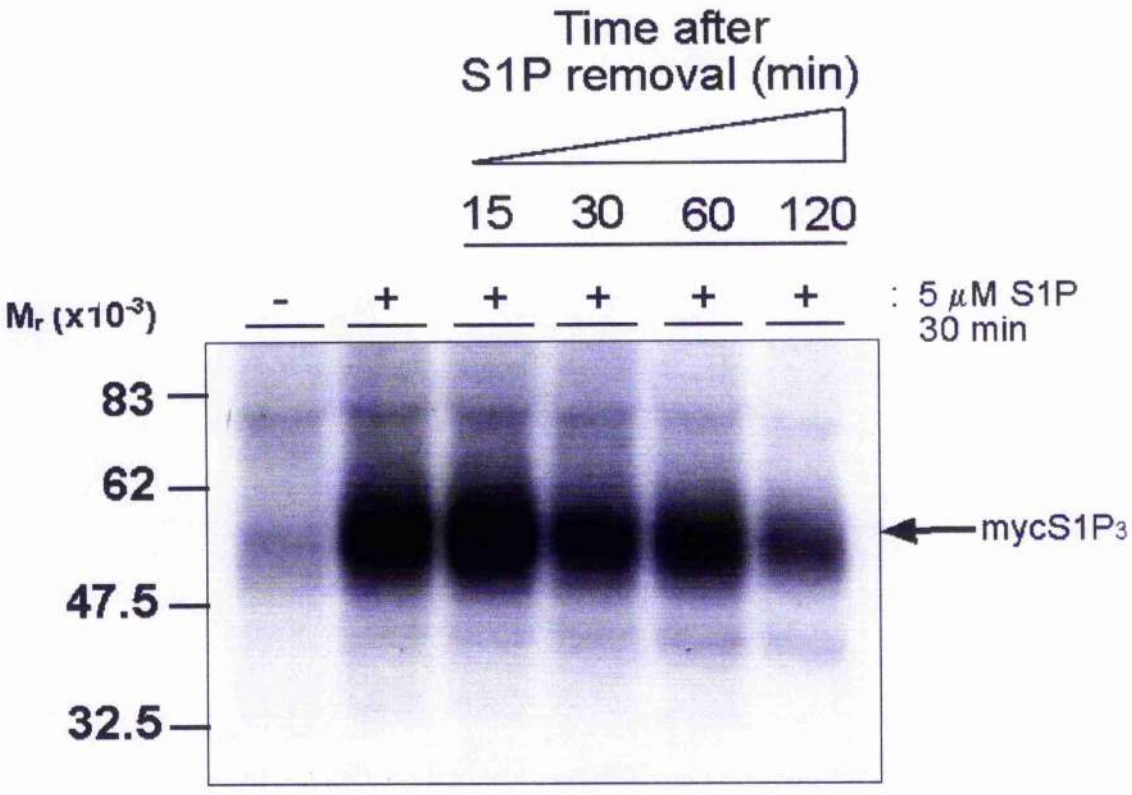

nM
μM
0
2
20
0.2
2
20
: [S1P]
30 min
Mr (x10-3)
83
62
47.5
32.5
mycS1P3
PHOSPHORYLATION
(% of maximum)
100
80
60
40
20
0
1
10
100
1000
10000
100000
[S1P] (nM)

Figure 3.9: The effect of S1P Removal on S1P-Mediated Phosphorylation in CCL-39/mycS1P$_3$ cells

^{32}P-labelled serum starved stably transfected CCL-39/mycS1P$_3$ cells were treated with either vehicle or 5μM of S1P at 37°C for 30min as indicated prior to removal of the medium, washing the cell monolayers twice with 3 ml/well of pre-warmed medium to wash out residual S1P, and incubation in 3 ml/well free S1P-free medium for the indicated times prior to analysis of S1P$_3$ phosphorylation by immunoprecipitation followed by SDS-PAGE and phosphorimaging. Maximal S1P-induced S1P$_3$ phosphorylation was set at 100% and other results were expressed relative to this level. These data represent the mean ± SEM of three experiments. Graph of quantitation from n=3. Astrixes indicate significant reduction in phosphorylation *versus* S1P-treated cells without washout.


Time after
S1P removal (min)
15 30 60 120
M_r (x10^{-3})
- + + + + +
: 5 μM S1P
30 min
83
62
47.5
32.5
mycS1P3

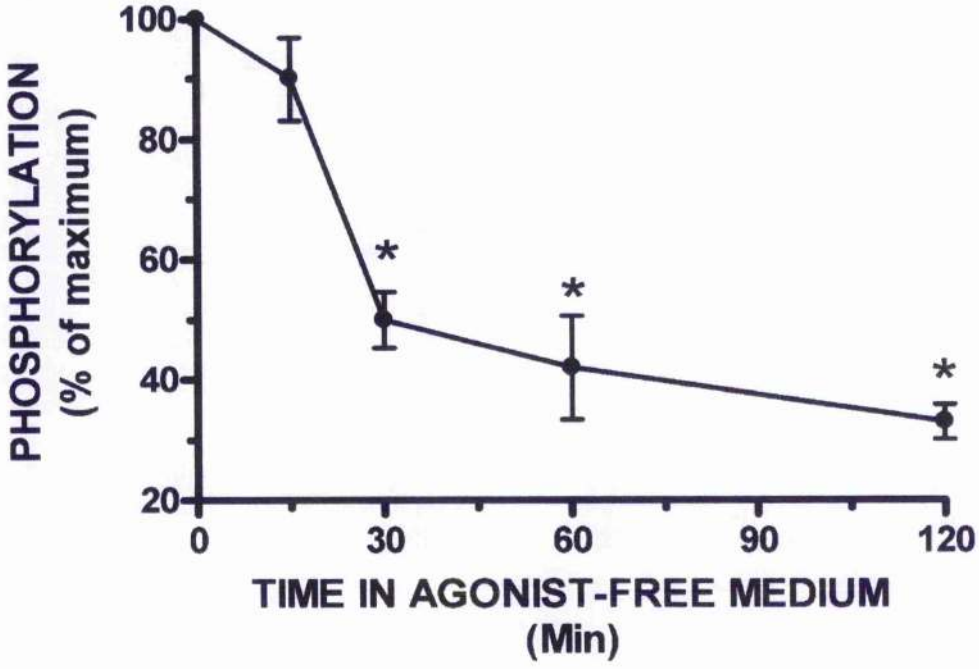

PHOSPHORYLATION
(% of maximum)
100
80
60
40
20
*
*
*
0
30
60
90
120
TIME IN AGONIST-FREE MEDIUM
(Min)

Figure 3.10: Reconstitution of S1P receptor phosphorylation *in vitro* with GRK2

In vitro phosphorylations were performed as described in Materials and Methods. Membranes from mycS1P$_1$- and mycS1P$_3$- expressing CCL39 cells were prepared and incubated with or without 5μM S1P in the absence or presence of 50nM purified recombinant GRK2 as indicated. Preparations of solubilised cell membrane extracts were immunoprecipitated with 9E10 to analyse receptor phosphorylation. Figure represents one of three experiments.

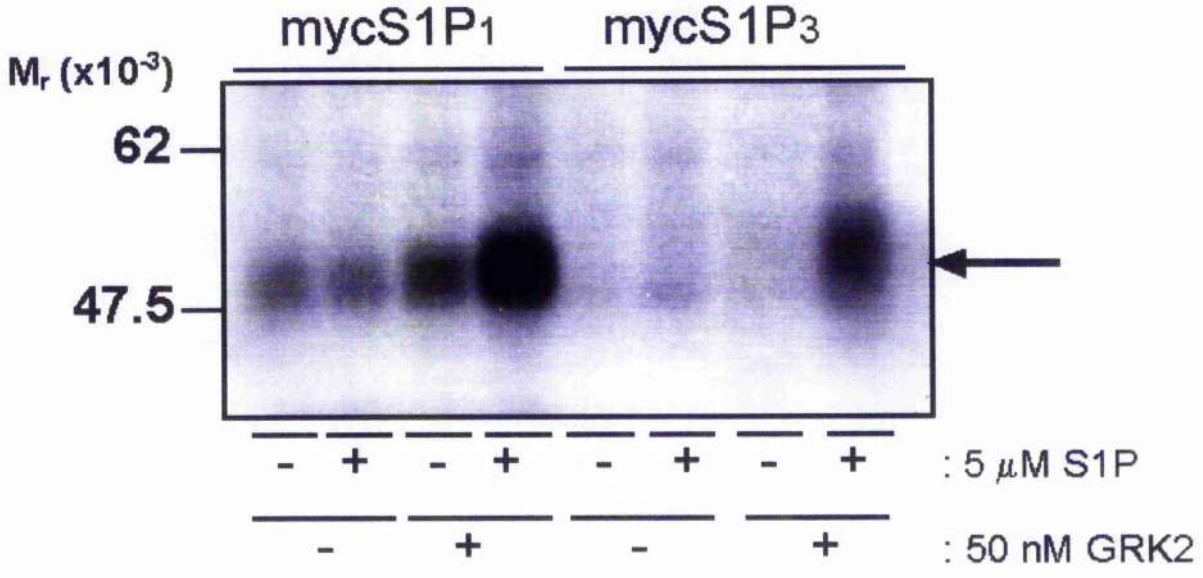

mycS1P1
mycS1P3
Mr (x10-3)
62
47.5
- + - + - + - +
: 5 μM S1P
- + - +
: 50 nM GRK2

Figure 3.11: Comparison of $S1P_3$ Phosphorylation by GRK2 and GRK5 *in vitro*

In vitro phosphorylations were performed as described in Materials and Methods. Membranes from myc$S1P_3$- expressing CCL39 cells were prepared and incubated with or without 5μM S1P in the absence or presence of 50nM purified recombinant GRK2 or GRK5 as indicated. Cell membranes were then solubilised and immunoprecipitated with 9E10 to analyse receptor phosphorylation. Figure represents one of three experiments.

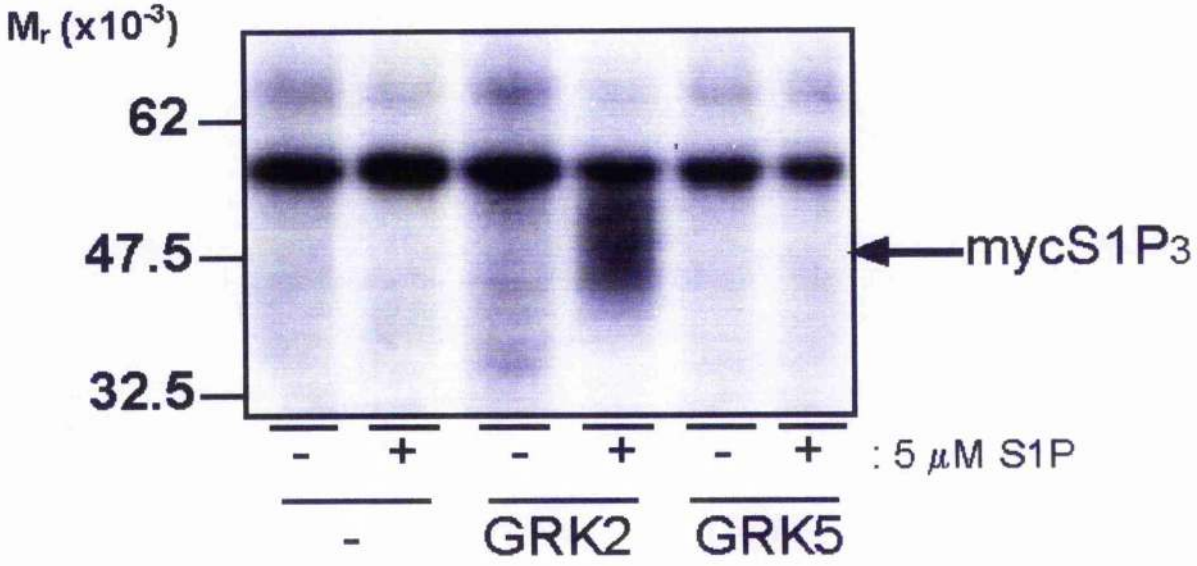

Mr (x10-3)
62
47.5
32.5
mycS1P3
- + - + - +
: 5 μM S1P
-
GRK2
GRK5

Figure 3.12: Rhodopsin Phosphorylation by GRK2 and GRK5 *in vitro*

In vitro rhodopsin phosphorylation assays were performed on urea-treated rod outer segments treated with or without light for 30min at room temperature in the presence of the indicated GRK isoforms as previously described in Section 2.4.6

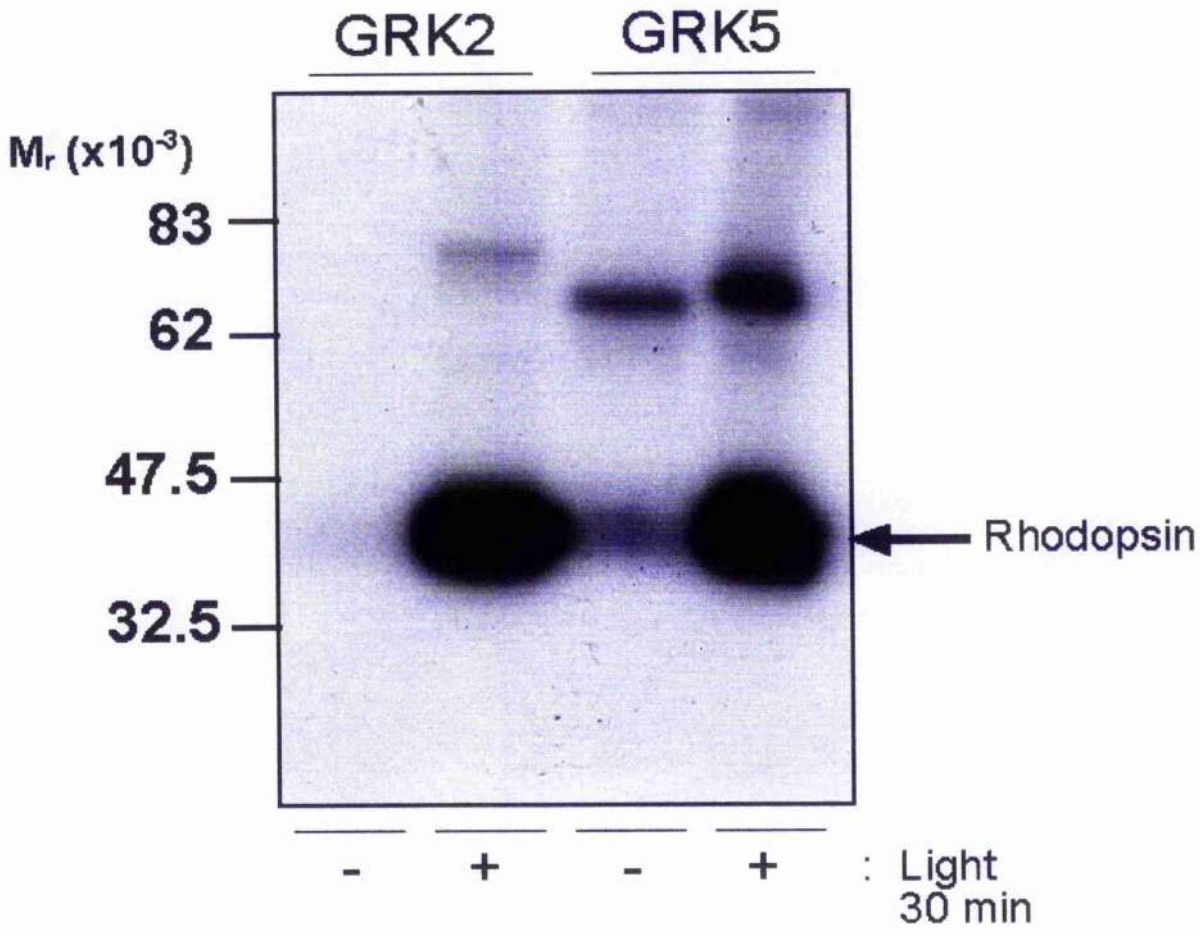
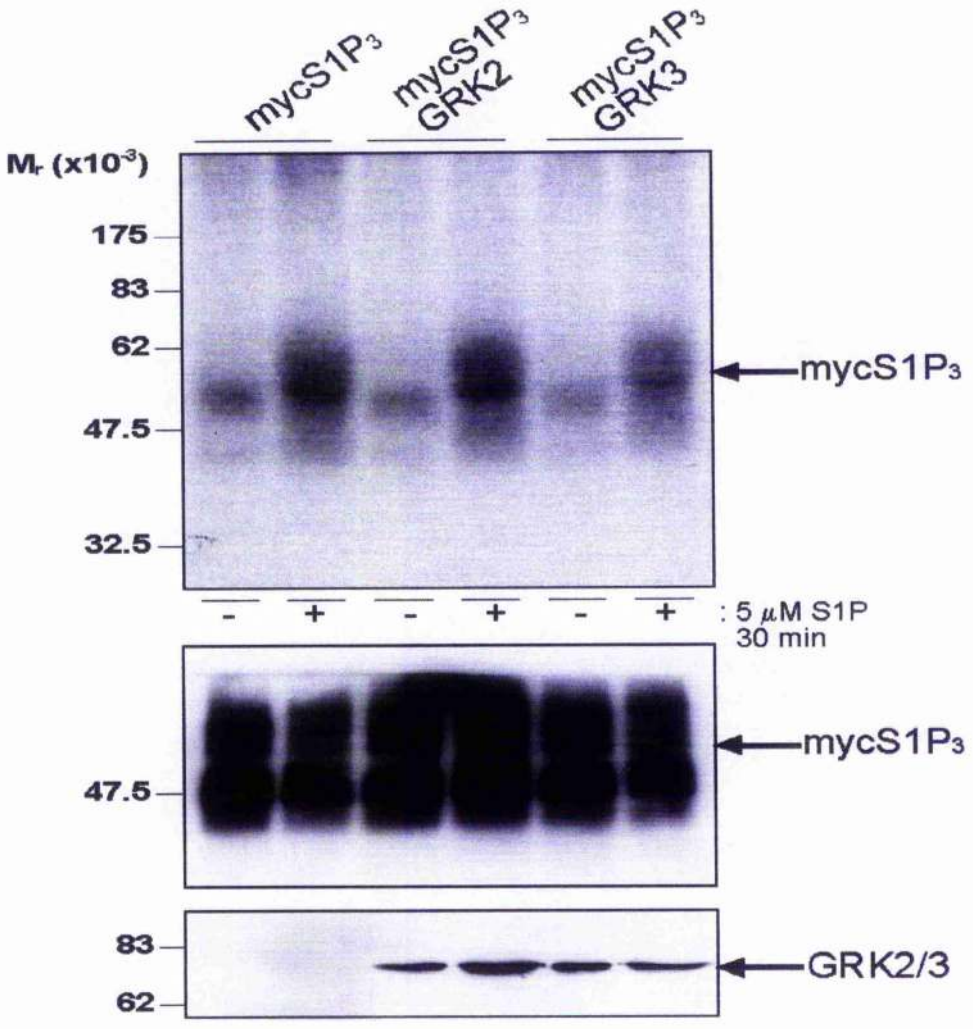

GRK2
GRK5
Mr (x10-3)
83
62
47.5
32.5
Rhodopsin
-
+
-
+
: Light
30 min

Figure 3.13: Lack of potentiation of $S1P_3$ phosphorylation upon overexpression of GRK2 and GRK3 in whole cells

^{32}P-prelabelled HEK293 cells transiently co-expressing myc$S1P_3$ and either GRK2 or GRK3 as indicated were incubated in the absence or presence of 5μM S1P for 30min prior to solublised cell extract preparation and receptor immunoprecipitation with 9E10 as described previously. Confirmation of receptor and GRK expression was made by immunoblotting with anti-myc 9E10 and anti-GRK2/3 monoclonal antibodies as indicated. Graphical analysis shows quantative analysis of data normalised to the level of agonist-stimulated WT $S1P_3$ phosphorylation (set at 100%, n=3). There was no statistically significant difference ($p>0.05$) in $S1P_3$ phosphorylation between each of the conditions.


mycS1P3
mycS1P3 GRK2
mycS1P3 GRK3
Mr (x10-3)
175
83
62
47.5
32.5
mycS1P3
- + - + - +
: 5 μM S1P
30 min
47.5
mycS1P3
83
62
GRK2/3

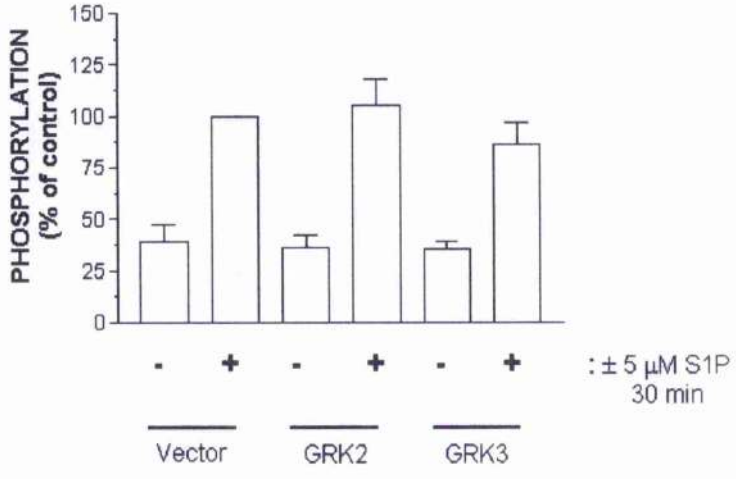

PHOSPHORYLATION
(% of control)
150
125
100
75
50
25
0
- + - + - +
: ± 5 μM S1P
30 min
Vector
GRK2
GRK3

Figure 3.14: Mutational analysis of $S1P_3$ Receptor C-Tail Truncations

The primary sequence of the carboxyl terminal domain of $S1P_3$ following Asn294 is shown. Potential phosphoacceptor sites are highlighted and the positions of the two truncations that were created are indicated by arrows at Δ45 and Δ27.

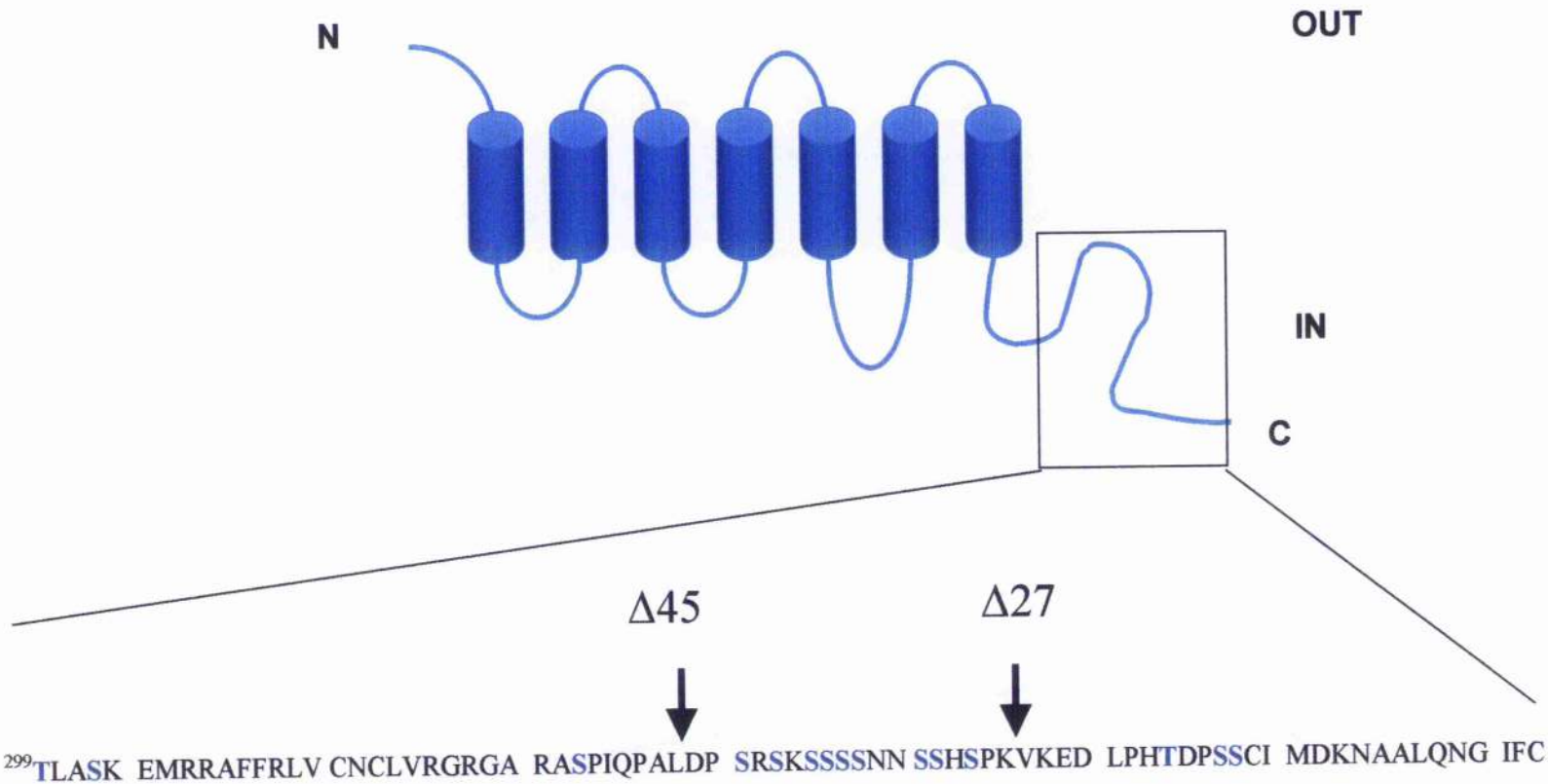

N
OUT
IN
C
Δ45
Δ27
299TLASK EMRRAFFRLV CNCLVRGRGA RASPIQPALDP SRSKSSSSNN SSHSPKVKED LPHTDPSSCI MDKNAALQNG IFC

Figure 3.15: Immunoblot Analysis of the MycS1P$_3$, MycS1P$_3$Δ27 and MycS1P$_3$Δ45

HEK293 cells transiently expressing either the mycS1P$_3$, MycS1P$_3$Δ27 or MycS1P$_3$Δ45 were solubilised and then analysed by SDS-PAGE and immunoblotting with the anti-myc monoclonal antibody 9E10. None of the bands labelled are observed in vector-transfected cells, hence they must be due to differentially processed forms of the receptor.

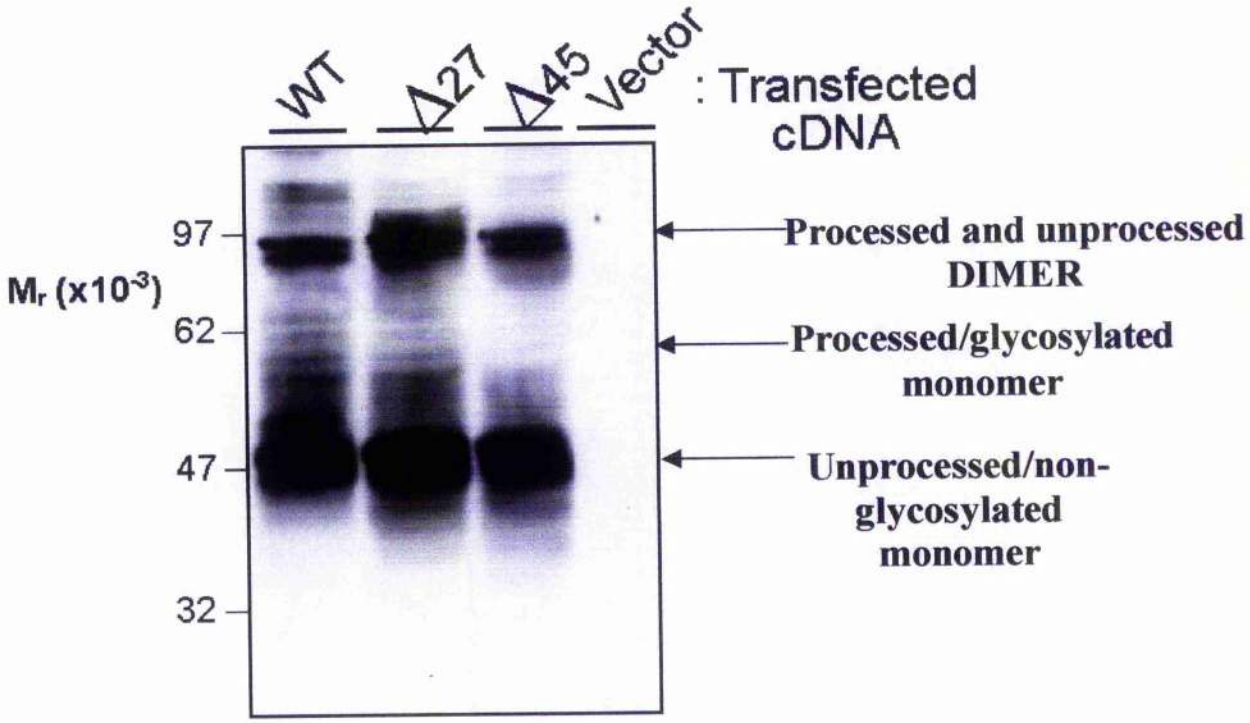
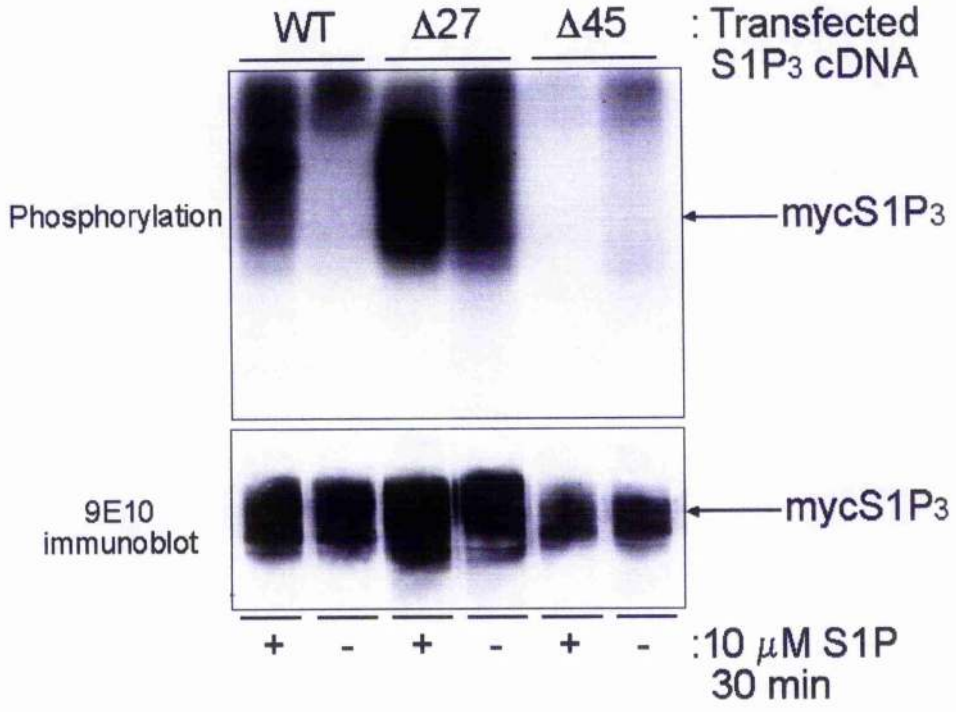

WT
Δ27
Δ45
Vector
: Transfected cDNA
M_r (x10^{-3})
97
62
47
32
Processed and unprocessed DIMER
Processed/glycosylated monomer
Unprocessed/non-glycosylated monomer

Figure 3.16: Whole cell phosphorylation of WT and mutant $S1P_3$ receptors

^{32}P-prelabelled HEK293 cells transiently expressing WT myc$S1P_3$ or mutant $S1P_3$ receptors were incubated in the absence or presence of 10μM S1P for 30min as indicated prior to solubilised cell extract preparation and receptor immunoprecipitation with 9E10 as described previously. Following fractionation of the immunoprecipitates by SDS-PAGE, phosphoproteins were visualised by autoradiography. Graphical analysis shows quantative analysis of data normalised to the level of agonist-stimulated WT $S1P_3$ phosphorylation (set at 100%, n=3). * indicates a significant increase ($p<0.01$) while ** indicates a significant decrease ($p<0.05$) versus the level of agonist-stimulated phosphorylation observed for WT $S1P_3$ under the same conditions (set at 100%).


WT
Δ27
Δ45
: Transfected S1P3 cDNA
Phosphorylation
mycS1P3
9E10 immunoblot
mycS1P3
+ - + - + -
:10 μM S1P 30 min

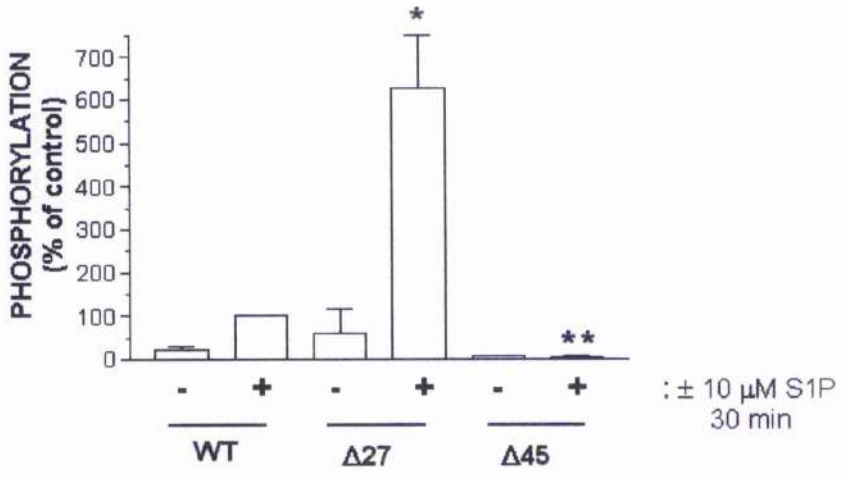
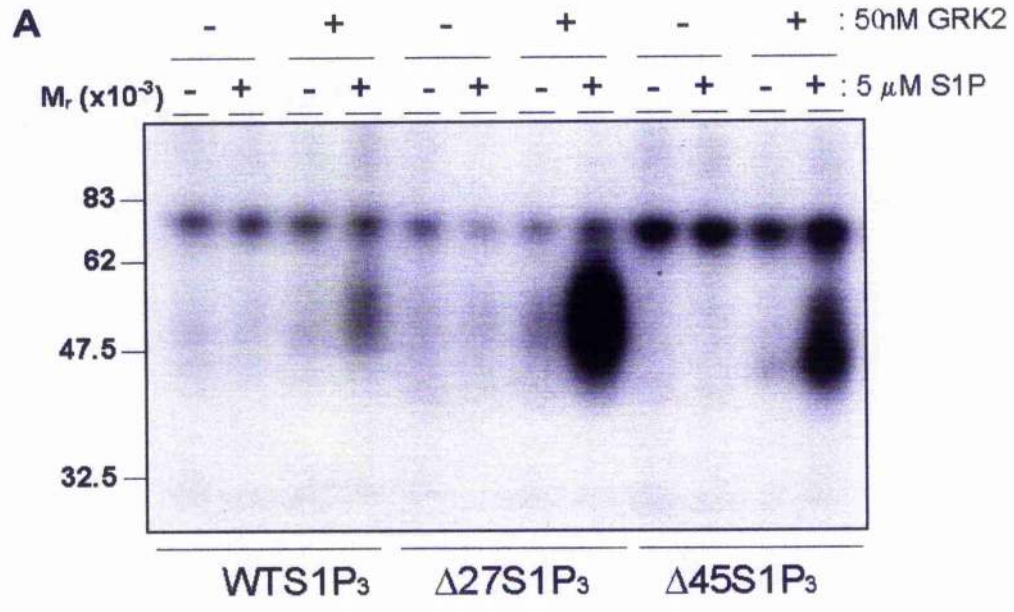

PHOSPHORYLATION (% of control)
700
600
500
400
300
200
100
0
*
**
- + - + - +
: ± 10 μM S1P 30 min
WT
Δ27
Δ45

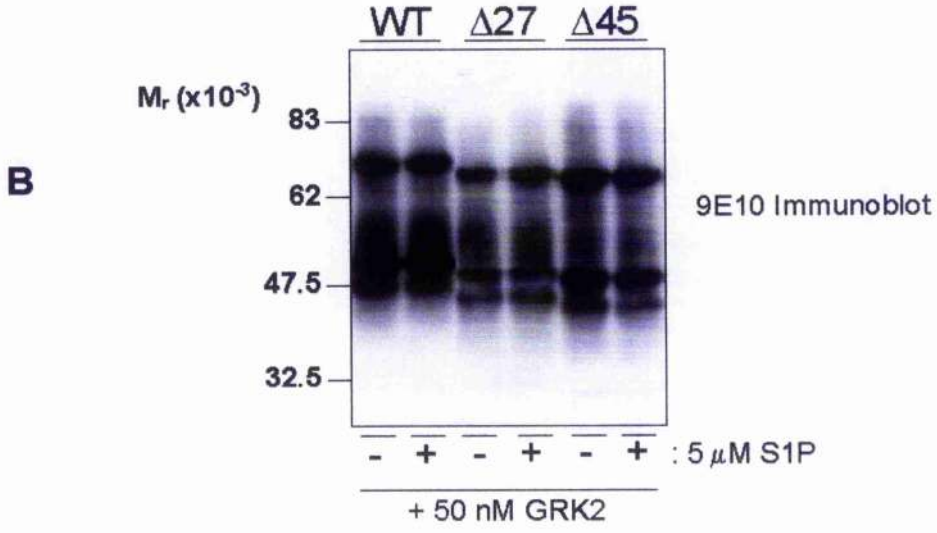
Figure 3.17: *In vitro* phosphorylation of WT and mutant $S1P_3$ receptors with GRK2

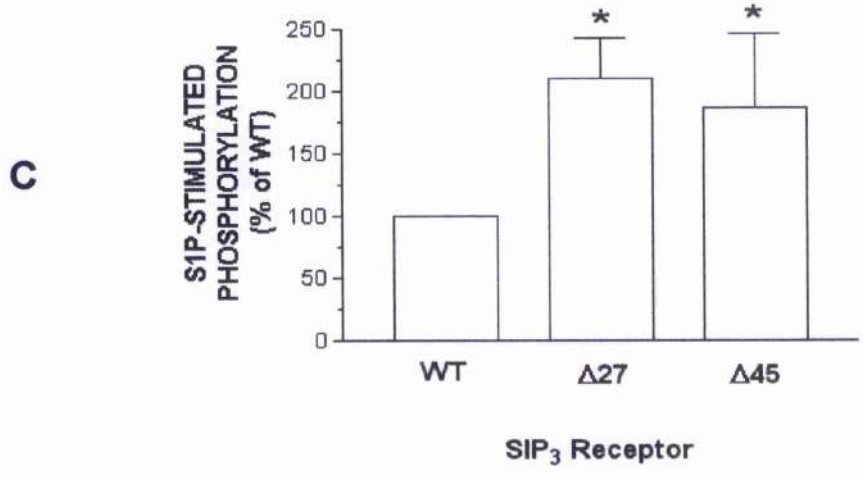
A: Membranes from serum-starved HEK293 cells transiently expressing WT myc$S1P_3$ or mutant myc$S1P_3$ receptors were incubated with [γ-^{32}-P] ATP in the absence or presence of 5μM S1P in the absence or presence of 50nM purified recombinant GRK2 prior to solubilised membrane extract preparation and receptor immunoprecipitation with 9E10 as described previously.

B: Confirmation of receptor expression was made by immunoblotting with anti-myc 9E10.

C: Quantative analysis of data normalised to the level of agonist-stimulated WT $S1P_3$ phosphorylation (set at 100%, n=3). * indicates a significant decrease ($p<0.05$) versus the level of agonist-stimulated phosphorylation observed for WT $S1P_3$ under the same conditions. Statistical significance was determined using the one-way Analysis of Variance (ANOVA) Dunnett multiple comparisons test.


A
- + - + - + : 50nM GRK2
M_r (x10^{-3}) - + - + - + - + - + - + : 5 μM S1P
83
62
47.5
32.5
WTS1P$_3$
Δ27S1P$_3$
Δ45S1P$_3$


B
WT Δ27 Δ45
M_r (x10^{-3})
83
62
47.5
32.5
9E10 Immunoblot
- + - + - + : 5 μM S1P
+ 50 nM GRK2


C
S1P-STIMULATED PHOSPHORYLATION (% of WT)
250
200
150
100
50
0
*
*
WT
Δ27
Δ45
SIP$_3$ Receptor

CHAPTER 4

Characterisation and Analysis of Human $S1P_3$ Receptor Subcellular Distribution

Characterisation and Analysis of $S1P_3$ Subcellular Distribution

4.1 Introduction

To understand how a receptor is localised at the plasma membrane is the first step in characterising the manner in which a receptor is internalised by a cell. Internalisation of GPCRs is an integral part of receptor regulation. The majority of GPCRs require phosphorylation as a precursor to their internalisation, as evidenced by β_2AR and thrombin receptors, amongst others [Lefkowitz, 2004]. Generally, receptor phosphorylation increases affinity for arrestins, which uncouple GPCRs from their associated G-proteins. As arrestin is an adaptor protein, it also functions directly in receptor trafficking and targeting of receptors to clathrin-coated vesicles from where the receptor is sequestered from the membrane into the cell [Goodman *et al.*, 1997; Krueger *et al.*, 1997; Ostrom, 2002]. By bringing a receptor closer to an endosome-associated phosphatase, GPCR internalisation is believed to promote dephosphorylation [Zhang *et al.*, 1996; Koenig and Edwardson, 1997]. Dephosphorylation and recycling of receptors back to the plasma membrane contributes to the reversal of desensitisation [Krueger *et al.*, 1997]. This process of resensitisation is required for complete recovery of cellular signalling following agonist withdrawal [Krueger *et al.*, 1997; Krupnick and Benovic, 1998]. Alternatively, the internalised receptor can be targeted to lysosomes for degradation [Koenig and Edwardson, 1997; Innamorati *et al.*, 2001; Fuertes *et al.*, 2003].

As described in Chapter 3, $S1P_3$ is phosphorylated in the presence of agonist, and the requires the presence of serine and threonine residues within the last 45 amino acids of the receptor's C-terminal tail. As a precedent, $S1P_1$ has already been shown to be phosphorylated at serine and threonine residues within the C-terminal tail and internalises maximally after two hours exposure to S1P [Van Brockyln *et al.*, 1998; Watterson *et al.*, 2002]. In this Chapter, the internalisation of human myc$S1P_3$ receptor stably expressed in CCL-39 hamster lung fibroblasts was characterised using cell surface biotinylation and confocal microscope analysis, while desensitisation was investigated through calcium mobilisation assays in Rat1a fibroblasts.

The β_2AR receptor was the first GPCR where internalised receptors could be distinguished from cell surface populations. This was shown using astrocytoma cells administered with catecholamine which resulted in a decrease in stimulated adenylate cyclase activity and a corresponding differential sedimentation on sucrose gradients compared with un-stimulated cells [Harden *et al.*, 1980]. The internalised receptors were found to be associated with a "light vesicle" fraction that could be separated from a "heavy vesicle" plasma membrane

fraction that was associated with the cell surface receptors [Harden *et al.*, 1980]. Previously, it has been reported that $S1P_1$ is targeted to plasmalemmal caveolae upon agonist stimulation [Igarashi and Michel, 2000]. Although this finding could not be reproduced by several members of the lab, it was useful to see how $S1P_3$ was distributed at the plasma membrane with and without agonist stimulation using a similar sucrose density system. It has been shown in a recent study that in $S1P_1$-expressing HEK293 cells, S1P exposure results in the translocation of β-arrestin 2 (arrestin 3) towards activated $S1P_1$ receptor [Hobson *et al.*, 2001]. It is likely that as the $S1P_3$ receptor also interacts with PDGF, *via* a distinct mechanism (Figure 1.14), that $S1P_3$ once activated by S1P also results in arrestin 3 translocation [Baudhuin *et al.*, 2004; Payne *et al.*, 2004; Tanimoto *et al.*, 2004; Waters *et al.*, 2004].

In this Chapter the investigations of subcellular localisation are described in succession from biotinylation and confocal laser microscopy studies, sedimentation sucrose density gradient assays to arrestin co-localisation analysis and intracellular calcium mobilisation assays.

4.2 Results

The $S1P_3$ receptor is phosphorylated in the presence of S1P, as described in the previous Chapter. That $S1P_3$ is phosphorylated in the presence of S1P allowed the enquiry of whether $S1P_3$ phosphorylation results in $S1P_3$ internalisation. Stable myc$S1P_3$ expressing CCL-39 cells provide a suitable model to characterise $S1P_3$ internalisation. A cell surface receptor biotinylation assay was performed on myc$S1P_3$-expressing CCL-39 cells which were exposed to 10μM S1P over a time course of two hours. The underlying premise of the biotinylation assay is that it makes use of the extreme size and abundance of biotin molecules which has a very high binding affinity with streptavidin ($10^{13}m^{-1}$), this conjugation is a stable interaction where streptavidin binds four molecules of biotin. Initially, sodium periodate oxidizes the glycoproteins present in the $S1P_3$ receptor to form aldehydes which can spontaneously react with hydrazides. Biotin-hydrazide is used to attach biotin onto the oxidized glycoproteins. Biotinylated proteins can then be detected by addition of streptavidin conjugated to horseradish peroxidase for luminol-based detection using ECL™ reagents. This shows that $S1P_3$ is not significantly internalised over time in CCL39 cells (Figure 4.1). After establishing a lack of internalisation in CCL39 cells the next clarification was to assert whether internalisation was dependent on agonist concentration. As seen with the time course experiment, these experiments demonstrate that no internalisation was seen at any of the concentrations used (Figure 4.2).

To allow visualisation of any S1P-induced changes in $S1P_3$ receptor trafficking, a myc$S1P_3$-GFP construct was generated and stably expressed in CCL-39 hamster lung fibroblasts as described in Materials and Methods Section 2.3.7 (Figure 4.3). Stable expression of the myc$S1P_3$-GFP receptor was confirmed by immunoblotting using the anti-myc monoclonal antibody 9E10 and a monoclonal anti-GFP antibody (Figure 4.4). Both receptors were shown to be expressed at similar levels as visualised by the 9E10 antibody. Incubation with the anti-GFP antibody selectively identified the $S1P_3$-GFP protein. The observed difference in molecular mass between the wild type $S1P_3$ receptor and the $S1P_3$-GFP receptor (around 28kDa) was consistent with the addition of the GFP tag. In previous similar studies of the $S1P_1$ receptor, it was confirmed that addition of the GFP tag does not influence receptor function. This has also been shown for $S1P_1$GFP [Watterson *et al.*, 2002] and other tagged GPCRs, such as β_2AR [Kallal and Benovic, 2000].

Based on the assumption that $S1P_1$ and $S1P_3$ share significant homology it was deemed appropriate to follow the same method of experimental analysis as observed in previous work carried out on the $S1P_1$ receptor [Watterson *et al.*, 2002] where the addition of a GFP tag to the C

terminus of the receptor did not appear to cause obstruction to receptor internalisation. The CCL-39/myc$S1P_3$-GFP cell line represented a relevant model for studies of $S1P_3$ receptor trafficking upon exposure to S1P. As initially observed, the CCL-39/myc$S1P_3$-GFP cells are present on the cell surface in unstimulated cells. When CCL-39/myc$S1P_3$-GFP cells were exposed to 10μM S1P over a two hour time course and studied with confocal microscopy, a two hour treatment of 10μM S1P does not result in a significant translocation of myc$S1P_3$-GFP from the cell surface as compared to untreated myc$S1P_3$-GFP (Figure 4.5). These data correlate with the cell surface biotinylation experiments, where myc$S1P_3$-expressing cells are shown not to internalise over time or increasing concentration of agonist. Also, this would suggest that there is no gross re-distribution of $S1P_3$ into microdomains at the cell surface. Currently, it is unknown how $S1P_3$ receptors are distributed within the plasma membrane. To determine $S1P_3$ receptor distribution and how it may change with agonist exposure a series of sucrose density gradient assays were performed (Figure 4.6) to identify whether $S1P_3$ is localised to lipid rafts after exposure to agonist, as observed for other GPCRs [Simons and Ikonen, 1997]. The gradients observed with agonist stimulation did show some redistribution of $S1P_3$ from plasma membrane fractions (~ fraction 9 to fraction 12), to vesicle fractions (fraction 1 to fraction 8) upon stimulation with S1P which suggests that recruitment of the receptor at the surface is possibly being blocked by a protein adaptor complex.

If $S1P_3$ is not localised at clathrin coated pits after agonist stimulation it is possibly because it is not recruited there by arrestin. This could be due to a number of reasons, for example, steric hindrance of the receptor due to other protein-$S1P_3$ receptor interactions may cause obstruction of arrestin binding. In order to observe what is preventing $S1P_3$ from internalising in CCL-39 cells it was important to determine whether arrestin would complex with the $S1P_3$ receptor. Preliminary findings from studies of co-expression of the $S1P_3$ receptor with arrestin-3 in CCL-39 cells indirectly suggest that agonist-stimulation of the $S1P_3$ receptor promotes downstream arrestin recruitment as observed by the increase in $S1P_3$ association after co-immunoprecipitation with arrestin 3 over a time course (Figure 4.7). However, due to the limited data collated in this experimentation further analyses are required to fully confirm this hypothesis.

However, further investigations with the Rat1a cell type have shown that $S1P_3$ can internalise upon S1P exposure (Figure 4.8), which further supports the concept of dynamic regulation of the $S1P_3$ receptor *via* differential internalisation. Biotinylation and confocal scanning laser microscopy studies of internalisation (Figure 4.1, 4.2 and 4.5) have shown that

CCL39 cells over-expressing myc-$S1P_3$ were unable to internalise upon exposure to S1P, and this itself was in contrast to investigations of internalisation for myc-$S1P_1$ in CCL39 cells [Watterson *et al.*, 2002]. These findings, taken with the internalisation observed for HEK293 cells over-expressing myc-$S1P_3$, suggest an as yet unobserved level of $S1P_3$ receptor regulation which has potential ramifications for downstream signalling, whereby differential internalisation between celltypes regulates variation in cell migration, proliferation and survival, and provides a novel mechanism that could be used to selectively control pathological conditions, such as angiogenesis.

Agonist-stimulated phosphorylation by GRKs of many GPCRs is followed by the removal of the phosphorylated receptor away from the cell surface into endosomal vesicles [Zhang *et al.*, 1997; Laporte *et al.*, 2002]. For example, it has been demonstrated that $S1P_1$ is internalised immediately following GRK2 phosphorylation of the receptor protein [Liu *et al.*, 1999; Watterson *et al.*, 2002]. Whether $S1P_3$ regulation is comparable with $S1P_1$ despite evidence for phosphorylation by a GRK-independent process was assessed *via* biotinylation assays which quantitated the levels of cell surface WT and mutant $S1P_3$ in HEK293 cells after S1P exposure. Following agonist treatment for 2hr, a significant loss of WT $S1P_3$ from the cell surface was noticed. Furthermore, successive truncations of the C-terminal tail of the receptor failed to significantly decrease the extent to which internalisation occurred, posing the hypothesis that prior phosphorylation of $S1P_3$ is not required for internalisation to occur (Figure 4.8).

GPCRs classically undergo rapid functional desensitisation of heterotrimeric G-protein signalling after they have been phosphorylated [Zhang *et al.*, 1997]. From the evidence for GRK-independent phosphorylation of $S1P_3$ in intact cells and its ability to internalise without receptor phosphorylation, it was important to determine the susceptibility of WT and mutant $S1P_3$ receptors to undergo functional desensitisation in intact cells. This was achieved by assessment of S1P-induced changes in $[Ca^{2+}]_i$ in Fura2-labelled Rat1a cells transiently expressing WT and non-phosphorylated Δ45 mutant $S1P_3$ receptors (Figure 4.9). Transfected cells chosen for study were identified by co-transfection with a GFP expression construct and recombinant protein expression was visualised by fluorescence microscopy. From initial experiments it was shown that as cells expressing GFP alone failed to significantly mobilise $[Ca^{2+}]_i$ after agonist exposure, cells co-expressing GFP and either WT or Δ45 $S1P_3$ receptors appeared to increase transient mobilisation of $[Ca^{2+}]_i$ after S1P challenge. Repeated challenge with a submaximal concentration of S1P induced a time-dependent desensitisation of agonist-

stimulated $[Ca^{2+}]_i$ mobilisation by both receptors. This emerged as a possible homologous desensitisation event that did not significantly deplete the intracellular calcium pool, as the response to endogenous α_{1B}-adrenoceptor activation by phenylephrine was identical between control GFP-expressing and $S1P_3$-expressing cells following S1P exposure (Figure 4.10 and 4.11).

There was no significant difference in desensitisation after S1P exposure between the WT receptor or the phosphorylation-resistant Δ45 $S1P_3$ (Figure 4.11), which intimates that functional desensitisation of $[Ca^{2+}]_i$ mobilisation in Rat1a cells progresses in a receptor phosphorylation- and internalisation-independent manner. A potential reason for the receptor phosphorylation independence of functional desensitisation of $[Ca^{2+}]_i$ mobilisation in Rat1a cells could be that the phospholipase C-β isoforms activated by $S1P_3$ are subject to negative regulation by PKC-mediated phosphorylation, which has been demonstrated for several G_i and $G_{q/11}$-coupled GPCRs, including the *N*-formyl-MetLeuPhe, P2Y2 purinergic and M3 muscarinic acetylcholine receptors [Ali *et al.*, 1998; Strassheim and Williams, 2000]. However, it was noted by personal communication [John Pediani], that pretreatment with a maximally effective concentration of the PKC inhibitor GF109203X failed to change WT and Δ45 mutant $S1P_3$ receptor desensitisation kinetics, suggesting that desensitisation is PKC independent.

4.3 Discussion

This Chapter has established through cell surface biotinylation and confocal microscopy studies that in CCL-39 cells the $S1P_3$ receptor, unlike the $S1P_1$ receptor, is not internalised upon agonist-stimulated phosphorylation in CCL39 cells. This behaviour appears to be cell-type specific as experiments on HEK293 cells have shown that $S1P_3$ can internalise in these cells. In contrast to many G-protein-coupled receptors, $S1P_3$ is not lost from the cell surface following sustained agonist exposure for up to two hours in CCL-39 cells.

In attempting to characterise $S1P_3$ as a classic GPCR this research has discovered the observation that $S1P_3$ possesses unique cell-type specific resistance to internalisation. Examples abound to highlight that individual subtypes of the S1P family are expressed differently from one cell type to another depending on their specific functions. For example, human cardiac S1P receptors are differentially expressed within cardiovascular tissues. The human cardiac $S1P_3$ receptor is localised predominantly in the aorta and is also found in SMCs of cardiac vessels whereas $S1P_1$ is present in cardiomyocytes and cardiac vessel endothelial cells, and is usually co-expressed with $S1P_2$ and $S1P_3$ [Mazurais *et al.*, 2002]. Another study has also shown that the expression of $S1P_3$ and $S1P_2$ receptors is 4-fold higher in murine cerebral artery compared with aorta [Coussin *et al.*, 2002], suggesting species variation.

In relation to cardiovascular disease, it is possible that dysfunctional expression of $S1P_1$ and/or $S1P_3$ would prevent the specificity of receptor internalisation required, for example, to balance blood vessel development or bradycardia and that this would ultimately create a negative feedback of downstream signalling that could result in angiogenesis, athlerosclerosis or ischemia, conditions for which both of these receptors have been implicated [Licht *et al.*, 2003; Forrest *et al.*, 2004; Sanna *et al.*, 2004]. Additionally, the finding that RGS_{1-4} differentially regulate signalling through $S1P_{1-3}$ receptors, combined with the effects of RGS proteins in AT-1 and ET_A receptor signalling suggest additional levels of specificity in regulation of S1P receptors [Cho *et al.*, 2003]. Subsequently, from the findings of cell type specificity in $S1P_3$ regulation in this research, it is possible that in fibroblast cells receptors are required for longer-term signalling at the cell-surface, for example, in the immune system, whereas in vascular cells the receptors are orchestrated at a faster pace, and this fine tuning of S1P receptor turnover allows rapid signalling events that are necessary in the cardiovascular system. CCL39, HEK293 and Rat1a cell lines are all fibroblast or fibroblast-like cells. When each of these celltypes overexpresses the $S1P_3$ receptor, and is treated with agonist, $S1P_3$ appears to show differential internalisation between cell types. This suggests that dynamic regulation of S1P receptors is dependent on celltype.

It has been suggested for $S1P_1$ that its expression in embryonic development in mice allows the S1P activated endothelial cells to become permissive to smooth muscle cells and pericytes to vessel walls [Allende and Proia, 2002]. Further results from Allende's group have indicated that the $S1P_1$, $S1P_2$ and $S1P_3$ receptors have redundant or cooperative functions for the development of a stable and mature vascular system during embryonic development [Kono *et al.*, 2004]. Redundant and cooperative functions of S1P receptors suggests that with regard to cell type, the greater level of expression of cooperative functioning S1P receptors will be preferred above those receptors that have redundant functions in development. $S1P_1$ is critical for S1P-induced, Gi-dependent migration, but not for PDGF-BB-induced, receptor tyrosine kinase-dependent chemotaxis in vascular smooth muscle cells [Kluk *et al.*, 2003]. Together this supports the model of distribution of receptor subtypes in endothelial cells having specific impact on the activity of the cells in relation to their environment.

Similar to $S1P_1$ [Igarashi and Michel, 2000] evidence from sucrose density gradient supports localisation of $S1P_3$ with caveolin-1. From studies of sucrose density gradients it has been observed that upon agonist exposure there is a significant change in localisation of $S1P_3$ within the plasma membrane. After stimulation with agonist, $S1P_3$ appears to localise to with caveolin-1. The significance of this localisation to lipid rafts has yet to be realised. This could be achieved through investigations of the actions of constituents of the lipid rafts by using statins to disrupt cholesterol levels.

From the results of subcellular distribution investigations it appeared important to discover if $S1P_3$ was able to interact at all with arrestin in receptor recruitment. Arrestins, as mentioned in Chapter 1, assist in recruiting receptors to clathrin-coated pits in order for them to become internalised. If this event requires agonist exposure, it is reasonable to assume that once S1P is delivered to the cell the localisation of $S1P_3$ at the plasma membrane would predominate in areas rich in caveolin, such as lipid rafts, in order for $S1P_3$ to become internalised. If however, as is proposed, a unique SH3 binding domain (or another interacting protein), obstructs interaction with arrestin, the receptor may not internalise and localisation within the plasma membrane would appear non-specific, i.e. no recruitment to lipid rafts. Preliminary findings have suggested an interaction between arrestin and the $S1P_3$ receptor upon agonist-stimulation. As $S1P_3$ does appear to co-localise with arrestin it is feasible that if internalisation does not occur there must be another interacting protein that restricts internalisation. This suggests that there may be an arrestin-dependence but does not explain the absence of internalisation.

As there appears to be no relation between phosphorylation and desensitisation or internalization it is suggested that other aspects of $S1P_3$ function are regulated by internalisation

and phosphorylation perhaps tyrosine kinase activity or activation of JNK. Evidence of precedents for resistance to internalisation despite receptor phosphorylation is provided by the impact of MAP kinase isoforms ERK1/2 on the internalization of DORs expressed in HEK293 cells. In a recent study, δ opiod receptors (DORs) were found to elicit different responses after ERK/MAP kinase phosphorylation was blocked [Eisinger and Schulz, 2004]. The study showed that DOR activation by etorphine transiently phosphorylated ERK/MAP kinases and brought about DOR internalization within 20min. In contrast, prolonged exposure of HEK293 cells to morphine excited persistent phosphorylation of ERK/MAP kinases, and those cells failed to internalize the opioid receptor. When ERK/MAP kinase phosphorylation was blocked by 2'-Amino-3'-methoxyflavone (PD98059), morphine gained the ability to strongly induce DOR endocytosis. The importance of activated MAP kinases for DOR internalization was further demonstrated by substances that induce phosphorylation of ERK1/2 and concomitantly prevent DOR sequestration by etorphine. Receptor internalization by morphine was also facilitated by inhibition of protein kinase C and opioid-mediated transactivation of epidermal growth factor receptor (EGFR), both activating ERK/MAP kinases by opioids. The mechanism permitting DOR internalization by PD98059 may relate to arrestin, which uncouples GPCRs and thus triggers receptor internalization. Arrestin considerably translocates toward the cell membrane upon DOR activation by morphine in presence of the MAP kinase blocker, but it fails in the absence of PD98059. The study concluded that ERK/MAP kinase activity prevents opioid receptor desensitization and sequestration by blocking arrestin 2 interaction with activated DORs [Eisinger and Schulz, 2004]. Evidence is gathering to suggest that ceramide and its metabolites sphingosine and S1P represent a new class of intracellular second messengers that mediate a variety of cellular functions. Sphingosine and S1P have been shown to induce mitogenesis in a wide range of cell types. PDGF, a potent mitogen, increases cellular levels of sphingosine and S1P, and inhibition of the PDGF-induced increase in S1P levels markedly decreased PDGF-induced cellular proliferation.

Correspondingly, $S1P_3$ may be prevented from internalisation by a kinase that blocks the machinery of the multi-complex that includes the interaction between arrestin2 and the activated $S1P_3$ receptor. It is possible that inhibiting PDGF receptor signalling in CCL39 cells may facilitate $S1P_3$ sequestration by S1P in the same way that DORs internalize when EGF is inhibited by PD98059.

Disruption of the PDGF-BB or PDGFR-β genes in mice resulted in defective ensheathment of nascent blood vessels. Dysfunctional migration of $S1P_1$ null embryonic

fibroblasts toward a gradient of PDGF links these two phenotypes at the final steps of vascular development, underscoring the importance of $S1P_1$ and endothelial cell-pericyte communication in vascular maturation and angiogenesis. This study revealed novel cross-talk between a receptor tyrosine kinase, PDGFR, and a GPCR, $S1P_1$ [Usui *et al.*, 2004].

Binding of PDGF to its receptor activates and recruits sphingosine kinase to the leading edge of the cell. This localized formation of S1P spatially and temporally stimulates $S1P_1$, resulting in activation and integration of downstream signals essential for cell locomotion, such as FAK and Src, necessary for turnover of focal complexes, and the small guanosine triphosphatase Rac, important for protrusion of lamellipodia and forward movement. These results shed light on the proposed vital role of $S1P_1$ in vascular maturation and angiogenesis. Further support for such receptor cross-communication recently emerged from the demonstration that PDGFR is tethered to $S1P_1$ providing a platform for integrative signaling by these two types of receptors [Alderton *et al.*, 2001b].

Alderton et al., [2001] concluded that growth factor receptor-GPCR complexes provide a platform for integrating signals from different receptor classes. A mechanistic model was provided that may account for the co-mitogenic effect of GPCR agonists with growth factors. More specifically, the proposed model provided a mechanism that may account for PDGF-stimulated cell motility being $S1P_1$-dependent [Hobson *et al.*, 2001]. S1P released from cells (not HEK293 cells) in response to PDGF could act back on S1P1-PDGF receptor complexes to induce more efficient downstream stimulation of effector pathways in response to PDGF. This might be specific to certain cell types where S1P functions as an autocrine with PDGF [Alderton *et al.*, 2001b]. Likewise, $S1P_3$ involvement in Akt activation has been discussed in relation to crosstalk with PDGF and integrative and sequential models of signalling [Baudhuin *et al.*, 2004] as mentioned previously, Figure 1.15.

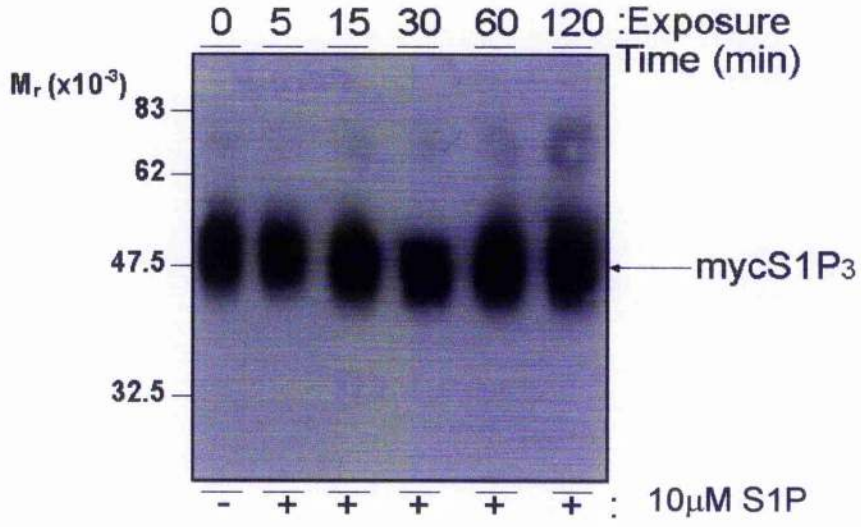
In contrast, it was recently proposed that tyrosine kinase receptors, such as the insulin-like growth factor-1 receptor, transactivate $S1P_1$ through Akt-dependent phosphorylation that does not require the sphingosine kinase pathway [Alderton *et al.*, 2001a]. Possibly, $S1P_3$ could interact with PDGF in a comparable way to $S1P_1$ binding IGF-1-activated Akt. It has been demonstrated for $S1P_1$ that residue Thr236 on the third intracellular loop is sensitive to phosphorylation by Akt, essential for $S1P_1$ activation of Rac which induced lamellapodia formation and migration of vascular endothelial cells [Lee *et al.*, 2001]. The third intracellular loop of $S1P_3$ (Figure 1.10) contains five serine residues, two of which have direct proximity to residue 236 but these do not impart the same sensitivity to Akt for $S1P_3$ as Thr236 does to $S1P_1$ [Lee *et al.*, 2001]. Further studies are necessary to validate the generality of this concept of S1P-

independent activation of S1PRs.

In conclusion, through mutagenesis studies $S1P_3$ has been shown to be rapidly and reversibly phosphorylated in whole cells within an area of 18 residues on the C-terminal domain in response to increased S1P concentrations. However, unlike $S1P_1$ and many other GPCRs, receptor phosphorylation is not required to trigger sequestration of $S1P_3$ away from the plasma membrane. One possible explanation is that constitutive association of a cytosolic protein or interaction motif within the carboxyl-terminal domain of $S1P_3$ is preventing the binding of arrestin proteins, which promote the clustering of GRK phosphorylated GPCRs within clathrin-coated pits prior to their internalisation. Functional analysis of wild type and phosphorylation resistant $S1P_3$ receptor-stimulated calcium mobilisation indicates that sensitivity to agonist-induced phosphorylation has no influence on the kinetics of $S1P_3$ receptor desensitisation. The question of whether a non-phosphorylated receptor can still bind arrestins to cause desensitisation is one that could be answered by mutagenesis. Point mutations of specific $S1P_3$ phosphorylation sites could be devised so that investigations of arrestin translocation can be made.

Figure 4.1: Detection of the Lack of S1P-Mediated Time-Dependent $S1P_3$ Receptor Internalisation in CCL-39 cells

Serum-starved stably transfected CCL-39/myc$S1P_3$ cells were treated at 37°C with vehicle or 10 μM S1P for the times indicated. The cells were examined for internalisation by cell surface labelling with biotin-LC-hydrazide, followed by immunoprecipitation of solubilised receptors with anti-myc 9E10 antibody. Biotin labelling of vehicle-treated CCL-39/myc$S1P_3$ cells was set at 100% and the results following agonist treatment expressed relative to the control. The data represents the mean ± SEM of three similar experiments.


0 5 15 30 60 120 :Exposure Time (min)
M_r (x10^{-3})
83
62
47.5
32.5
mycS1P3
- + + + + + : 10μM S1P

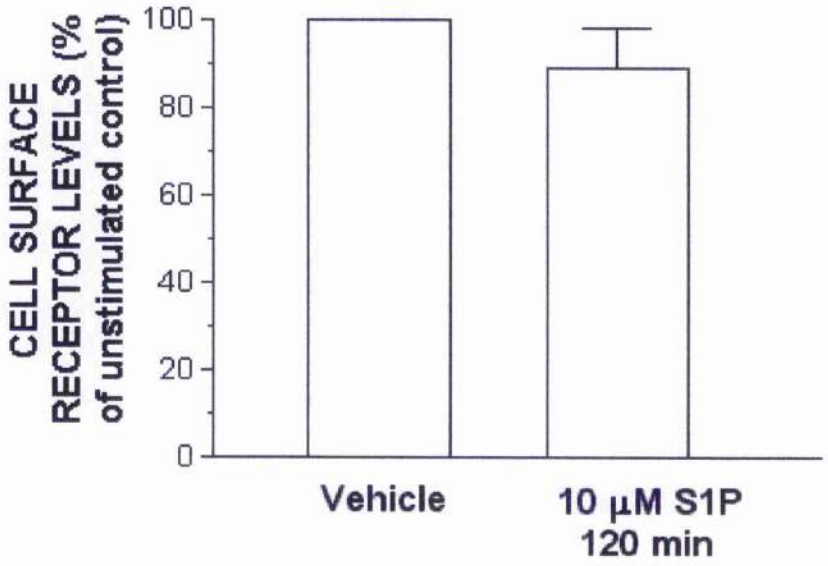

CELL SURFACE RECEPTOR LEVELS (% of unstimulated control)
100
80
60
40
20
0
Vehicle
10 μM S1P 120 min

Figure 4.2: Detection of the Lack of S1P-Mediated Dose-Dependent $S1P_3$ Receptor Internalisation in CCL-39 cells

Serum-starved stably transfected CCL-39/myc$S1P_3$ cells were treated at 37°C with vehicle or increasing concentrations of S1P for two hours. The cells were examined for internalisation by cell surface labelling with biotin-LC-hydrazide, followed by immunoprecipitation of solubilised receptors with anti-myc 9E10 antibody. The data shown represents one of three similar experiments.

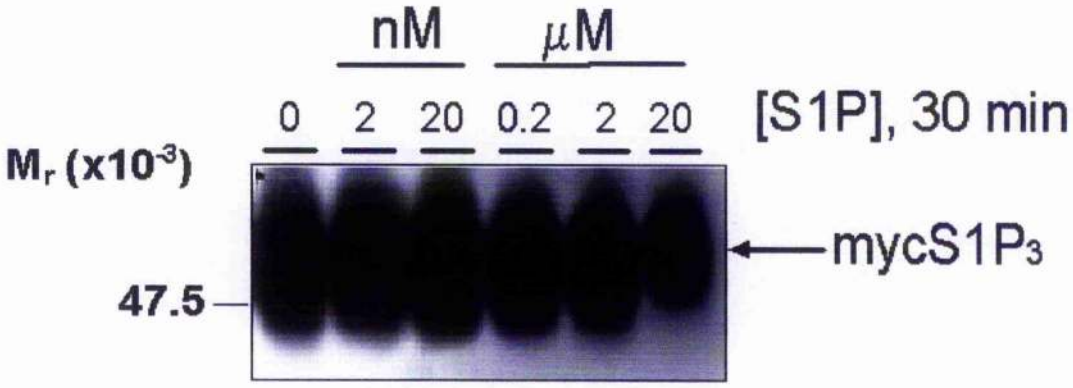

nM
μM
0
2
20
0.2
2
20
[S1P], 30 min
Mr (x10-3)
47.5
mycS1P3

Figure 4.3: Schematic of the mycS1P$_3$-GFP Receptor

The human mycS1P$_3$ receptor was tagged with green fluorescent protein using a pcDNA/human mycS1P$_3$ template. The mycS1P$_3$ receptor was ligated into the multiple cloning site of pEGFP-N1 at *Hind*III/*Bam*HI. The addition of the GFP tag allowed visualisation of any movement of the cell surface S1P$_3$ receptor following sustained agonist exposure.

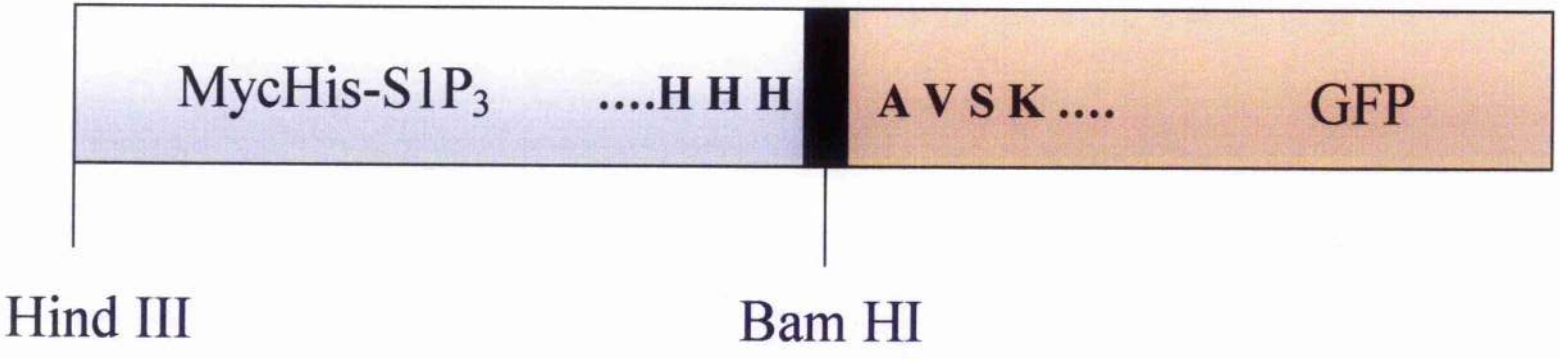

MycHis-S1P3
....H H H
A V S K ....
GFP
Hind III
Bam HI

Figure 4.4: Stable Expression of mycS1P$_3$ and mycS1P$_3$-GFP Receptors in CCL-39 cells

Cell extracts prepared from non-transfected CCL-39 fibroblasts or CCL-39 cells stably expressing either mycS1P$_3$ or mycS1P$_3$-GFP receptor were solubilised, normalised for protein content and analysed by SDS-PAGE and immunoblotting with either an anti-myc monoclonal or anti-GFP antibody as indicated. mycS1P$_3$ and mycS1P$_3$-GFP bands are indicated.

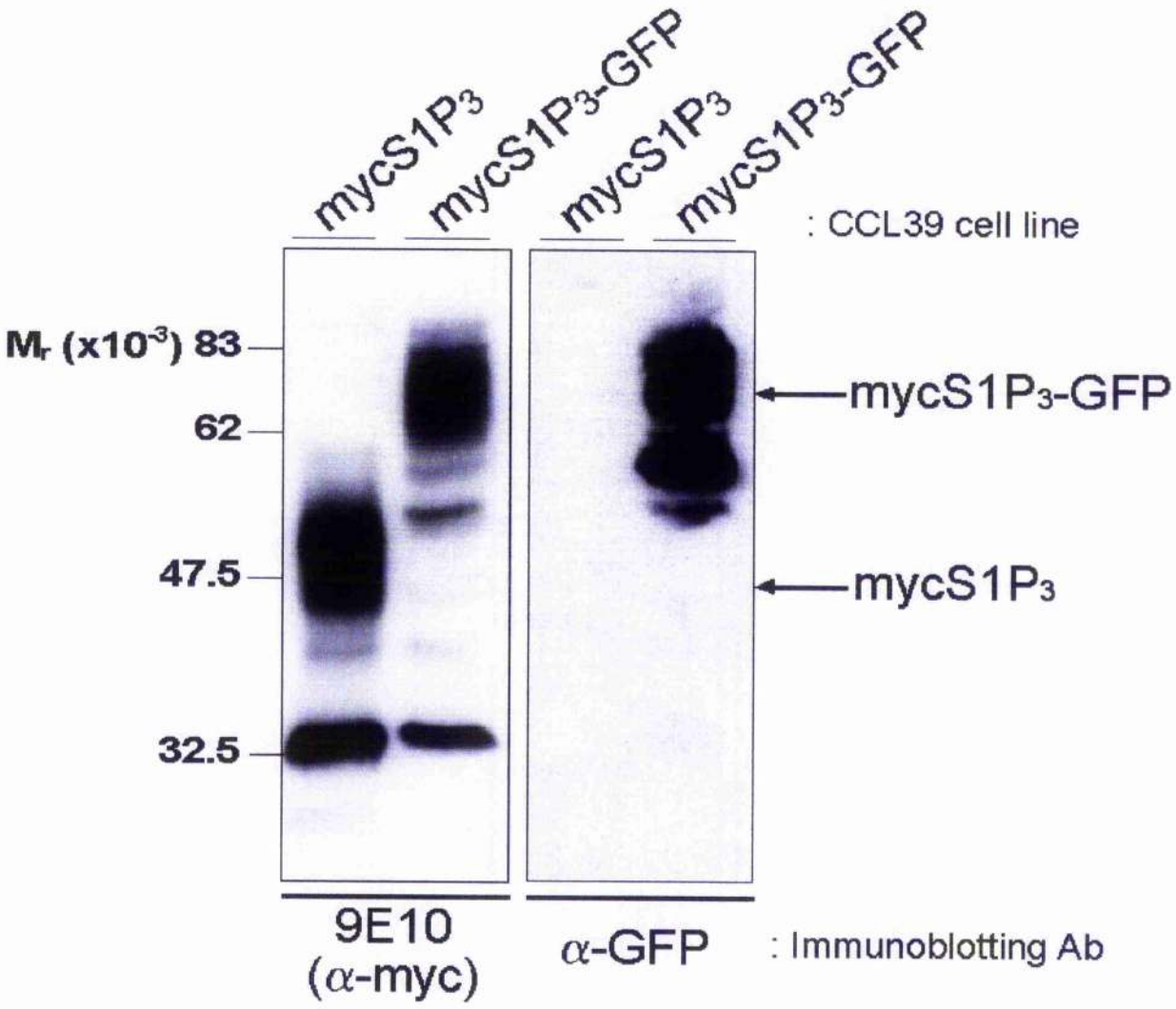
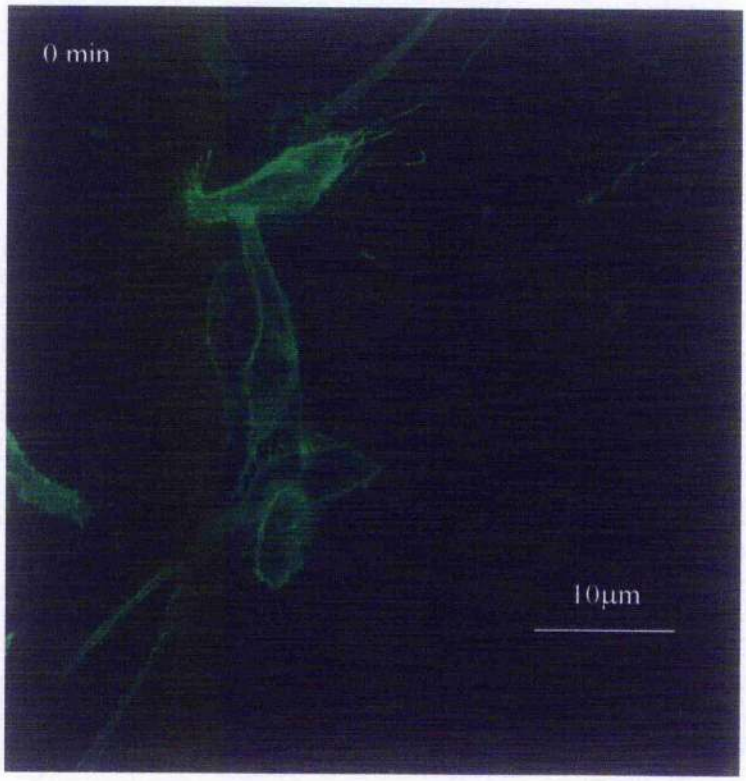

mycS1P3
mycS1P3-GFP
mycS1P3
mycS1P3-GFP
: CCL39 cell line
Mr (x10-3) 83
62
47.5
32.5
mycS1P3-GFP
mycS1P3
9E10
(α-myc)
α-GFP
: Immunoblotting Ab

Figure 4.5: Visualisation By Confocal Microscopy Of The Resistance To S1P-Mediated Internalisation Of mycS1P$_3$-GFP Receptors expressed in CCL-39 cells.

A: Serum-starved CCL39/mycS1P$_3$-GFP cells were plated onto coverslips and then washed and fixed in paraformaldehyde prior to visualisation of receptor distribution by confocal microscopy. Under conditions of no agonist treatment, the mycS1P$_3$-GFP construct was expressed on the cell surface.

B: Serum-starved CCL39/mycS1P$_3$-GFP cells plated onto coverslips and exposed to 20μM S1P for either, 15, 30, 60, 90 or 120min at 37°C. The cells were then washed and fixed in paraformaldehyde prior to visualisation of receptor distribution by confocal microscopy. From 0min to 120min there was no variation in distribution of receptors at the cell surface, as observed. Thus, no significant trafficking of the mycS1P$_3$-GFP receptor was seen after 2 hours in the presence of 20μM S1P.

A



B

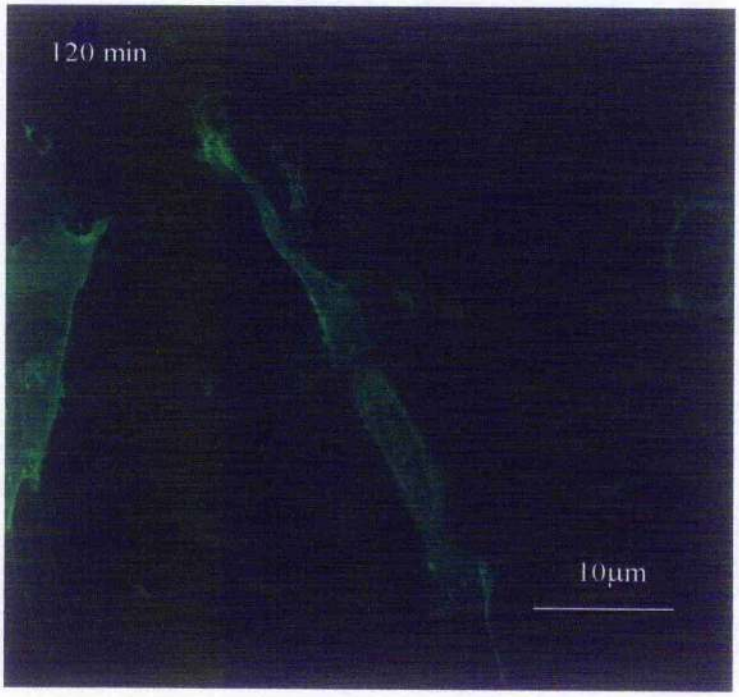


Figure 4.6: Sucrose Density Gradient Analysis of $S1P_3$ receptor distribution in the plasma membrane of CCL-39 cells

Stably expressing CCL-39/myc$S1P_3$ cells were treated with either vehicle or 10μM S1P in DMEM. Samples were analysed by sucrose density gradient ultracentrifugation as described in Section 2.6. Fraction samples were resolved on a 12.5% SDS gel. Gels were transferred to nitrocellulose and probed for either myc$S1P_3$ receptor or caveolin-1 (22K). Caveolin-1 and $S1P_3$ were observed at samples 3 and 4 and 9-12.

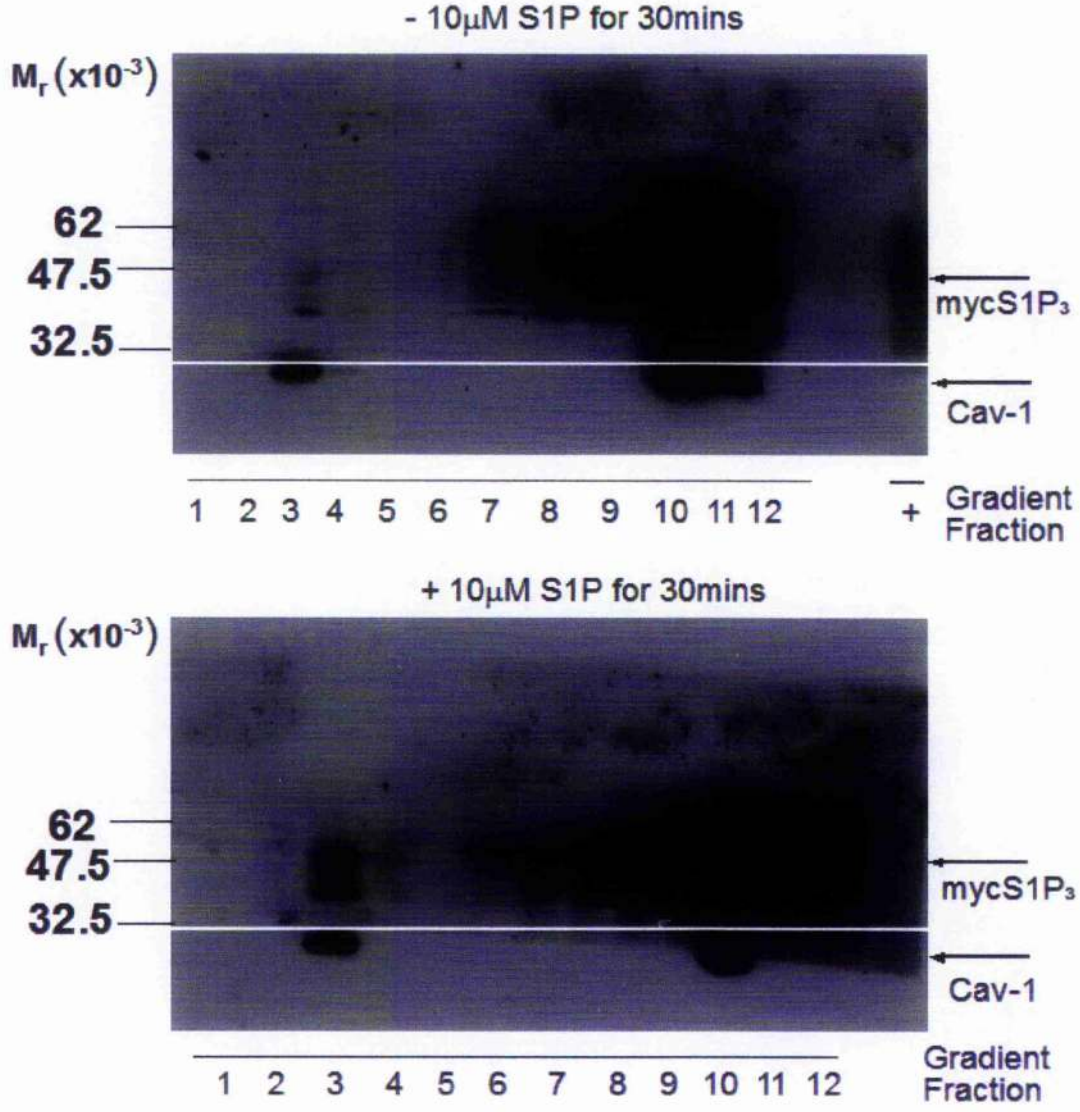

- 10μM S1P for 30mins
M_r (x10^{-3})
62
47.5
32.5
mycS1P$_3$
Cav-1
1 2 3 4 5 6 7 8 9 10 11 12
— +
Gradient Fraction
+ 10μM S1P for 30mins
M_r (x10^{-3})
62
47.5
32.5
mycS1P$_3$
Cav-1
1 2 3 4 5 6 7 8 9 10 11 12
Gradient Fraction

Figure 4.7: Preliminary study of myc-$S1P_3$ receptor association with arrestin-3

Myc$S1P_3$ stably transfected CCL39 cells were co-transfected with arrestin-3 and, following serum starvation for 24hrs, treated with 10μM S1P agonist for up to 2 hours. All subsequent procedures were carried out at 4°C. The cells were solubilized and analysed for protein content as described in Section 2.4.3. Proteins were normalised and solubilised cell membrane extracts were immunoprecipitated with anti-arrestin-3 to detect co-localisation. Following incubation for 1 hour, immune complexes were analysed by SDS-PAGE and transfered to nitrocellulose. Membranes were incubated with monoclonal antibody 9E10 followed by HRP-conjugated anti-mouse, as detailed in section 2.4.4. Since there is no negative control sample included (i.e. non-transfected CCL39s) it is not possible to assign what the multiple bands in the $S1P_3$ lane are. Bands in the co-IP lanes are mostly from HRP-conjugated second Ab recognition of the IgG used to immunoprecipitate arrestin-3 (the $S1P_3$ band is clearly present below the IgG heavy chain and its presence increases with time after time zero, but it is considerably weaker than the IgG bands).

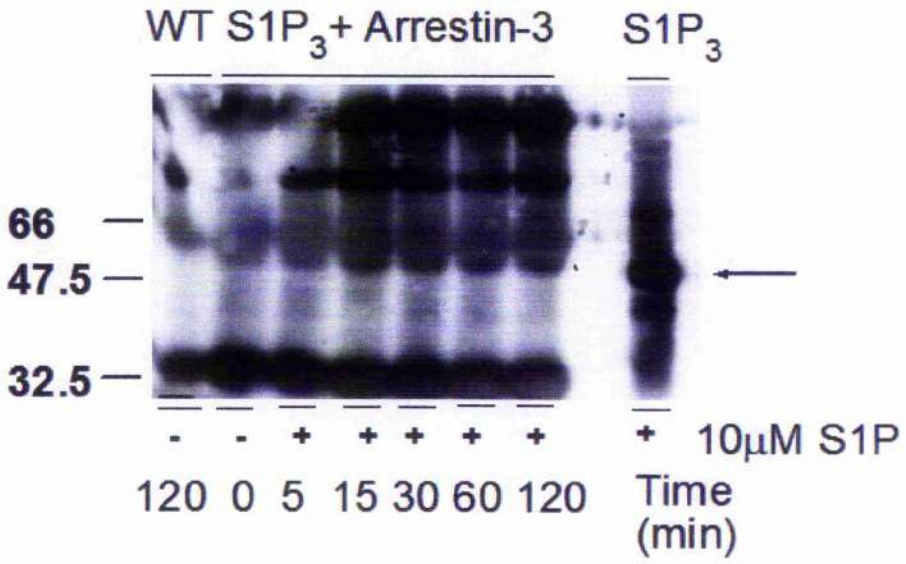

WT S1P3+ Arrestin-3
S1P3
66
47.5
32.5
- - + + + + + + 10μM S1P
120 0 5 15 30 60 120 Time (min)

Figure 4.8: Effect of agonist exposure on WT and mutant $S1P_3$ receptor cell surface expression in HEK293 cells

A: HEK293 cells transiently expressing the indicated myc epitope-tagged $S1P_3$ receptors were incubated with 5μM S1P for two hours. Following biotinylation of cell surface glycoproteins, soluble cell extracts normalised for protein content were prepared for receptor immunoprecipitation. Following fractionation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane for probing with HRP-conjugated streptavidin as described in Section 2.4.8.

B: Quantitative analysis from three such experiments at time point = 2 hr. Statistical analysis revealed no significant difference ($p>0.05$) in extent of internalisation between WT and mutant receptors at this time point.

C: Quantitative analysis of time-courses of WT and Δ45 $S1P_3$ receptor internalisation. Data normalised to the level of cell surface receptor observed in unstimulated cells (set at 100%).

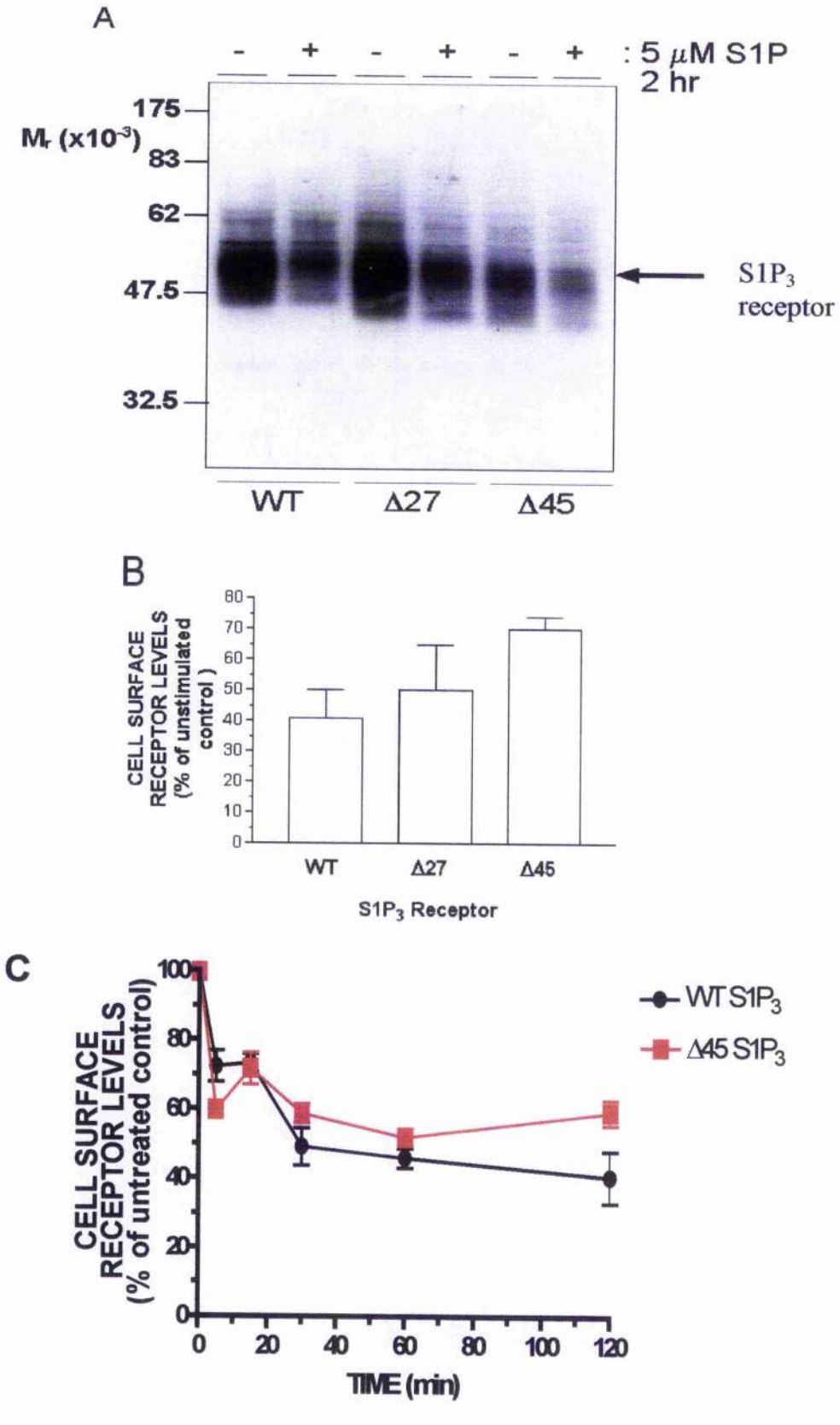

A
- + - + - + : 5 μM S1P
2 hr
175
M_r (x10^-3)
83
62
47.5
32.5
S1P3 receptor
WT
Δ27
Δ45
B
CELL SURFACE RECEPTOR LEVELS (% of unstimulated control)
80
70
60
50
40
30
20
10
0
WT
Δ27
Δ45
S1P3 Receptor
C
CELL SURFACE RECEPTOR LEVELS (% of untreated control)
100
80
60
40
20
0
0
20
40
60
80
100
120
TIME (min)
WT S1P3
Δ45 S1P3

Figure 4.9: Determination of $S1P_3$ receptor expression in Rat1a cells

Rat1a cells were transiently transfected with expression constructs encoding the indicated $S1P_3$ receptors or empty vector. Soluble cell extracts were then processed for immunoblotting with anti-myc 9E10 antibody.

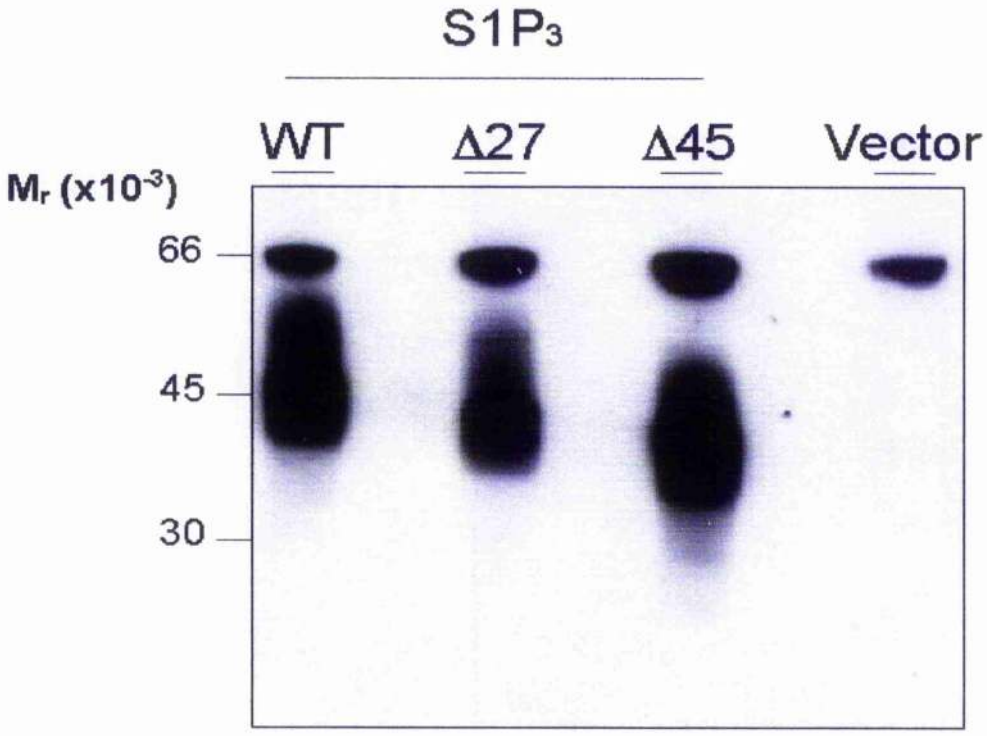

S1P3
WT
Δ27
Δ45
Vector
Mr (x10-3)
66
45
30

Figure 4.10: Functional Desensitisation of $[Ca^{2+}]_I$ Mobilisation in WT and Mutant $S1P_3$ Receptors

Calcium mobilisation experiments were performed on quiescent Rat1a cells transiently expressing either GFP alone (Control) or in combination with WT and Δ45 $S1P_3$ receptors were treated for 1min periods with 0.5μM S1P every 6min as indicated (arrows). The response to activation of stably expressed α_{1b}-adrenoceptors following administration of 3μM phenylephrine (Phe) is also indicated. These traces are from one experiment that is representative of four separate transfections.

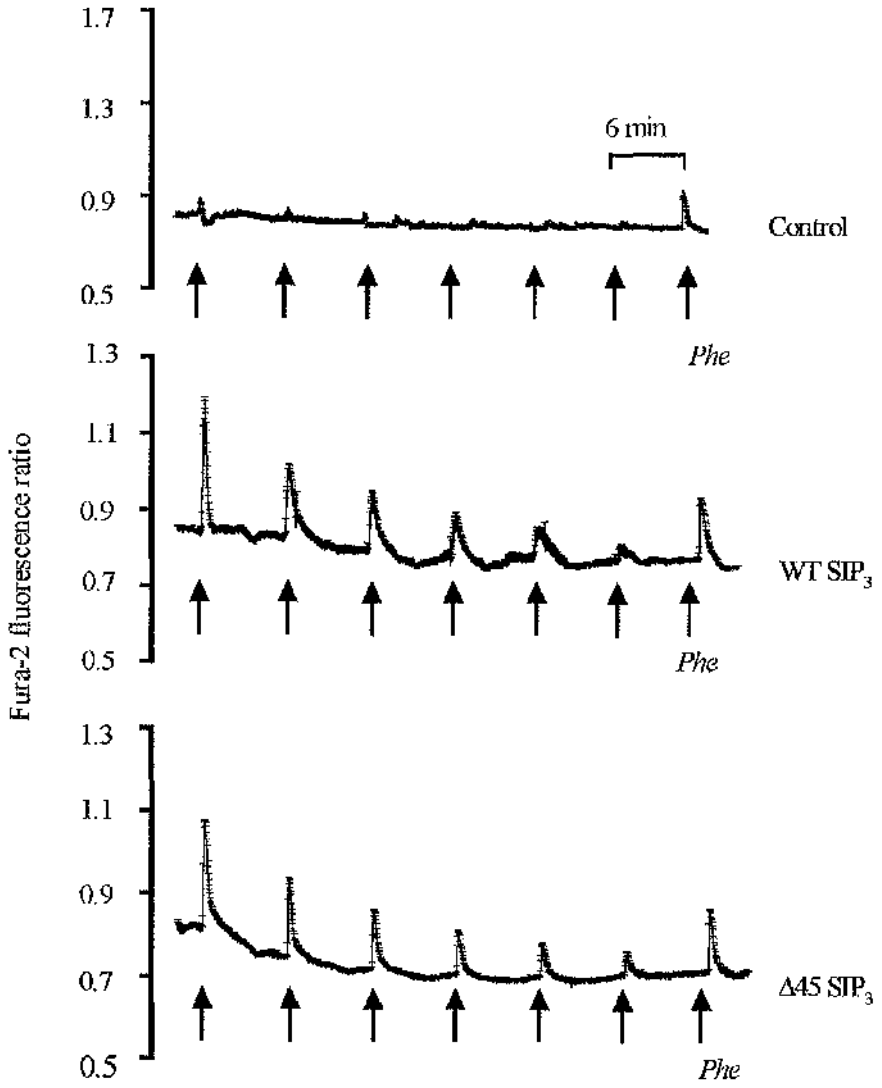
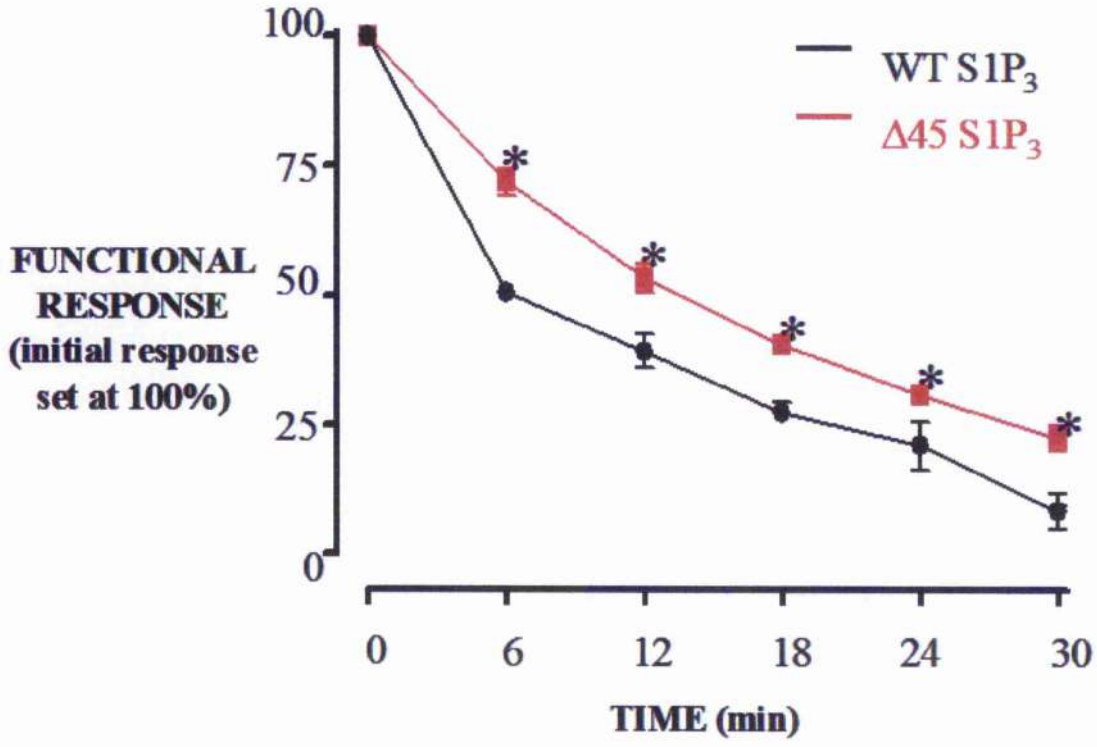

1.7
1.3
0.9
0.5
6 min
Control
Phe
1.3
1.1
0.9
0.7
0.5
WT SIP3
Phe
Fura-2 fluorescence ratio
1.3
1.1
0.9
0.7
0.5
Δ45 SIP3
Phe

Figure 4.11: Rates of WT and Mutant $S1P_3$ Receptor Desensitisation of $[Ca^{2+}]_I$ Mobilisation

Quantitative analysis of WT and Δ45 $S1P_3$ receptor desensitisation of $[Ca^{2+}]_I$ over three experiments. In each case, responses have been normalised to the mobilisation observed upon initial cellular challenge with 0.5μM S1P. Astrixes indicate significant difference ($p<0.05$). Mutant actually desensitises by slightly less at each time point examined compared to WT, even though it is not phosphorylated at all.


100
75
50
25
0
FUNCTIONAL RESPONSE (initial response set at 100%)
WT S1P3
Δ45 S1P3
*
*
*
*
*
0
6
12
18
24
30
TIME (min)

CHAPTER 5

Strategies to Identify Novel Human $S1P_3$ Receptor-Interacting Proteins

Strategies to Identify Novel Human $S1P_3$ Receptor-Interacting Proteins

5.1 Introduction

After establishing for the first time that $S1P_3$ is phosphorylated in the presence of S1P, that $S1P_3$ internalisation is cell-type specific and that a kinase, unlikely to be GRK2 or similar is involved in phosphorylation and additionally, that arrestin may associate with the receptor at the onset of phosphorylation, it would seem that the next step would be to identify the regulatory mechanism of $S1P_3$ that confers $S1P_3$ as distinct from the other S1P receptors. The ability of the Δ45 truncated $S1P_3$ mutant receptor to undergo agonist-induced internalisation and desensitisation to the same extent as the WT receptor suggests that phosphorylation of the carboxyl-terminal domain may regulate novel aspects of $S1P_3$ function. The novelty of $S1P_3$ compared to the other S1P receptors might be provided by the presence of a class I SH3 domain interaction motif (RxxPxxP) between Arg^{324} and Pro^{330} (R^{324}ASPIQP) within the receptors C-terminal domain. It is possible that this may control specific downstream interaction that makes it distinct from other S1P receptors.

To identify how the SH3 interaction motif plays a role in $S1P_3$ signalling it is important to determine which proteins may interact with it [Jahn *et al.*, 1997; Simpson *et al.*, 1999]. To search for potential binding partners for the C-terminal tail of the $S1P_3$ receptor several approaches are available. Both a biochemical approach and the yeast two-hybrid system were used [Bartel and Fields, 1997; Jahn *et al.*, 1997]. Parallel studies of the SH3 domain interaction motif using the GST-fusion pull-down assays, a protein purification strategy using a C-terminal SH3 domain binding motif within $S1P_3$ as an affinity probe, provides supporting evidence for potential $S1P_3$-interacting proteins. In the biochemical approach, GST fusion proteins encompassing the SH3 domain binding motif of the C-terminal tail of the $S1P_3$ receptor were purified from bacteria, conjugated to glutathione Sepharose beads, and then incubated separately with cell extracts. Research has proffered candidates for $S1P_3$-interaction, as previously, it has been shown that fodrin, Fyn, phosphoinositide-3 kinase (PI3K) interact similarly to Src. Thus, a study was performed with $S1P_3$ and this selection of proteins to observe potential interactions. Fodrin, Fyn, Src and PI3K were chosen as they each have different functions in distinct pathways. The Src Family of protein tyrosine kinases, of which Src was the first example, includes Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk and Yrk. To date, all cells that are studied have at least one of these kinases which act in cellular control. Each family member is characteristised by a src-homology (SH) domain structure, SH1 is a kinase domain, SH2 and SH3 domains, and

SH4 which is a domain that has myristoylation and membrane-localisation sites. Inter-domain interactions regulated by phosphorylation control the activity of each kinase.

Fodrin, is a tetrameric protein (α 240 kD, β 235 kD) found in the brain, and it is an isoform of spectrin. Spectrin is a membrane-associated dimeric protein (240 and 220 kD) of erythrocytes, which forms a complex with ankrin, actin and probably other components of the 'membrane cytoskeleton', so that there is a meshwork of proteins underlying the plasma membrane, potentially restricting the lateral mobility of integral proteins. Spectrin contains the EF hand motif which is a very common calcium motif that consists of a twelve amino acid loop with a twelve amino acid α -helix at each end. This provides octahedral coordination for the calcium ion. Fyn is a non-receptor type tyrosine kinase of the Src family, that is strongly expressed in the central nervous system (CNS), suggesting that it plays an important role in brain function. Fyn-deficient mutant mice have a variety of abnormal signs such as impaired long-term potentiation in the hippocampus, with deficits in spatial learning, abnormality in suckling behavior in neonatal mutants, increased fearfulness and enhanced sensitivity to audiogenic seizures [Kitazawa *et al.*, 1998]. Recent findings have suggested that the disassembly of hemidesmosomes mediated by Fyn is a prerequisite for normal cell migration and tumor invasion [Mariotti *et al.*, 2001]. Members of the PI3K family control several cellular responses including cell growth, survival, cytoskeleton remodelling and the trafficking of intracellular organelles in many different types of cell. In particular, PI3K has important functions in the immune system [Koyasu, 2003].

Primarily, for the high proportion of GPCRs, agonist-mediated phosphorylation and β-arrestin interaction is directed at the C-terminal domain. Alterations within this region have been shown to influence the processes of desensitisation [Smith *et al.*, 1998; Pizard *et al.*, 1999; Blaukat *et al.*, 2001], sequestration [Innamorati *et al.*, 2001; Laporte *et al.*, 2002], and resensitisation [Oakley *et al.*, 1999; Innamorati *et al.*, 2001]. Many investigations make use of point mutations and/or receptor truncations as the most common strategies for the study of C-tail function. Alternatively, chimeric receptors can be generated, this approach facilitiates the predicted outcome as either the retention of receptor function or the conferral of donor receptor properties to the recipient.

A study of PDE4 enzymes exemplifies the rationale behind the use of GST fusion proteins in identifying potential interactors for the SH3 domain bind motif [Beard *et al.*, 1999]. The PDE4D gene encodes five distinct isoenzymes. PDE4D4 and PDE4A5 shows a distinct levels of interaction with protein SH3 domains, including lyn, fyn and src itself as well as the

cytoskeletal protein fodrin and abl tyrosyl kinase which implies that such an interaction occurs by virtue of the unique proline-rich N-terminal region characteristic of the PDE4D4 isoenzyme. The selectivities of these two enzymes might reflect differences in the form of the proline-rich segments of their N-terminal regions. PDE4A5 is characterised by three PxxPxxR motifs, whereas PXXP stretches predominate in PDE4D4. This is suggested to be likely due to differences reflected in the range of partners these PDE4 enzymes select in various cell types and which may promote functional differences in the roles of these two PDE4 species [Beard *et al.*, 1999].

Thus, the potential of these two enzymes to interact with SH3 domain-containing proteins may be related to a major functional role. It was suggested that the functional importance of any binding of these enzymes to SH3 domain-containing proteins may be in targeting the PDE4 species to a specific location within the cell and thus controlling local cAMP levels [Beard *et al.*, 1999]. This may be the case for the proteins that interact with the SH3 domain binding motif in $S1P_3$ receptors.

Two parallel mechanistically distinct approaches have been used in the research of this chapter to address a role for the $S1P_3$ RXXPXXP motif that is predicted to act as an SH3 domain interaction motif. Using an *in vitro* approach with GST-SH3 domains, it was found that the RxxPxxP motif is functional and exhibits a marked degree of specificity for FynSH3. Concomitantly, positive clones were isolated *via* a genetic screen to identify $S1P_3$ C-terminal domain interacting proteins, although these could not be rescued from yeast and were not characterised any further. Finally, two chimeras were generated in an attempt to test the importance of any identified interactors for receptor function in the context of an intact GPCR in the future.

5.2 Results

Preliminary analysis of the alignment of C-terminal residues of several S1P receptors identified $S1P_3$ as having a unique SH3 interaction motif, and $S1P_2$ a PDZ interaction motif (Figure 5.1). Concurrently with the GST fusion protein assay, the yeast two-hybrid system was used to search for potential binding partners for the $S1P_3$ SH3 domain interaction motif. After creating a construct incorporating the SH3 interaction motif *via* oligonucleotides and PCR, the resulting DNA was cloned into the plasmid PGBKT7 and transformed into yeast cells to confirm expression. The addition of appropriate size ~8kDa, consistent with the fusion of the $S1P_3$ C-terminal domain to the GAL4-DNA binding domain can be seen. (Figure 5.2). The $S1P_3$ SH3 domain interaction motif within the C-terminal was fused to the GAL4 DNA binding domain and was used as bait to screen a human cDNA library in the yeast strain Y187. Of the 6.4×10^6 independent colonies contained within the library, seven clones exhibited moderate to strong growth on −HIS or −ADE media. Each potential interacting colony was assessed for binding to the $S1P_3$CT peptide through multiple stages of screening using X-α Gal to identify the positive interactors by expression of blue colonies (Figure 5.3). Once positive colonies were discovered they were grown as cultures over 1-2 days and then rescued from plasmid. The purity of DNA from each colony was assessed by running samples on a 1% agarose gel with the appropriate DNA marker (Figure 5.4).

Once the DNA was purified it was amplified by PCR for sequencing to be carried out (Figure 5.5). Unfortunately, despite rigorous screening no proteins were identified by sequencing. It should have been possible to rescue the clones containing the interactors from yeast and transform them into bacteria conventionally. As this did not work an attempt to PCR out the clone was made using primers designed to anneal to the either side of the insert within the vector employed to construct the library, this also proved to be unsuccessful.

In parallel to test whether $S1P_3$ could specifically interact with SH3 domains *in vitro*, pull-down assays were performed to assess the ability of $S1P_3$ to bind to a panel of GST fusion proteins containing the SH3 domains from Fyn, Src, fodrin and PI3K. Proteins were expressed in BL21 *E.coli* and immobilised on glutathione beads to assess purity (Figure 5.6). Interestingly, these demonstrated that $S1P_3$ was able to bind exclusively to the SH3 domain derived from Fyn (Figure 5.7).

Phosporylation sites are situated downstream from the RXXPXXP motif, to test whether prior $S1P_3$ phosphorylation could either alter the association of $S1P_3$ with Fyn-SH3 or facilitate interaction with any of the other SH3 domains, the ability of each of the GST-SH3 domains to

bind $S1P_3$ was compared between extracts derived from control cells and cells treated with 5μM S1P for 30min, which is sufficient to induce maximal receptor phosphorylation (Figure 3.7). Under these conditions, agonist pretreatment failed to alter the extent to which $S1P_3$ could interact with Fyn-SH3 and it did not promote subsequent interaction with the other SH3 domains tested (Figure 5.8). Thus, it appears that Fyn interaction is constitutive and not agonist-dependent.

A rationale for making chimeric receptors was to design an approach whereby it would be possible to test the functional significance of any SH3 domain interaction within the context of an intact S1P GPCR. The simplest way to do this was to swap the C-terminal domains of $S1P_1$ (which does not have a RxxPxxP motif) and $S1P_3$ following the predicted palmitoylation sites. Both constructs were generated and transfected into HEK293 cells to see of they encoded full length proteins: anti-myc immunoblots showed positive expression of each construct. Receptor phosphorylation assays also showed that A) they were accessible to extracellularly applied agonist, and thus present on the cell surface, B) capable of binding agonist, since each responded positively to S1P and C) appropriately regulated by phosphorlyation upon agonist binding in a manner similar to the parental WT receptors $S1P_1$ and $S1P_3$ (Figure 5.9).

While far from conclusive it is possible to put forward a tentative model of the regulation of the $S1P_3$ receptor (Figure 5.10), which encompasses all of the findings of this research and highlights the areas of future research.

5.3 Discussion

In this chapter several steps have been made to examine the potential SH3 binding domain of $S1P_3$ using the two hybird technique and GST fusion pull-down assays. The yeast two hybrid technique yielded seven positive clones which could not be rescued conventionally from yeast, sequenced or cloned by PCR. A comparison of $S1P_3$ C terminal tail SH3 domain binding motif interacting proteins could not be made between results from the yeast two hybrid assay and the GST fusion pull down assay as a consequence.

The yeast two hybrid technique or GST pulldown approaches are each prone to limitations and an interaction indicated by either technique is not unequivocal proof that this interaction occurs *in vivo*. Additional strategies can be carried out to gain further evidence for an interaction by using affinity chromatography followed by protein identification to discover which proteins can bind to a known protein. Detection of proteins can be made using gel overlay assays which are similar to western blots, and further confirmation can be found by co-immunoprecipitation. Rapid advances in proteomic technology and improved sensitivity of mass spectrometry achieved during the course of this thesis now makes it feasable to identify proteins bound either to immunoprecipitated GPCRs or GST's encoding cytoplasmic GPCR domains following pull downs of cell extracts. For example, 15 proteins, containing synaptic multidomain proteins with PDZ domains, have been found to interact with the C-tail of $5\text{-}HT_{2C}$ receptors using a proteomic approach [Becamel *et al.*, 2002].

As mentioned in Chapter 4, CCL-39 cells over-expressing the $S1P_3$ receptor do not internalise the receptor after agonist stimulation. As Fyn was shown to interact with the SH3 domain binding motif of $S1P_3$, one possible scenario is of interaction with Fyn or a related protein and the SH3 binding motif of the $S1P_3$ receptor competitively preventing arrestin-receptor interaction, thus imposing a barrier as well as a conformational restriction on the receptor which confines the receptor to the plasma membrane without removal *via* clathrin coated pits. A non-GPCR precedent for lack of internalisation exists in apoER2. As described in Chapter 1 the TM domain and PXXP motifs of the apoER2 exclude it from carrying out clathrin-mediated endocytosis [Sun and Soutar, 2003].

The potential role for Fyn or a similar interacting protein, although not fully elucidated in this research could be to function as a blockade to arrestin associating with a multicomplex. This would support the observation in CCL39 cells where $S1P_3$ receptors appear to have resistance to internalisation. The expression levels of Fyn in CCL39 cells has not been recorded in this lab, so it is possible that this is a novel regulatory mechanism in $S1P_3$ transduction, studies of Fyn in relation to signal transduction and cytoskeleton remodelling have been made in the olfactory

system and also T cell activation.

Synaptic transmission in the granule cells of the olfactory bulb of the homozygous Fyn (a nonreceptor type tyrosine kinase)-deficient (fyn^{2}/fyn^{2}) and heterozygous Fyn-deficient ($+/fyn^{2}$) mice was studied by using slice preparations from the olfactory bulb. The results demonstrated that the functions of $GABA_A$ and NMDA receptors in the olfactory system of Fyn-deficient mice are altered [Kitazawa *et al.*, 1998], which infers that tyrosine phosphorylation by Fyn kinase is required in synaptic transmissions between cells and receptors. Fyn has also been shown to play a specific role in the mechanism and functional significance of hemidesmosome disassembly during normal epithelial cell migration and squamous carcinoma invasion. The findings of this study indicated that a fraction of the EGF receptor (EGF-R) combines with the hemidesmosomal integrin α6ß4 in both normal and neoplastic keratinocytes. EGF-R activation causes tyrosine phosphorylation of the ß4 cytoplasmic domain and disruption of hemidesmosomes. The Src family kinase inhibitors PP1 and PP2 prevent tyrosine phosphorylation of ß4 and disassembly of hemidesmosomes without interfering with the activation of EGF-R. Coimmunoprecipitation experiments indicate that Fyn and, to a lesser extent, Yes, another receptor tyrosine kinase, combine with α6ß4. $Fyn^{+/-}$ was shown to prevent tyrosine phosphorylation of ß4 and disassembly of hemidesmosomes. This allows the suggestion that the EGF-R causes disassembly of hemidesmosomes by activating Fyn, which in turn phosphorylates the ß4 cytoplasmic domain. Neoplastic cells expressing $Fyn^{-/-}$ display increased hemidesmosomes and migrate poorly *in vitro* in response to EGF. Additionally, $Fyn^{+/-}$ decreased the ability of squamous carcinoma cells to invade through Matrigel *in vitro* and to form lung metastases following intravenous injection in nude mice. These results suggested that disruption of hemidesmosomes mediated by Fyn is a prerequisite for normal keratinocyte migration and squamous carcinoma invasion [Mariotti *et al.*, 2001]. This has implications for the findings of this thesis, where interaction with Fyn has been suggested to occur with the $S1P_3$ receptor C-terminal. It is tempting to suggest that whichever way the interaction of the SH3 domain binding motif of $S1P_3$ affects internalisation, it is possible that $S1P_3$ may assist in developing the cytoskeleton using this route. Although $S1P_3$ appears to co-localise with arrestin, arrestin interaction may not be as a recruitment to clathrin coated pits, but as a scaffolding protein which requires $S1P_3$ to complex with Fyn. Investigations of this characteristic by controlled disruption of the constituents of lipid rafts could help to elucidate this potential interaction.

Tyrosine phosphorylation can modulate $GABA_A$ receptor function, and deletion of the *fyn*-kinase gene has been shown to alter GABAergic function in olfactory bulb neurons [Kitazawa *et al.*, 1998]. One study determined whether the *fyn* gene deletion may have altered

behavioural and functional actions of compounds that act on $GABA_A$ receptors, as this may suggest a role for fyn-kinase in modulating $GABA_A$ receptor function, possibly *via* direct interactions between the kinase and receptor. The results of this investigation suggested that fyn-kinase may alter the function of $GABA_A$ receptors, perhaps via actions on β2 and/or β3 receptor subunits [Boehm *et al.*, 2004]. Data that lends itself to interpretation for the $S1P_3$/Fyn interaction model where, depending on celltype, fyn may interact with the $S1P_3$ receptor to alter its conformation and thus regulate internalisation. This is particularly interesting, when one considers the processes and the physiological locations that $S1P_3$ and Fyn are implicated in. So far, due to lack of binding partner identification, research has been limited as to the function of fyn in immunology [Filipp and Julius, 2004].

It has been well established that SH3 binding site serve important functional roles in many GPCRs. For example, a recent study [Beard *et al.*, 1999] has shown that P*XX*P motifs in the third intracellular loop and the carboxyl-terminal tail of the β_3 adrenergic receptor (β3AR) interact directly with Src and are required for ERK1/2 activation [Cao *et al.*, 2000b]. P*XX*P motifs in the β1 adrenergic receptor interact with endophilins, SH3 domain-containing proteins, and are involved in receptor internalization and receptor coupling to G proteins [Tang *et al.*, 1999]. In the dopamine D4 receptor, P*XX*P motifs have been implicated in the control of receptor internalization as well as coupling to adenylyl cyclase and MAP kinase [Oldenhof *et al.*, 1998]. Also the SH3 binding sites in the $P2Y_2$ nucleotide receptor carboxyl-terminal tail interact directly with Src and regulate activities of Src, proline-rich tyrosine kinase 2 (Pyk2), and growth factor receptors [Liu *et al.*, 2004].

Data from Bivona *et al.* [2003], defined a new pathway for phospholipase Cγ that activates Ras on the Golgi apparatus by means of RasGRP1. This Src/PLC-γ1/RasGRP1-dependent pathway is distinct from the protein tyrosine kinase receptor/Shc (present or absent)/Grb2/SOS pathway that activates Ras on the plasma membrane. Moreover, by activating both RasGRP1 and the Ras GTPase-activating protein CAPRI, Ca^{2+} can regulate Ras in opposite directions on different subcellular compartments. The binary GTP/GDP switch that constitutes Ras as a signalling element cannot explain the variety of outcomes of Ras activation. By expressing Ras on different sub-cellular compartments and by using distinct modes of regulation, the cell gains increased capacity to modulate its output [Bivona *et al.*, 2003]. A mechanism similar to this, which allows regulation on different levels would permit each S1P receptor subtype to act at various stages of development of vessels, organs, etc., by signalling multiple downstream pathways. A potential complex that reflects this behaviour could occur between

$S1P_3$/Fyn or a similar kinase/PDGF. $S1P_3$ forms a similar multiprotein complex which requires the presence of a tyrosine kinase as findings from a study of PDGFβ and $S1P_1$ have shown that c-Src is involved in regulating signal transmission from PDGFβ receptor–GPCR(s) complexes in mammalian cells [Waters *et al.*, 2004; Waters *et al.*, 2005]. Another study was the first to show that $S1P_3$ mediates the crosstalk between S1P and PDGFR [Baudhuin *et al.*, 2004], and this provides a mechanism for tyrosine phosphorylation in $S1P_3$ signalling.

Many G protein-coupled receptors activate growth factor receptors, although the mechanisms controlling this transactivation are unclear. Comparative studies to those carried out in this research were made when two proline-rich, SH3 binding sites (P*XX*P) in the carboxyl-terminal tail of the human $P2Y_2$ nucleotide receptor were identified that directly associated with Src in protein binding assays [Liu *et al.*, 2004]. $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors couple to $G_{q/11}$ and activate phospholipase C, resulting in increased inositol 3-phosphate formation and mobilization of intracellular Ca^{2+} [Lee *et al.*, 2003]. Src was found to co-precipitate with the $P2Y_2$ receptor in 1321N1 astrocytoma cells stimulated with the $P2Y_2$ receptor agonist uridine triphosphate (UTP). A mutant $P2Y_2$ receptor lacking the P*XX*P motifs was found to stimulate calcium mobilization and serine/threonine phosphorylation of the Erk1/2 mitogen-activated protein kinases, like the wild-type receptor, but was defective in its ability to stimulate tyrosine phosphorylation of Src and Src-dependent tyrosine phosphorylation of the proline-rich tyrosine kinase 2, epidermal growth factor receptor (EGFR), and platelet-derived growth factor receptor [Liu *et al.*, 2004]. Dual immunofluorescence labeling of the $P2Y_2$ receptor and the EGFR indicated that UTP caused an increase in receptor co-localization in the plasma membrane that was prevented by the Src inhibitor, pyrazole pyrimidine-type 2 (PP2). Both these results indicate that agonist-induced binding of Src to the SH3 binding sites in the $P2Y_2$ receptor may facilitate Src activation, which recruits the EGFR into a protein complex with the $P2Y_2$ receptor and allows Src to efficiently phosphorylate the EGFR [Liu *et al.*, 2004]. Although this research has shown through GST-fusion constructs that Src does not interact with the C terminal domain of $S1P_3$ (Figure 5.8), it is possible that another member of the Src family, possibly Fyn, could become activated through agonist-induced binding of Fyn to the SH3 binding site in the $S1P_3$ receptor, in turn recruiting PDGF into a protein complex with the $S1P_3$ receptor to allow Fyn to efficiently phosphorylate PDGF.

Initially, the $S1P_1$ and $S1P_3$ C-tail exchange mutants were created to investigate whether altered Fyn interaction would be observed when the $S1P_3$ C-tail was removed. These experiments have yet to be performed, however, the results from mutant phosphorylation studies

have shown that $S1P_3CT_1$ has similar levels of phosphorylation to WT $S1P_1$ and that this in itself is an indicator that the $S1P_3$ C-tail hinders phosphorylation of the WT $S1P_3$ receptor. Perhaps involvement of an intracellular loop that encourages heightened phosphorylation in the $S1P_1$ receptor can occur more readily with the addition of the $S1P_3$ CT and this may have evolutionary consequences that support $S1P_1$ as the dominant S1P receptor. If the $S1P_1$ receptor is damaged at the C-terminal end, or agonist stimulation is not prevalent or there is interaction with a pathway whereby a downstream protein alters the conformation of $S1P_1$ then possibly increased phosphorylation of the receptor could be enabled by the involvement of an agonist-imitating region of an intracellular loop on the $S1P_1$ receptor. It has been reported that a KRX-725 peptide created from a region of the second intracellular loop of $S1P_3$ can mimic the effects of S1P to cause receptor phosphorylation [Licht *et al.*, 2003].

In summary, through the results discovered by the investigations reported in this thesis, a tentative model for $S1P_3$ receptor signalling has been proposed, (Figure 5.10). The model shows that $S1P_3$ undergoes S1P-induced phosphorylation, and that this phosphorylation is most likely an agonist-activated non-GRK kinase. The model also shows that an SH3 containing protein, possibly Fyn, interacts with the SH3 domain binding motif of the C-terminal domain of the $S1P_3$ receptor. This interaction may alter correct complex formation, presumably for the cell types studied, this obstruction only occurs in CCL39 cells where internalisation is not achieved. This suggests that in certain cell-types, it may be a possibility that $S1P_3$ receptor regulation could be orchestrated through interaction with a tyrosine kinase. This itself implies that the $S1P_3$ receptors involvement with PDGF could regulate $S1P_3$ signalling in certain cell types.

Figure 5.1: Alignment of the C-tails of $S1P_{1,2,3,5}$, highlighting the SH3-interaction motif on $S1P_3$

A schematic representation of the C-terminal tails of S1P receptors $S1P_{1,2,3}$ and $S1P_5$ showing the differences of amino acid residues (numbered) between each receptor. $S1P_3$ possesses an SH3 interaction motif unlike its fellow S1P receptors. $S1P_2$ possesses a PDZ interaction motif. Amino acids highlighted in yellow are homologous between receptors. * denotes potential palmitylation sites. Potential sites of phosphorylation in $S1P_3$ are underlined.

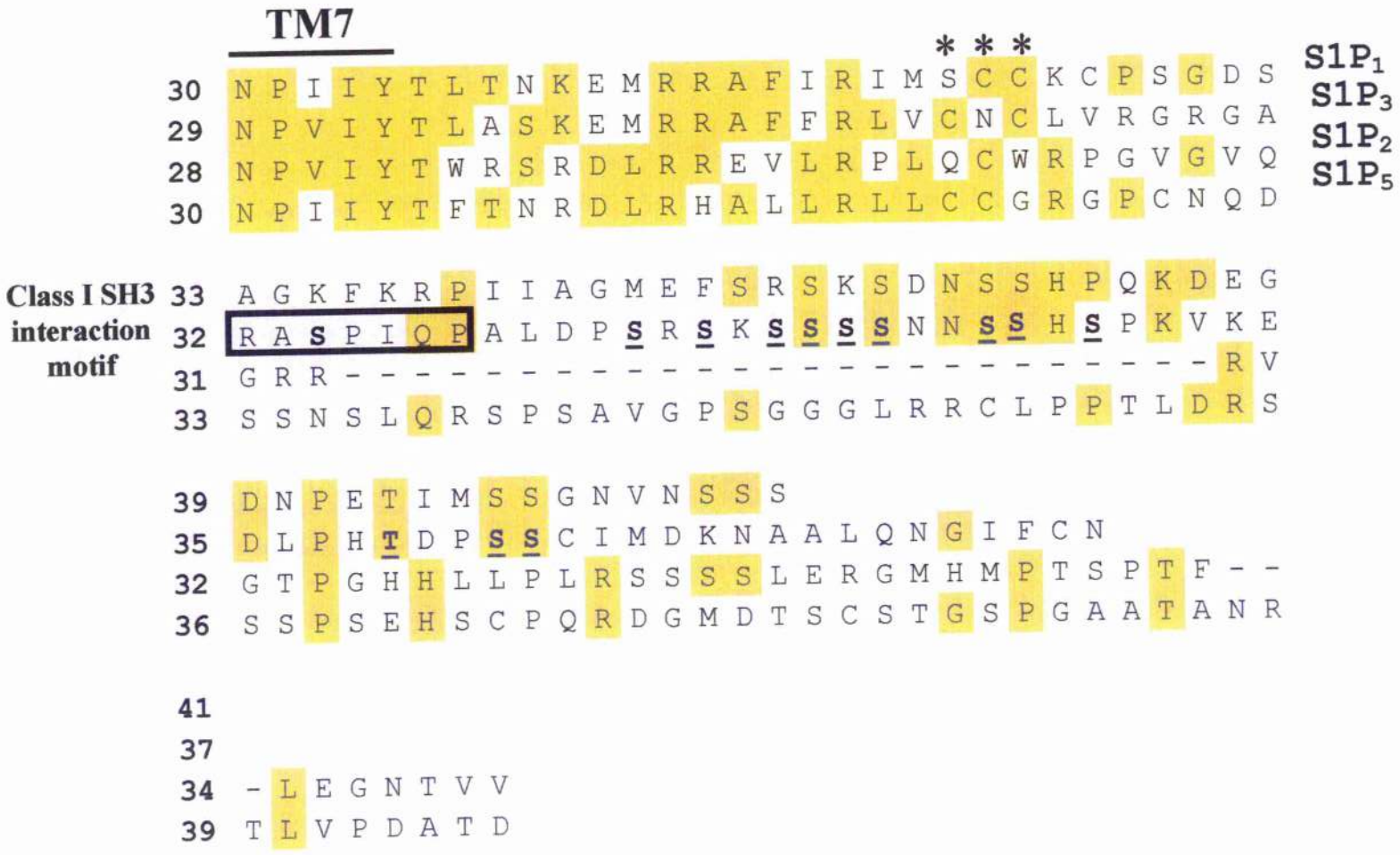

TM7
* * *
S1P1
S1P3
S1P2
S1P5
30 N P I I Y T L T N K E M R R A F I R I M S C C K C P S G D S
29 N P V I Y T L A S K E M R R A F F R L V C N C L V R G R G A
28 N P V I Y T W R S R D L R R E V L R P L Q C W R P G V G V Q
30 N P I I Y T F T N R D L R H A L L R L L C C G R G P C N Q D
Class I SH3 interaction motif
33 A G K F K R P I I A G M E F S R S K S D N S S H P Q K D E G
32 R A S P I Q P A L D P S R S K S S S S N N S S H S P K V K E
31 G R R - R V
33 S S N S L Q R S P S A V G P S G G G L R R C L P P T L D R S
39 D N P E T I M S S G N V N S S S
35 D L P H T D P S S C I M D K N A A L Q N G I F C N
32 G T P G H H L L P L R S S S S L E R G M H M P T S P T F - -
36 S S P S E H S C P Q R D G M D T S C S T G S P G A A T A N R
41
37
34 - L E G N T V V
39 T L V P D A T D

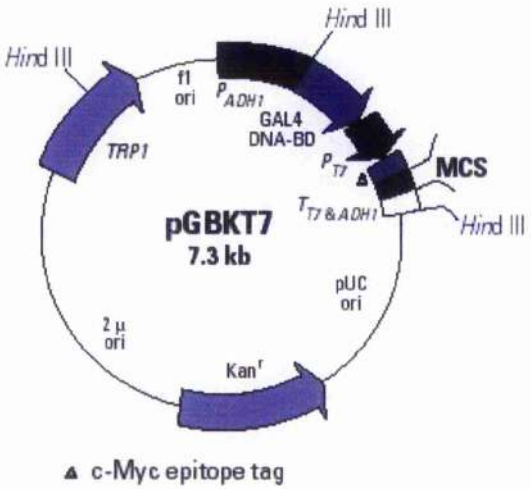
Figure 5.2: Schematic and immunoblot of Y2H bait protein

A: Schematic of the vector PGBKT7 showing the multiple cloning site (MCS) where the $S1P_3$ receptor C-terminal (SIP_3CT) peptide was inserted for use as the Y2H bait protein, Δ the c-Myc epitope tag, kanamycin resistance and the GAL4 DNA binding domain.

B: The 76 amino acid peptide Lys^{303}-Asn^{378}, taken from the $S1P_3$ cytoplasmic C-terminal ($S1P_3CT$), which was used as the molecular bait that includes the SH3 domain binding motif, RASPIQP (red).

C: Cell extracts of HEK293 cells transfected with either the vector PGBKT7 alone or the $S1P_3CT$/PGBKT7 construct were solubilised, normalised for protein content and analysed by SDS-PAGE and immunoblotting with anti-myc monoclonal 9E10.

A



B

KEMRRAFFRL VCNCLVRGRG ARASPIQPAL DPSRSKSSSS
NNSSHSPKVK EDLPHTDPSS CIMDKNAALQ NGIFCN

C

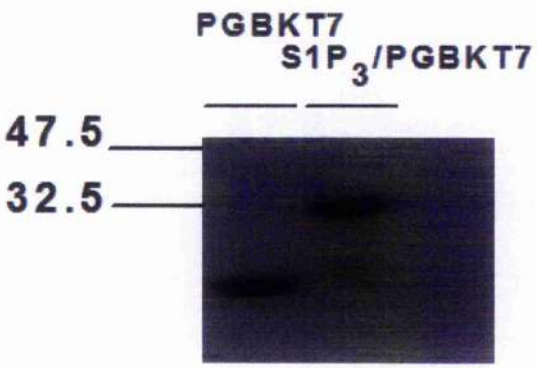


Figure 5.3: Screening of true binding partners using the X-αGal assay

A selection plate of minimal medium without Trp, Leu, Ade, His. It shows the two $S1P_3$/$pGBKT_7$ constructs and several alternative yeast strain positive controls. X-gal expression appears blue.

Anticlockwise:

1: $S1P_3$/$pGBKT_7$ and $pGADT_7$

2: $S1P_3$/$pGBKT_7$ and $pGADT_7$

3: $pGBKT_7$ and $pGADT_7$

4: pGM20 and $pGADT_7$

5: pGM20 and pGM22

6: pGM47

pGADT7, pGM20, pGM22 and pGM47 were used as known positive controls for plasmid interaction and X-gal expression.

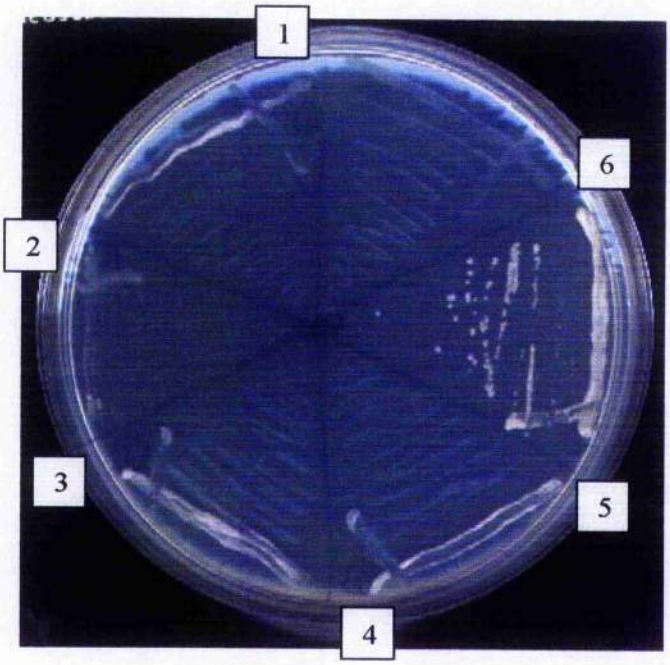

1
2
3
4
5
6

Figure 5.4: Detection of Positive Clones of Interaction with the $S1P_3$ receptor SH3 Binding Domain Motif

Lanes 1-14 show seven positive colonies in duplicate which were rescued from the PGBKT7 plasmid and amplified with appropriate primers to allow identification of potential binding partner DNA.

M refers to a 1 Kbp ladder, ++ refer to positive controls pGAD C1 and PGAD + YSC84.

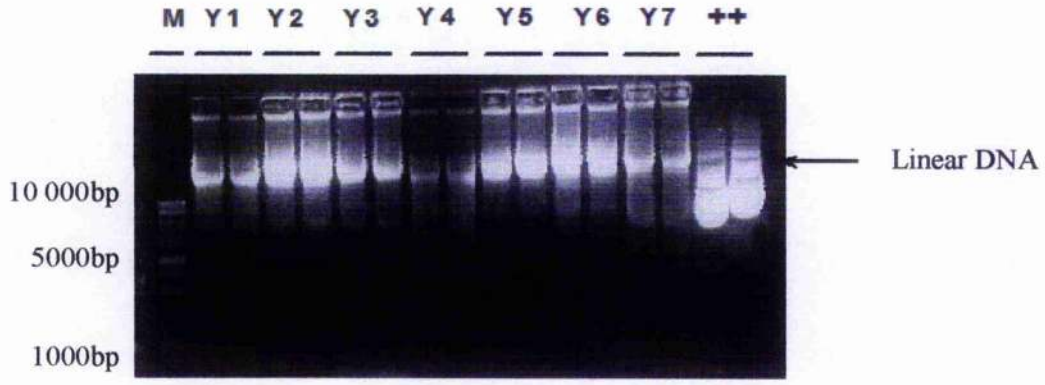

M
Y1
Y2
Y3
Y4
Y5
Y6
Y7
++
10 000bp
5000bp
1000bp
Linear DNA

Figure 5.5: Digestion of Positive Clones of Interaction with the $S1P_3$ receptor SH3 binding domain motif

Of the seven positive clones, the five clone PCR products most strongly amplified were purified and then digested with EcoRI and BamHI. Each digested clone was then run on a 1% agar gel with a 1kbp DNA marker.

The symbols + and ++ refers to positive controls PGAD C1 and PGAD + YSC84.

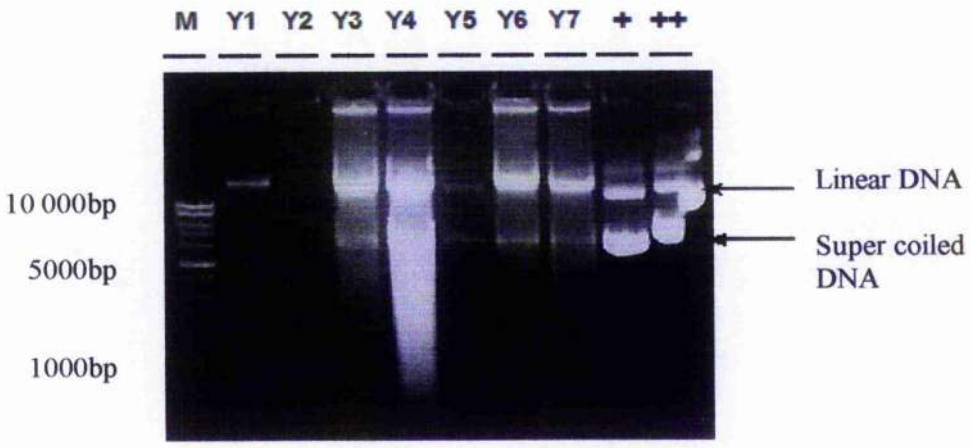

M
Y1
Y2
Y3
Y4
Y5
Y6
Y7
+
++
10 000bp
5000bp
1000bp
Linear DNA
Super coiled DNA

Figure 5.6: The Purification of GST-SH_3 Fusion Proteins from E.coli

Fyn, fodrin, Src and PI3K were expressed in BL21 *E.coli* and immobilised on glutathione beads. Samples were run on SDS-PAGE and transferred to nitrocellulose for analysis of protein purity. Lane 2 = GST alone. Gel has NOT been transferred to nitrocellulose and blotted with any Ab. The gel was run and stained with Coomasie Blue to ensure purity and integrity of the isolated fusion proteins.

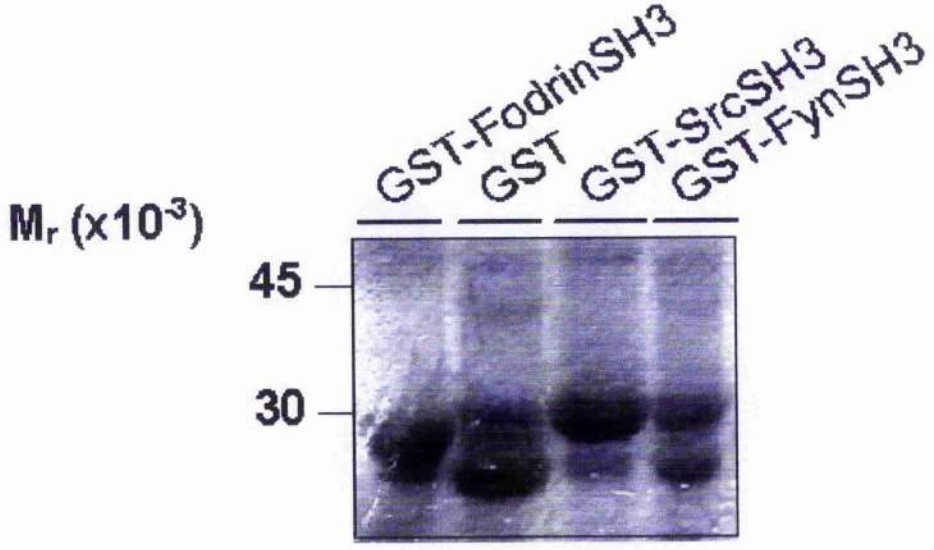

GST-FodrinSH3
GST
GST-SrcSH3
GST-FynSH3
Mr (x10-3)
45
30

Figure 5.7: A Specific Pull-Down Assay of Fyn-SH_3 with $S1P_3$

A panel of GST fusion proteins containing the SH3 domains from Fyn, Src, fodrin and PI3K were assayed using a GST fusion protein assay, as described in section 2.7 to test the ability of $S1P_3$ to bind to them *in vitro*.

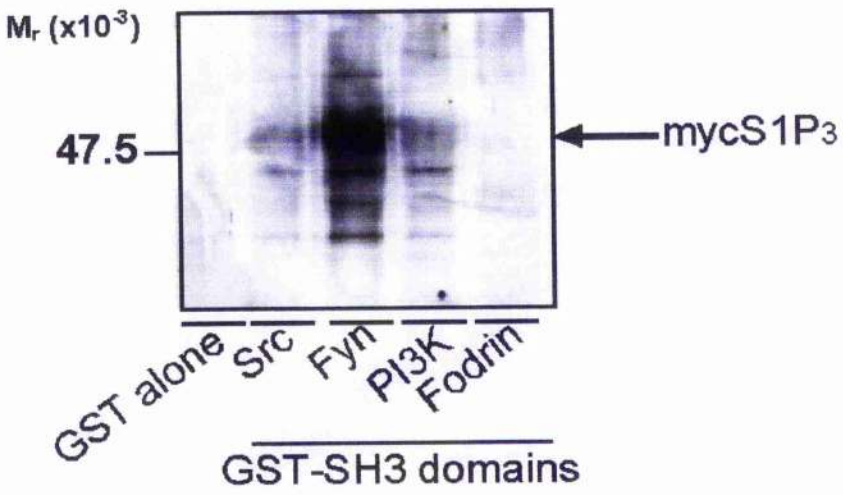
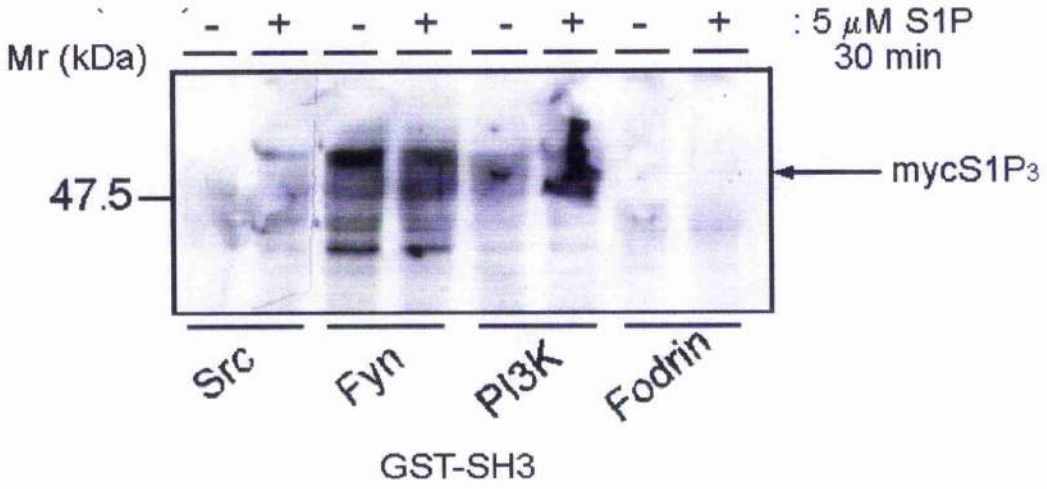

Mr (x10-3)
47.5
mycS1P3
GST alone
Src
Fyn
PI3K
Fodrin
GST-SH3 domains

Figure 5.8: Effect of agonist treatment with Fyn-SH3/$S1P_3$ interaction

Following treatment of CCL39/myc$S1P_3$ cells with 5 μM S1P for 30 min, cells were solubilised and equalised for protein content prior to incubation for 1 hr with rotation with glutathione-Sepharose beads to which 10 μg of the indicated GST-SH3 domain fusion proteins had been immobilised, as described in Section 2.7. Following isolation of GST-SH3-bound proteins by brief centrifugation and washing, the presence of $S1P_3$ was determined by SDS-PAGE and immunoblotting with anti-myc antibody 9E10. This is one of four experiments, quantification from which is presented. There was no statistically significant difference in the recovery of $S1P_3$ pulled down with GST-FynSH3 in control *versus* agonist-treated cells.


- + - + - + - + : 5 μM S1P
30 min
Mr (kDa)
47.5
mycS1P3
Src
Fyn
PI3K
Fodrin
GST-SH3

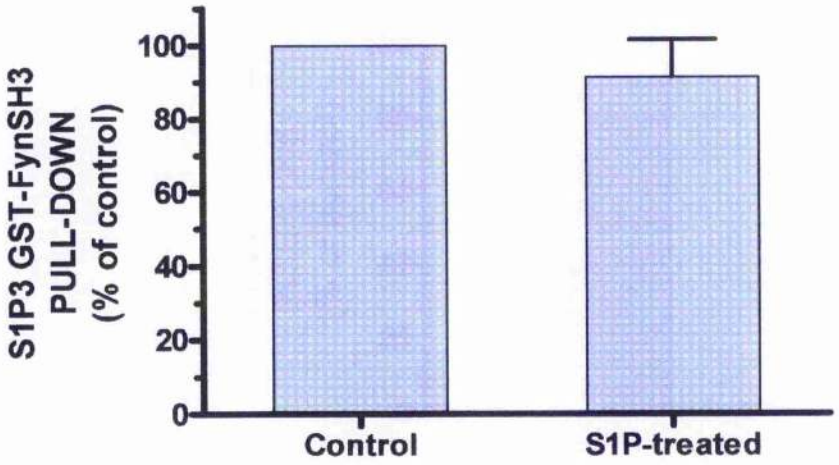
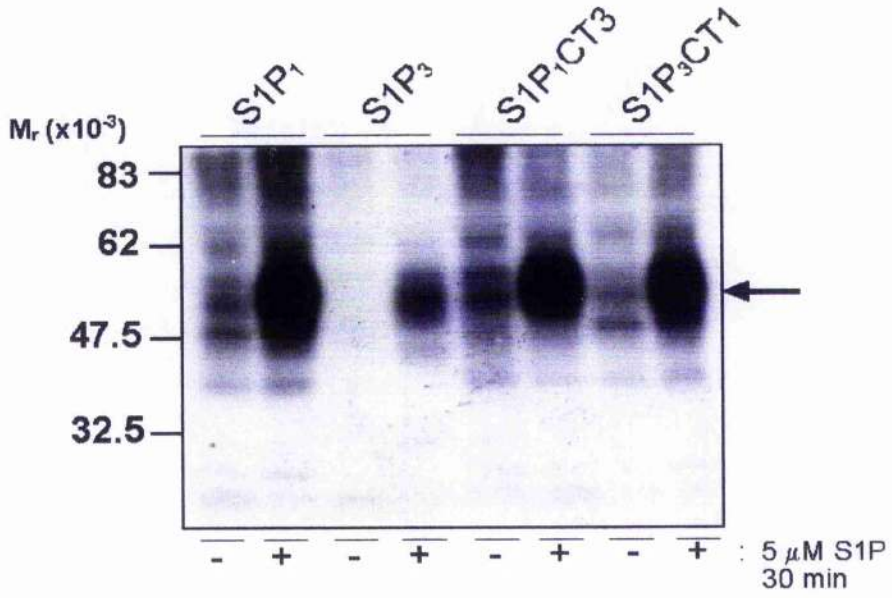

S1P3 GST-FynSH3
PULL-DOWN
(% of control)
100
80
60
40
20
0
Control
S1P-treated

Figure 5.9: C-tail exchange mutations, mycS1P$_1$CT$_3$ and mycS1P$_3$CT$_1$ undergo agonist-stimulated phosphorylation

A: Two constructs were created by double PCR that possessed the WT full-length receptor of either S1P$_1$ or S1P$_3$, from the N-terminal through to the last transmembrane region and with the construct exchanging the C-terminal for that of the opposite receptor. ^{32}P-labelled serum starved stably transfected CCL-39/mycS1P$_1$ or CCL-39/mycS1P$_3$ or transiently transfected CCL-39/mycS1P$_1$CT$_3$ or CCL-39/mycS1P$_3$CT$_1$ cells were treated with either vehicle or 5μM of S1P at 37°C for 30min. The cells were then solubilised for analysis of phosphorylation by immunoprecipitation followed by SDS-PAGE and visualised by autoradiography.

B: CCL-39 cells expressing each receptor were solubilised and then analysed by SDS-PAGE and immunoblotting with the anti-myc monoclonal antibody 9E10.


S1P1
S1P3
S1P1CT3
S1P3CT1
Mr (x10-3)
83
62
47.5
32.5
- + - + - + - +
: 5 μM S1P
30 min

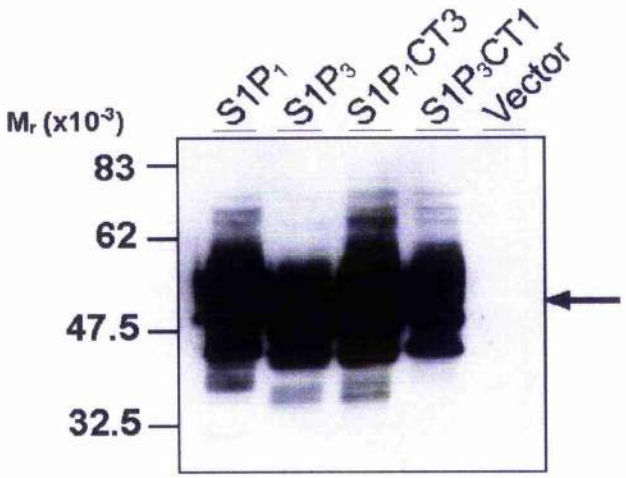
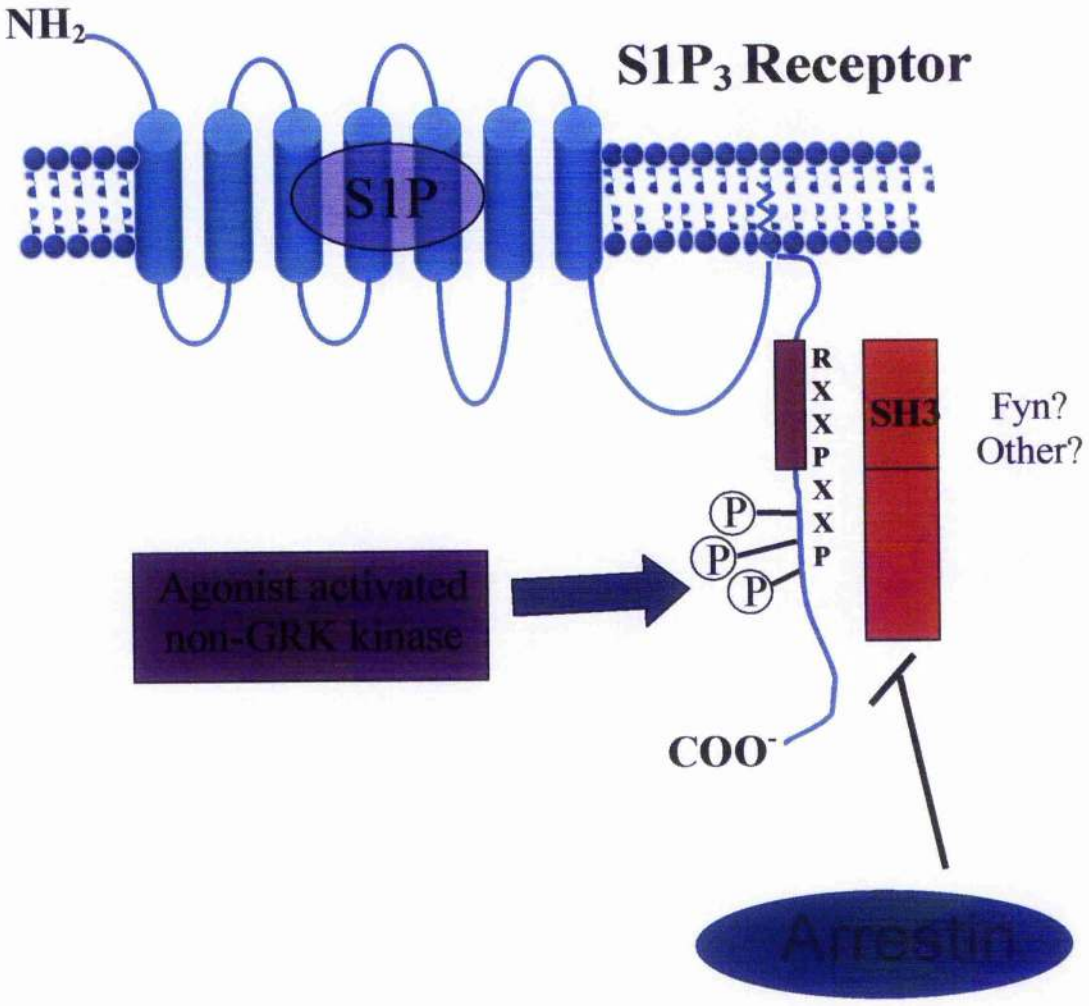

S1P1
S1P3
S1P1CT3
S1P3CT1
Vector
Mr (x10-3)
83
62
47.5
32.5

Figure 5.10: Tentative Model of Human $S1P_3$ Receptor Regulation

A potential signalling model for the human $S1P_3$ receptor. This model incorporates the possibility of phosphorylation of the $S1P_3$ receptor by a kinase similar to GRK2, involvement of arrestin and protein interaction at the SH3 interaction motif which may be Fyn or a related protein.


NH2
S1P3 Receptor
S1P
R
X
X
P
X
X
P
SH3
Fyn?
Other?
P
P
P
Agonist activated
non-GRK kinase
COO-
Arrestin

CHAPTER 6
Final Discussion

Summary

Regulation of receptor phosphorylation, internalisation and desensitisation is critical to GPCR transduction. The classical model of GPCR phoshorylation and internalisation is the β_2AR which provided the basic model to begin this research [Bouvier *et al.*, 1989; Freedman *et al.*, 1995; January *et al.*, 1997; Bouvier *et al.*, 1998; Mayor Jr. *et al.*, 1998; Seachrist *et al.*, 2000]. Over the course of the last three chapters, it has been demonstrated that upon agonist exposure the S1P receptor $S1P_3$ is phosphorylated and that $S1P_3$ phosphorylation cannot be duplicated by activation of second messenger-activated kinases. A number of characteristics of $S1P_3$ regulation were identified as distinct from previously described regulation of the related receptor $S1P_1$, which has been the subject of indepth characterisation [Liu *et al.*, 1999; Lee *et al.*, 2001; Watterson *et al.*, 2002]. Initially, $S1P_3$ phosphorylation is solely agonist-dependent, whereas $S1P_1$ is also phosphorylated upon activation of second messenger-activated kinases PKB and PKC [Lee *et al.*, 2001; Watterson *et al.*, 2002]. Currently, the effects of PKC phosphorylation are unknown, however, sensitivity of $S1P_1$ to phosphorylation by PKB is considered to be a pre-requisite for activation of Rac and subsequent S1P-mediated chemotaxis [Lee *et al.*, 2001]. Seemingly, $S1P_1$ sensitivity to multiple kinases activated by different receptor systems enables it to act as a signal integration point for angiogenic stimuli in endothelial cells, while $S1P_3$ function is controlled entirely by S1P, its physiological activator. Additionally, though GRK2 phosphorylates both $S1P_1$ and $S1P_3$ *in vitro*, it is improbable that $S1P_3$ regulation by this kinase exists in intact cells since a mutant $S1P_3$ construct, with apparent resistance to phosphorylation in intact cells, displays the equivalent GRK2-mediated phosphorylation as the WT receptor *in vitro*, and GRK2 or GRK3 overexpression fails to potentiate $S1P_3$ phosphorylation in intact cells. Furthermore, $S1P_3$ desensitisation of Ca^{2+} mobilisation does not appear to be affected by receptor phosphorylation as both WT and phosphorylation-resistant $S1P_3$ responses desensitise at indistinguishable rates, which opposes the mechanism of the majority of GPCRs. As a final point, in HEK293 cells the receptor can internalise upon agonist exposure under conditions where mutant $S1P_3$ receptor phosphorylation is undetectable.

This research poses a number of questions concerning $S1P_3$ regulation, beginning with what is the kinase responsible for receptor phosphorylation in intact cells? Albeit improbable that GRK2 and the closely related kinase GRK3 control $S1P_3$ phosphorylation in intact cells, mutagenesis studies elucidate a region of the carboxyl-terminal domain spanning Leu^{332} and Val^{352} as most probably containing the sites of receptor phosphorylation. Deleting this region does not reduce the ability of the $S1P_3$ receptor to stimulate $[Ca^{2+}]_i$ mobilisation, denoting

that the resistance to phosphorylation does not reflect a gross detrimental change in receptor conformation produced be the truncation. Furthermore, this region is almost bereft in acidic residues, which is a common hallmark of GRK2/3 substrates including $S1P_1$ [Onorato *et al.*, 1991; Prossnitz *et al.*, 1995]. Implausible too is the concept that $S1P_3$ is a substrate for the GRK4-6 subfamily, as GRK5, a prototypical member of this group cannot reconstitute $S1P_3$ phosphorylation *in vitro*. Hence, it is plausible that $S1P_3$ is phosphorylated in an agonist-dependent manner by a kinase distinct from the GRK family. Reports are increasing which describe agonist-dependent, GRK-independent regulation of GPCR phosphorylation, [Budd *et al.*, 2000; Blaukat *et al.*, 2001; Hanyaloglu *et al.*, 2001]. However, an acidotropic kinase that typically phosphorylate clusters of Ser and/or Thr residues in a sequential fashion similar to GRK2 or GKR3 would be an unlikely candidate for agonist-activated $S1P_3$ phosphorylation [Tobin, 2002].

A compelling aspect of this research is the evidence supporting a role for the $S1P_3$ receptor's extreme carboxyl-terminus in regulating the inhibition of $S1P_3$ phosphorylation. Initial removal of the last 27 residues of the carboxyl-terminal results in a receptor markedly phosphorylated with the absence of agonist and receptor phosphorylation in the presence of S1P is some five-fold greater than observed in the WT receptor. Accordingly, the extreme carboxyl-terminus (or any receptor-bound protein) could sterically hinder interaction of a $S1P_3$ kinase with the receptor, such that when removed significant agonist-independent receptor phosphorylation can occur. Endorsement for this model is given by the observation that agonist-stimulated phosphorylation of both $S1P_3$ receptor truncation mutants by GRK2 *in vitro* is increased in comparison to the WT receptor. Although GRK2 is most probably not the kinase responsible for $S1P_3$ phosphorylation in intact cells, the observations discussed advocate a model for WT $S1P_3$ receptor having reduced kinase accessibility as a result of its extreme 27 residue component.

The uniqueness of $S1P_3$ regulation is also exemplified by its ability to internalise in HEK293 cells despite the loss of agonist-stimulated phosphorylation upon deletion of the region between Leu^{332} and Val^{352}, although $S1P_3$ receptor internalisation is not apparent in CCL-39 cells intimating a cell-type specific role for S1P receptors where subcellular distribution of S1P receptors is dependent on the pathways required for activation by the cell [Hedemann *et al.*, 2004]. An ability to internalise regardless of loss of phosphoacceptor sites in the carboxyl-terminal domain is characteristically associated with family 2 GPCRs, like the secretin receptor [Holtmann *et al.*, 1996; Walker *et al.*, 1999], instead of family 1 receptors like $S1P_3$ although μ-opioid receptor internalisation in response to etorphine treatment occurs independently of

receptor phosphorylation [Qiu *et al.*, 2003]. Together with evidence against a role for GRK2 in mediating $S1P_3$ phosphorylation, it may be predicted that $S1P_3$ phosphorylation could have consequences for receptor function which are not seen in classical internalisation and functional desensitisation pathways. The observation that a phosphorylation-resistant $S1P_3$ truncation mutant desensitises at a rate indistinguishable from the WT receptor, indicates that phosphorylation is not required for this aspect of $S1P_3$ receptor regulation which is consistent with the hypothesis. A potential role for the C-terminal domain of $S1P_3$ was put forward by the presence of a RxxPxxP class I SH3 domain interaction motif as a scaffold for the phosphorylation-regulated assembly of signalling complexes. Motifs akin to the SH3 binding motif are found in several GPCRs, including the A_1 adenosine, dopamine D4 and β_3-adrenergic receptors [Oldenhof *et al.*, 1998; Cao *et al.*, 2000a]. For β_3-adrenergic receptors, its motifs mediate receptor interaction with Src, which is essential to observe receptor activation of the ERK signalling cascade [Cao *et al.*, 2000a]. A panel of GST fusion proteins encoding SH3 domains from multiple sources have been compared by their ability to interact and precipitate $S1P_3$ from cell extracts. The function of the SH3 binding motif within $S1P_3$ is currently unknown, but in this study it has been shown that it is functional and displays a high degree of specificity, as evidenced by the ability of a recombinant SH3 domain from Fyn, but not those from PI3K, fodrin or Src to bind to $S1P_3$ *in vitro*. However, Fyn is probably not a *bona fide* $S1P_3$-interacting protein as it was not possible to detect any association between myc$S1P_3$ and endogenous Fyn by co-immunoprecipitation in either vehicle- or agonist-treated CCL39 cells (W.A. Sands & T.M. Palmer, *unpublished observations*).

Although only conjecture at this point, it would be interesting to discover how celltype influences the interaction of fyn with $S1P_3$ and whether there is potential for it to assist in mediating TcR/CD3 signalling. However, it has been proposed that the required kinase specific regulatory mechanisms necessary for interaction with fyn reside within lipid rafts, and subcellular localisation of $S1P_3$, identified in this thesis presents the receptor within the PM, and not uniquely or abundantly within the areas of lipid rafts. Yet, if one reverses this hypothesis, the role of fyn in T cell signalling is well-known. In the resting T cell 98% of Fyn is localized in lipid rafts. Following TCR activation, Lck-associated CD4 is activated and then translocates to lipid rafts. Evidence has shown that raft-associated Lck activates Fyn, inferring that Fyn functions downstream of Lck in TCR signalling. Recently, it was shown that Fyn is required for tyrosine phosphorylation of Wiskott-Aldrich syndrome protein (WASp), a critical regulator of the Arp2/3 complex and actin polarization in T cells, which is regulated by activated Cdc42-GTP. Mutation

of Tyr^{291}, the Fyn phosphorylation site on WASp, severely inhibited TCR-induced actin polymerization and prevented the rescue of WASp deficient cells, suggesting that Fyn contributes to the regulation of cytoskeletal remodelling. Phosphorylation of this site was still observed in a mutant WASp that lacked a Cdc42 binding site [Cannons and Schwartzberg, 2004]. Although somewhat tenuous, it is possible to perceive that a complex of $S1P_3$ and arrestin recruits to where Fyn localises and this provides a platform where cytoskeletal remodelling can be regulated, this may have benefits in diseases such as atherosclerosis and COPD. And if fyn is not the specific kinase, this putative extension of the proposed model of $S1P_3$ regulation goes someway to encouraging new explanations of its roles in various celltypes and physiological processes.

Although $S1P_3$ and $S1P_1$ share similar homology they employ markedly different molecular mechanisms to govern signal output compared to other GPCRs. These differences can be exploited to specifically inhibit angiogenic signalling pathways in disease states. The unique regulation of $S1P_3$ phosphorylation may be useful in designing selective drugs. If selective drugs were designed to inhibit or promote phosphorylation, then downstream signalling could be regulated enabling the processes of angiogenesis, cell growth, differentiation to be accelerated or decelerated accordingly. For example the S1P agonist FYT720 is in trials for use as drug in transplant rejection [Rosen and Liao, 2003], and more recently a novel immunomodulator, KRP203, similar to FTY720 is being developed for use in organ transplantation [Shimizu *et al.*, 2005].

Amongst the S1P family, the presence of an SH3 domain binding motif is unique to $S1P_3$. The ability of $S1P_3$ to avoid sequestration under certain conditions can be compared to the apolipoprotein E receptor 2 (apoER2), which comprises, among others, an SH3 domain binding motif. ApoER2 is expressed mainly in the brain, and the first evidence for its physiological role came from genetically modified mice [Sun and Soutar, 2003]. The human brain expresses two major splice variants of apoER2 mRNA, one of which includes an additional exon that encodes 59 residues in the cytoplasmic domain. The LDL receptor does not contain the exon containing the three proline-rich (*PXXP*) motifs that may allow apoER2 to function as a signal transducer. LDL, VLDL and apoER2 all have an amino-terminal region composed of multiple cysteine-rich repeats that mediates ligand binding in the LDL receptor. Adjacent to this is a region with homology to the EGF precursor followed by a serine- and threonine-rich region that is heavily glycosylated in the LDL receptor. This is followed by a single TM segment and finally followed by a carboxyl-terminal cytoplasmic domain containing an NPVY motif that comprises the internalization signal of the LDL receptor [Sun and Soutar, 2003]. Investigations with chimeras

comprising the ectodomain and TM domain of the LDL receptor fused to the cytoplasmic domain of apoER2 lacking the P*XX*P-containing residues have shown chimeras are capable of mediating clathrin-dependent endocytosis of LDL as effectively as cells expressing the LDL receptor but not if the P*XX*P insert is present in the protein [Sun and Soutar, 2003]. Although expressed on the cell surface, the P*XX*P-containing chimeric receptor is excluded from clathrin vesicles as judged by its failure to co-localize with AP-2 possibly due to interaction with intracellular adaptors or scaffolding proteins. Chimeras with the TM domain of apoER2, predicted to be longer than that of the LDL receptor by several residues, failed to mediate endocytosis of LDL or to co-localize with AP-2 regardless of the presence or absence of the P*XX*P insert. Features that render apoER2 a signalling receptor rather than an endocytosis receptor, such as the LDL receptor, reside in or near the TM domain and in the PXXP motifs. The VLDLR and apoER2 can bind lipoproteins containing apoE, but their primary role *in vivo* does not appear to involve lipoprotein catabolism, and their pattern of expression differs from that of the LDL receptor.

ApoE has proved to be an important factor in the repair of neural injury, and it also appears to interact with β-amyloid proteins. The P*XX*P insert in apoER2 has also been shown to bind to scaffolding proteins, for example, the c-Jun amino-terminal kinase-interacting proteins JIP-1 and JIP-2, also known to be involved in cell signalling It has previously been shown that apoER2 cannot bind LDL and is unable to internalize and degrade even apoE-containing lipoproteins, which is consistent with the view that the main function of apoER2 is not endocytosis of lipoproteins. While the LDL receptor localizes with clathrin-containing membrane fragments apoER2 tends to associate with lighter, caveolin-containing membranes' It appears that both the TM domain and the cytoplasmic domain of apoER2 influence the properties of the protein probably because they determine its ability to localize in different regions of the cell membrane [Sun and Soutar, 2003].

Another study of apoE isoforms demonstrated that inhibition of apoptosis by lipoproteins depends partly on the apoE genotype, with lipoproteins from the apoE4/4 genotype inhibiting apoptosis less than those from other apoE genotypes. Also, the antiapoptotic activity of HDL was suppressed by VLDL comprising the apoE4 isoform. These observations may help explain how apoE modifies disease expression, and may assist in new therapeutic strategies.[DeKroon *et al.*, 2003].

In recent studies myogenic differentiation was found to be accompanied by a profound variation of $S1P_2$ and $S1P_3$ receptor expression levels and the progressive uncoupling of S1P from PLD activation [Meacci *et al.*, 2003b]. This could explain the variation in internalisation

characteristics of $S1P_3$ between cell types. Depending on the requirement of the receptor to assist in proliferation of a cell, the $S1P_3$ receptor may be expressed differentially at the cell surface of different cell-types, and thus internalisation may not be easily observed in certain cell types, if it occurs at all.

As there is an abundance of potential phosphorylation sites within the C-terminal domain of $S1P_3$ (including Ser^{326} within the SH3 binding motif), it is important to determine whether phosphorylation blocks or primes receptor interaction with associated or alternative adapter proteins. This study can be made once two-hybrid screening and affinity purification using mutant $S1P_3$ C-terminal domain constructs in which phosphorylation sites can be mutated to either Asp or Glu to mimic the acquisition of negative charge and conformation changes occuring in phosphorylation.

A novel association of S1P receptors could come from its role in survival of T cells. A key initiating event in T-cell activation by antigen is the increased phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) tyrosines in T cell receptor (TCR) subunits by the Src family kinases Lck and Fyn. Exactly how the TCR couples antigen recognition to this phosphorylation remains to be determined [Cannons and Schwartzberg, 2004]. Proposed models include juxtaposition of Lck to the TCR mediated by CD4 or CD8, increased local concentrations of kinases and their substrates following receptor oligomerization and lipid raft coalescence and the active exclusion of Csk and PTPases. These mechanisms are not mutually exclusive, and probably operate in concert.

In vitro, both Lck and Fyn can phosphorylate ITAM tyrosines of the ζ chain. Fyn seems to phosphorylate only one site with high affinity, while Lck readily phosphorylates four or five other sites. It seems that Lck is the kinase responsible for much, if not all, of the ITAM phosphorylation that follows TCR triggering in T-cells. Fyn may catalyse some ITAM phosphorylation in the absence of Lck, but is largely unable to compensate for the loss of Lck [Cannons and Schwartzberg, 2004]. Based on this discrepancy of regulation between Lck and Fyn, it would imply that those receptors requiring interaction with fyn for transduction may be less significant than those interacting with Lck, and this would hold true for S1P receptors, where $S1P_1$ is the major interactor with T cells in lymphocyte recirculation. And this would reinforce the supporting role of $S1P_3$, or help explain a distinct celltype specific function for $S1P_3$, with regard to the immune system, which has been discovered by its expression in dendritic cells and macrophages

To reiterate, the characterisation of receptor sensitivity to phosphorylation and changes in subcellular distribution that regulate the angiogenic S1P receptor $S1P_3$ has been reported. Similar to the related receptor $S1P_1$, $S1P_3$ phosphorylation is rapid and reversible in intact cells within an 18 residue region in its C-terminal domain in response to increasing concentrations of agonist. Yet $S1P_3$ is dissimilar to $S1P_1$ and many other GPCRs in that there appears to be no requirement for receptor phosphorylation to trigger the sequestration of $S1P_3$ away from the plasma membrane. In addition, GRK2 selectively phosphorylates $S1P_3$ *in vitro*. When *in vitro* and intact cell phosphorylation assays are compared *via* mutated receptors, results indicate that it is improbably that GRK2 is the agonist-regulated $S1P_3$ kinase present in intact cells. Furthermore, functional analysis of WT and phosphorylation-resistant mutant $S1P_3$–stimulated calcium mobilisation indicated that sensitivity to agonist-induced phosphorylation was unable to influence $S1P_3$ receptor desensitisation kinetics. Whilst $S1P_3$ has the potential to initiate G-protein-independent signalling, suggested by an ability to interact *in vitro* with the FynSH3 domain, the insensitivity to receptor phosphorylation of this event implies that any interaction *in vivo* with the $S1P_3$-SH3 domain would be constitutive.

As described in Chapter 1 the apoER2 has novel internalisation characteristics. The FDNPVY motif within the apoER2 c-terminal has been predicted to form a reverse turn conformation, and it is believed that this conformation is important for directing the receptor to clathrin-coated pits [Sun and Soutar, 2003]. The 59 amino acids in the cytoplasmic domain of the chimeric LDLR-TM/apoER2+, which are located 26 amino acids downstream from FDNPVY, appeared to disrupt the function of this motif [Sun and Soutar, 2003]. Although the FDNPVY motif is not present in the C-terminal of the $S1P_3$ receptor, a similar acting motif could be inhibited by the presence of the SH3 domain binding motif that $S1P_3$ possesses.

The direction of future work should be in identifying $S1P_3$ binding partners whose receptor interaction is regulated by phosphorylation within the SH3 domain, and also defining the kinase responsible for $S1P_3$ phosphorylation. Viable β-arrestin knockout cell lines would be useful tools to delineate the sequestration pathways utilised by $S1P_3$ receptors. Furthermore, assessment of the intracellular localisation would provide greater insight into the mechanisms involved in the resensitisation of the full-length receptor and the carboxyl tail chimeras. Further understanding of $S1P_3$ receptor regulation could also be achieved by investigations of C-terminally GFP-tagged forms of chimeric $S1P_3$ receptors which would provide the opportunity to directly monitor the localisation, and trafficking of receptors in response to extracellular stimuli.

In a recent study, a stable knock-down of $S1P_1$ was generated in human endothelial cell lines derived from human pulmonary microvascular endothelial cells (HPMEC-ST1.6R) and human angiosarcoma (AS-M.5 and ISO-HAS.1) to show the influences of $S1P_1$ on an array of functions in endothelial cells [Krump-Konvalinkova *et al.*, 2005]. The research establishes for the first time a reliable system that allows long-term functional consequences of $S1P_1$ silencing to be studied in detail [Krump-Konvalinkova *et al.*, 2005]. Similar investigations could be performed using the $S1P_3$ receptor as a knock-down. This is achieved by generating $S1P_3$-specific small interfering RNA (siRNA) vectors, by cloning the sequences encoding the hairpin siRNA targeted to $S1P_3$ into the siRNA expression vector, and introducing plasmids encoding the $S1P_3$ targeted siRNA into endothelial cells. This would help to assertain how $S1P_3$ exerts its influence in endothelial cells, which would augment the findings made from $S1P_3$ overexpressed in fibroblasts in this thesis.

From the proposed model that this thesis puts forward for the characterisation of the $S1P_3$ receptor it is clear that the mechanisms involved in $S1P_3$ signal transduction are not as simple as originally perceived. It appears that S1P receptor signalling is intertwinned with various growth factor signalling mechanisms, and is associated with transactivation of a number of tyrosine kinases, not merely G-protein interactions. $S1P_3$ is an enigmatic receptor, and the discrete nature of its distribution in various physiological systems lends itself to dynamic regulation for a multitude of important processes which require further investigation to provide therapeutic tools for the treatment and control of many pathological diseases.

References

Ahn, S., Maudsley, S., Luttrell, L.M., Lefkowitz, R.J. and Daaka, Y. (1999). Src-mediated tyrosine phosphorylation of dynamin is required for ß2-adrenergic receptor internalisation and mitogen-activated protein kinase signalling. *J. Biol. Chem.* **274**: 1185-1188.

Akhtar, N. and Hotchin, N.A. (2001). RAC1 regulates adherens junctions through endocytosis of E-cadherin. *Mol. Biol. Cell.* **12**: 847-862.

Alderton, F., Darroch, P., Sambi, B., McKie, A., Ahmed, I.S., Pyne, N. and Pyne, S. (2001a). G-protein-coupled receptor stimulation of the p42/p44 mitogen-activated protein kinase pathway is attenuated by lipid phosphate phosphatases 1,1a, and 2 in human embryonic kidney 293 cells. *J. Biol. Chem.* **276**: 13452-13460.

Alderton, F., Rakhit, S., Kong, K.C., Palmer, T., Sambi, B., Pyne, S. and Pyne, N.J. (2001b). Tethering of the Platelet-derived Growth Factor beta Receptor to G-protein-coupled Receptors. A novel platform for integrative signalling by these receptor classes in mammalian cells. *J. Biol. Chem.* **276**: 28578-28585.

Ali, H., Sozzani, S., Fisher, I., Barr, A.J., Richardson, R.M., Haribabu, B. and Snyderman, R. (1998). Differential regulation of formyl peptide and platelet-activating factor receptors. Role of phospholipase Cbeta3 phosphorylation by protein kinase A. *J Biol Chem.* **273**: 11012-16.

Allende, M.L. and Proia, R. L. (2002). Sphingosine 1-phosphate receptors and the development of the vascular system. *Biochim Biophys Acta.* **1582**: 222-227.

Ammit, A.J., Hastie, A.T., Edsall, L.C., Hoffman, R.K., Amrani, Y., Krymskaya, V.P., Kane, S.A., Peters, S.P., Penn, R.B., Spiegel, S. and Panettieri, R.A. (2001). Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *FASEB J.* **15**: 1212-1214.

An, S., Bleu, T., Hallmark, O.G. and Goetzl, E.J. (1998). Characterization of a Novel Subtype of Human G Protein-coupled Receptor for Lysophosphatidic Acid. *J. Biol. Chem.* **273**: 7906-7910.

An, S., Bleu, T., Huang, W., Hallmark, O.G., Coughlin, S.R. and Goetzl, E.J. (1997). Identification of cDNAs encoding two G-protein coupled receptors for lysosphingolipids. *FEBS Lett.* **417**: 279-282.

An, S., Bleu, T. and Zheng, Y. (1999). Transduction of intracellular calcium signals through G protein-mediated activation of phospholipase C by recombinant sphingosine 1-phosphate receptors. *Mol. Pharmacol.* **55**: 787-794.

An, S., Zheng, Y. and Bleu, T. (2000). Sphingosine 1-phosphate-induced cell proliferation, survival, and related signalling events mediated by G protein-coupled receptors Edg3 and Edg5. *J. Biol. Chem.* **275**: 288-296.

Ancellin, N. and Hla, T. (1999). Differential pharmacological properties and signal transduction of the sphingosine 1-phosphate receptors EDG-1, EDG-3, and EDG-5. *J. Biol. Chem.* **274**: 18997-19002.

Anderson, R.G.W. (1998). The caveolae membrane system. *Annu. Rev. Biochem.* **67**: 199-225.

Anliker, B. and Chun, J. (2004a). Cell surface receptors in lysophospholipid signaling. *Seminars in Cell & Developmental Biology.* **15**: 457-465.

Anliker, B. and Chun, J. (2004b). Lysophospholipid G Protein-coupled Receptors. *J. Biol. Chem.* **279**: 20555-20558.

Appleyard, S.M., Celver, J., Pineda, V., Kovoor, A., Wayman, G.A. and Chavkin, C. (1999). Agonist-dependent desensitisation of the κ opioid receptor by G protein receptor kinase and β-arrestin. *J. Biol. Chem.* **274**: 23802-23807.

Araki, S., Kikuchi, A., Hata, Y., Isomura, M. and Takai, Y. (1990). Regulation of reversible binding of smg p25A, a ras p21-like GTP-binding protein, to synaptic plasma membranes and vesicles by its specific regulatory protein, GDP dissociation inhibitor. *J Biol Chem.* **265**: 13007-15.

Argraves, K. M., Wilkerson, B. A., Argraves, W. S., Fleming, P. A., Obeid, L. M. and Drake, C. J. (2004). Sphingosine-1-phosphate Signaling Promotes Critical Migratory Events in Vasculogenesis. *J. Biol. Chem.* **279**: 50580-50590.

Arikawa, K., Takuwa, N., Yamaguchi, H., Sugimoto, N., Kitayama, J., Nagawa, H., Takehara, K. and Takuwa, Y. (2003). Ligand-dependent inhibition of B16 melanoma cell migration and invasion via endogenous S1P2 G protein-coupled receptor. Requirement of inhibition of cellular RAC activity. *J Biol Chem.* **278**: 32841-51.

Arvanitakis, L., Geras-Raaka, E., Varma, A., Gershengorn, M.C. and Cesarman, E. (1997). Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation. *Nature.* **385**: 347-50.

Attramadal, H., Arriza, J.L., Aoki, C., Dawson, T.M., Codina, J., Kwatra, M.M., Snyder, S.H., Caron, M.G. and Lefkowitz, R.J. (1992). ß-arrestin2, a novel member of the arrestin/ß-arrestin gene family. *J. Biol. Chem.* **267**: 17882-17890.

Bartel, P.L. and Fields, S. (1997). The Yeast Two-Hybrid System, Oxford University Press.

Baudhuin, L.M., Jiang, Y., Zaslavsky, A., Ishii, I., Chun, J. and Xu, Y. (2004). S1P3-mediated Akt activation and cross-talk with platelet-derived growth factor receptor (PDGFR). *FASEB J.* **18**: 341-3.

Beard, M.B., O'Connell, J.C., Bolger, G.B. and Houslay, M.D. (1999). The unique N-terminal domain of the cAMP phosphodiesterase PDE4D4 allows for interaction with specific SH3 domains. *FEBS Lett.* **460**: 173-7.

Becamel, C., Alonso, G., Galeotti, N., Demey, E., Jouin, P., Ullmer, C., Dumuis, A., Bockaert, J. and Marin, P. (2002). Synaptic multiprotein complexes associated with 5-HT_{2C} receptors: A proteomic approach. *EMBO J.* **21**: 2332-2342.

Benard, V., Bokoch, G.M. and Diebold, B.A. (1999). Potential drug targets: small GTPases that regulate leukocyte function. *Trends Pharmacol. Sci.* **20**: 365-370.

Bencini, C., Squecco, R., Piperio, C., Formigli, L., Meacci, E., Nosi, D., Tiribilli, B., Vassalli, M., Quercioli, F., Bruni, P., Zecchi Orlandini, S. and Francini, F. (2003). Effects of sphingosine 1-phosphate on excitation-contraction coupling in mammalian skeletal muscle. Journal of Muscle Research and Cell Motility. **24:** 539-554.

Bivona, T.G., Perez de Castro, I., Ahearn, I.M., Grana, T.M., Chiu, V.K., Lockyer, P.J., Cullen, P.J., Pellicer, A., Cox, A.D. and Philips, M.R. (2003). Phospholipase Cγ activates Ras on the Golgi apparatus by means of RasGRP1. *Nature.* **424**: 694-698.

Bladt, F., Aippersbach, E., Gelkop, S., Strasser, G.A., Nash, P., Tafuri, A., Gertler, F.B. and Pawson, T. (2003). The Murine Nck SH2/SH3 Adaptors Are Important for the Development of Mesoderm-Derived Embryonic Structures and for Regulating the Cellular Actin Network. *Mol Cell Biol.* **23**: 4586–4597.

Blaukat, A., Pizard, A., Breit, A., Wernstedt, C., Alhenc-Gelas, F., Muller-Esterl, W. and Dikic, I. (2001). Determination of Bradykinin B2 Receptor in Vivo Phosphorylation Sites and Their Role in Receptor Function. *J. Biol. Chem.* **276**: 40431-40440.

Bockaert, J., Claeysen, S., Compan, V. and Dumuis, A. (2004). 5-HT4 receptors. *Curr Drug Targets CNS Neurol Disord.* **3**: 39-51.

Bockaert, J., Marin, P., Dumuis, A. and Fagni, L. (2003). The 'magic tail' of G protein-coupled receptors: an anchorage for functional protein networks. *FEBS Lett.* **546**: 65-72.

Boehm, Stephen L., II.,, Peden, L., Harris, R. A. and Blednov, Y. A. (2004). Deletion of the fyn-Kinase Gene Alters Sensitivity to GABAergic Drugs: Dependence on {beta}2/{beta}3 GABAA Receptor Subunits. *J Pharmacol Exp Ther.* **309**: 1154-1159.

Boguski, M.S. and McCormick, F. (1993). Proteins regulating Ras and its relatives. *Nature.* **366**: 643-654.

Boivin, P. and Lecomte, M.C. (1997). Protein domains homologous to pleckstrin repeats. *M S-Med. Sci.* **13**: 639-646.

Bouchard, C., Ribeiro, P., Dube, F. and Anctil, M. (2003). A new G protein-coupled receptor from a primitive metazoan shows homology with vertebrate aminergic receptors and displays constitutive activity in mammalian cells. *J Neurochem.* **86**: 1149-61.

Boujaoude, L., Bradshaw-Wilders, C., Mao, C., Cohn, J., Ogretmen, B., Hannun, Y.A. and Obeid, L.M. (2001). Cystic fibrosis transmembrane regulator regulates uptake of sphingoid base phosphates and lysophosphatidic acid - modulation of cellular activity of sphingosine 1-phosphate. *J. Biol. Chem.,.* **276**: 35258-35264.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. *Nature.* **348**: 125-132.

Bourne, H.R., Sanders, D.A., McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. *Nature.* **348**: 125-132.

Bouvier, M., Collins, S., O'Dowd, B.F., Cambell, P.T., de Blasi, A., Kobilka, B.K., MacGregor, C., Irons, G.P., Caron, M.G. and Lefkowitz, R.J. (1989). Two distinct pathways for cAMP-mediated down-regulation of the beta 2-adrenergic receptor. Phosphorylation of the receptor and regulation of its mRNA level. *J. Biol. Chem.* **264**: 16786-16792.

Bouvier, M., Hausdorff, W.P., De Blassi, A., O'Dowd, B.F., Kobilka, B.K., Caron, M.G. and Lefkowitz, R.J. (1998). Removal of phosphorylation sites from the ß2-adrenergic receptor delays the onset of agonist-promoted desensitisation. *Nature*. **333**: 370-373.

Brady, A.E. and Limbird, L.E. (2002). G protein-coupled receptor interacting proteins: Emerging roles in localization and signal transduction. *Cellular Signalling*. **14**: 297-309.

Bremnes, T., Paasche, J.D., Mehlum, A., Sandberg, C., Bremnes, B. and Attramadal, H. (2000). Regulation and intracellular trafficking pathways of the endothelin receptors. *J. Biol. Chem.* **275**: 17596-17604.

Brezillon, S., Lannoy, V., Franssen, J.D., Le Poul, E., Dupriez, V., Lucchetti, J., Detheux, M. and Parmentier, M. (2003). Identification of natural ligands for the orphan G protein-coupled receptors GPR7 and GPR8. *J Biol Chem.* **278**: 776-83.

Brinkmann, V., Cyster, J.G. and Hla, T. (2004). FTY720: Sphingosine 1-Phosphate Receptor-1 in the Control of Lymphocyte Egress and Endothelial Barrier Function. American Journal of Transplantation. **4:** 1019-1025.

Brodin, L., Low, P. and Shupliakov, O. (2000). Sequential steps in clathrin-mediated synaptic vesicle endocytosis. *Curr. Op. Neurobiol.* **10**: 312-320.

Buday, L. and Downward, J. (1993). Epidermal growth factor regulates p21ras through the formation of a complex of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell*. **73**: 611-620.

Budd, D. C., McDonald, J. E. and Tobin, A. B. (2000). Phosphorylation and Regulation of a Gq/11-coupled Receptor by Casein Kinase 1alpha. *J. Biol. Chem.* **275**: 19667-19675.

Budde, K. (2002). First human trial of FTY720, a novel immunomodulator, in stable renal transplant patients. *J. Am. Soc. Nephrol.* **13**: 1073–1083.

Buhl, A.M., Johnson, N.L., Dhanasekaran, N. and Johnson, GL. (1995). $G_{\alpha 12}$ and $G_{\alpha 13}$ stimulate Rho-dependent stress fiber formation and focal adhesion assembly. *J Biol Chem.* **270**: 24631-4.

Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P.J. and Gierschik, P. (1992). Isozyme-selective stimulation of phospholipase C-beta 2 by G protein beta gamma-subunits. *Nature*. **360**: 684-6.

Candelore, M.R., Wright, M.J., Tota, L.M., Milligan, J., Shei, G-J., Bergstrom, J.D. and Mandala, S.M. (2002). Phytosphingosine 1-phosphate: a high affinity ligand for the S1P4/Edg-6 receptor. *Biochemical and Biophysical Research Communications*. **Volume 297**: 600-606.

Cannons, J. L. and Schwartzberg, P.L. (2004). Fine-tuning lymphocyte regulation: what's new with tyrosine kinases and phosphatases? *Current Opinion in Immunology*. **16**: 296-303.

Cao, W., Luttrell, L.M., Medvedev, A.V., Pierce, K.L., Daniel, K.W., Dixon, T.M., Lefkowitz, R.J. and Collins, S. (2000a). *J Biol Chem.* **275**: 38131.

Cao, W., Luttrell, L.M., Medvedev, A.V., Pierce, K.L., Daniel, K.W., Dixon, T.M., Lefkowitz, R.J. and Collins, S. (2000b). Direct Binding of Activated c-Src to the beta 3-Adrenergic Receptor Is Required for MAP Kinase Activation. *J. Biol. Chem.* **275**: 38131-38134.

Carmeliet, P. and Jain, R.K. (2000). Angiogenesis in cancer and other diseases. *Nature*. **407**: 249-257.

Casey, P.J. and Seabra, M.C. (1996). Protein prenyltransferases. *J. Biol. Chem.* **271**: 5289-5292.

Cavalli, V., Corti, M. and Gruenberg, J. (2001). Endocytosis and signaling cascades: a close encounter. *FEBS Lett.*. **498**: 190-196.

Chae, S.S., Proia, R.L. and Hla, T. (2004). Constitutive expression of the S1P1 receptor in adult tissues. *Prostaglandins Other Lipid Mediat.* **73**: 141-50.

Chaung, T.T., LeVine, H. and De Blasi, A. (1995). Phosphorylation and activation of ß-adrenergic receptor kinase by protein kinase C. *J. Biol. Chem.* **270**: 18660-18665.

Chaung, T.T., Paolucci, L. and De Blasi, A. (1996). Inhibition of G protein-coupled receptor kinase subtypes by protein kinase C. *J. Biol. Chem.* **271**: 28691-28696.

Chen, C.A. and Manning, D.R. (2001). Regulation of G proteins by covalent modification. *Oncogene*. **20**: 1643-1652.

Chen, C.Y., Dion, S.B., Kim, C.M. and Benovic, J.L. (1993). ß-adrenergic receptor kinase. Agonist-dependent receptor binding promotes kinase activation. *J. Biol. Chem.* **268**: 7825-7831.

Chen, W., Hu, L.A., Semenov, M.V., Yangawa, S., Kikuchi, A., Lefkowitz, R.J. and Miller, W.E. (2001). β-Arrestin1 modulates lymphoid enhancer factor transcriptional activity through interaction with phosphorylated dishevelled proteins. *Proc. Natl. Acad. Sci.* **98**: 14889-94.

Cho, H., Harrison, K., Schwartz, O. and Kehrl, J.H. (2003). The aorta and heart differentially express RGS (regulators of G-protein signalling) proteins that selectively regulate sphingosine 1-phosphate, angiotensin II and endothelin-1 signalling. *Biochem. J.* **371**: 973–980.

Chuang, D-M. and Costa, E. (1979). Evidence for internalization of the recognition site of ß-adrenergic receptors during receptor subsensitivity induced by (-)-isoproterenol. *Proc Natl Acad Sci U S A.* **76**: 3024–3028.

Chun, J., Goetzl E.J., Hla, T., Igarashi, Y., Lynch K.R., Moolenaar, W., Pyne, S. and Tigyi, G. (2002). International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. *Pharmacol. Rev.* **54**: 265-269.

Clark, E.A. and Brugge, J.S. (1995). Integrins and Signal Transduction Pathways: The Road Taken. *Science*. **268**: 233-239.

Clark, R.B. (1986). Receptor desensitisation. *Adv. Cyclic Nuc. Prot. Phos. Res.* **20**: 151-209.

Clark, R.B., Knoll, B.J. and Barber, R. (1999). Partial agonists and G protein-coupled receptor desensitisation. *Trends Pharamacol. Sci.* **20**: 279-286.

Clark, R.B., Kunkel, M.W., Friedman, J., Goka, T.J. and Johnson, J.A. (1988). Activation of cAMP-dependent protein-kinase is required for heterologous desensitisation of adenylyl cyclase in S49 wild-type lymphoma cells. *Proc. Natl. Acad. Sci. USA.* **85**: 1442-1446.

Contos, J.J. and Chun, J. (2001). The mouse Lp(A3)/Edg7 lysophophatidic acid receptor gene: genomic structure, chromosomal localisation, and expression pattern. *Gene*. **267**: 243-253.

Contos, J.J.A. and Chun, J. (1998). Complete cDNA Sequence, Genomic Structure, and Chromosomal Localization of the LPA Receptor Gene, lpA1/vzg-1/Gpcr26. *Genomics*. **51**: 364-378.

Contos, J.J.A., Ishii, I. and Chun, J. (2000). Lysophosphatidic acid receptors. *Mol. Pharmacol.* **58**: 1188-1196.

Coussin, F., Scott, R.H., Wise, A. and Nixon, G.F. (2002). Comparison of sphingosine 1-phosphate-induced intracellular signaling pathways in vascular smooth muscles: differential role in vasoconstriction. *Circ Res.* **91**: 151-7.

Cross, M.J. and Claesson-Welsh, L. (2001). FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends Pharmacol. Sci.* **22**: 201-207.

Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P.G., Coso, O.A., Gutkind. S. and Spiegel, S. (1996). Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature*. **381**: 800-3.

Daaka, Y., Luttrell, L.M. and Lefkowitz, R.J. (1997a). Switching of the coupling of the ß2-adrenergic receptor to different G proteins by protein kinase A. *Nature*. **390**: 88-91.

Daaka, Y., Pitcher, J.A., Richardson, M., Stoffel, R.H., Robishaw, J.D. and Lefkowitz, R.J. (1997b). Receptor and Gbeta gamma isoform-specific interactions with G protein-coupled receptor kinases. *PNAS*. **94**: 2180-2185.

Davidson, J.S., Flanagan, C.A., Davies, P.D., Hapgood, J., Myburgh, D., Elario, R., Millar, R.P., Forrest Owen, W. and McArdle, C.A. (1996). Incorporation of an additional glycosylation site enhances expression of functional human gonadotropin-releasing hormone receptor. *Endocrine*. **4**: 207-212.

Davidson, J.S., Flanagan, C.A., Zhou, W., Becker, I.I., Elario, R., Emeran, W., Seaflon, S.C. and Millar, R.P. (1995). Identification of N-glycosylation sites in the gonadotrophin-releasing hormone receptor: role in receptor expression but not ligand binding. *Mol. Cell. Endocrinol.* **107**: 241-245.

Davis, S., Aldrich, T.H., Jones, P.F., Acheson, A., Compton, D.L., Jain, V., Ryan, T.E., Bruno, J., Radziejewski, C., Maisonpierre, P.C. and Yancopoulos, G.D. (1996). Isolation of Angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell.* **87**: 1161-1169.

De Ligt, R.A., Kourounakis, A.P. and IJzerman, A.P. (2000). Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery. *Br J Pharmacol.* **130**: 1-12.

De Weerd, W.F.C. and Leeb-Lundberg, L.M.F. (1997). Bradykinin sequesters B2 bradykinin receptors and the receptor-coupled Ga subunits Gaq and Gal in caveolae in DDT1 MF-2 smooth muscle cells.

DeFea, K.A., Zalevsky, J., Thoma, M.S, Dery, O., Mullins, R.D. and Bunnett, N.W. (2000). ß-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J. Cell. Biol.* **148**: 1267-1281.

DeKroon, R. M., Mihovilovic, M., Goodger, Z. V., Robinette, J. B., Sullivan, P. M., Saunders, A. M. and Strittmatter, W. J. (2003). ApoE genotype-specific inhibition of apoptosis. *J. Lipid Res.* **44**: 1566-1573.

Diviani, D., Lattion, A.L., Cotecchia, S. (1997). Characterisation of the phosphorylation sites involved in G protein-coupled receptor kinase- and protein kinase C-mediated desensitisation of the a1B-adrenergic receptor. *J. Biol. Chem.* **272**: 28712-28719.

Dohlman, H.G. and Thorner, J. (1997). RGS proteins and signalling by heterotrimeric G proteins. *J. Biol. Chem.* **272**: 3871-3874.

Donati, C., Meacci, E., Nuti, F., Becciolini, L., Farnararo, M. and Bruni, P. (2005). Sphingosine 1-phosphate regulates myogenic differentiation: a major role for S1P2 receptor. *FASEB J.* **19**: 449-51.

Downes, G.B. and Gautam, N. (1999). The G protein subunit gene families. *Genomics.* **62**: 544-552.

Drmota, T., Gould, G.W. and Milligan, G. (1998). Real time visualisation of agonist-mediated redistribution and internalisation of a green fluorescent protein-tagged form of the thyrotropin-releasing hormone receptor. *J. Biol. Chem.* **273**: 24000-24008.

Duong, C. Q., Bared, S. M., Abu-Khader, A., Buechler, C., Schmitz, A. and Schmitz, G. (2004). Expression of the lysophospholipid receptor family and investigation of lysophospholipid-mediated responses in human macrophages. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids.* **1682**: 112-119.

Dvorak, H.F. (1986). Tumours: wounds that do not heal. Similarities between tumour stroma generation and wound healing. *N. Engl. J. Med.* **315**: 1650-1659.

Eisinger, D. A. and Schulz, R. (2004). Extracellular Signal-Regulated Kinase/Mitogen-Activated Protein Kinases Block Internalization of {delta}-Opioid Receptors. *J Pharmacol Exp Ther.* **309**: 776-785.

Endo, A., Nagashima, K-I., Kurose, H., Mochizuki, S., Matsuda, M. and Mochizuki, N. (2002). Sphingosine 1-Phosphate Induces Membrane Ruffling and Increases Motility of Human Umbilical Vein Endothelial Cells via Vascular Endothelial Growth Factor Receptor and CrkII. *J. Biol. Chem.* **277**: 23747-23754.

Evers, E.E., Zondag, G.C.M., Malliri, A., Price, L.S., ten Klooster, J.-P., van der Kammen, R.A. and Collard, J.G. (2000). Rho family proteins in cell adhesion and cell migration. *Eur. J. Cancer.* **36**: 1269-1274.

Fagni, L., Chavis, P., Ango, F. and Bockaert, J. (2000). Complex interactions between mGluRs, intracellular Ca2+ stores and ion channels in neurons. *Trends in Neurosciences.* **23**: 80-88.

Felder, C. C. and Glass, M. (1998). Cannabinoid Receptors And Their Endogenous Agonists. *Annual Review of Pharmacology and Toxicology.* **38**: 179-200.

Ferguson, S.G.G. (2001). Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitisation and signalling. *Pharmacol. Rev.* **53**: 1-24.

Ferguson, S.S.G., Menard, L., Barak, L.S., Colapietro, A-M., Koch, W.J. and Caron, M.G. (1995). Role of Phosphorylation in Agonist-promoted -Adrenergic Receptor Sequestration. *J. Biol. Chem.* **270**: 24782-24789.

Feron, O., Smith, T.W., Michel, T. and Kelly, R.A. (1997). Dynamic targeting of the agonist-stimulated m2 muscarinic acetylcholine receptor to caveolae in cardiac myocytes. *J. Biol. Chem.* **272**: 17744-17748.

Filipp, D. and Julius, M. (2004). Lipid rafts: resolution of the "fyn problem"? *Molecular Immunology.* **41**: 645-656.

Forrest, M., Sun, S.-Y., Hajdu, R., Bergstrom, J., Card, D., Doherty, G., Hale, J., Keohane, C., Meyers, C., Milligan, J., Mills, S., Nomura, N., Rosen, H., Rosenbach, M., Shei, G.-J., Singer, I. I., Tian, M., West, S., White, V., Xie, J., Proia, R. L. and Mandala, S. (2004). Immune Cell Regulation and Cardiovascular Effects of Sphingosine 1-Phosphate Receptor Agonists in Rodents Are Mediated via Distinct Receptor Subtypes. *J Pharmacol Exp Ther.* **309**: 758-768.

Fredriksson, R., Lagerstrom, M.C., Lundin, L-G. and Schioth, H.B. (2003). The G-Protein-coupled Receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol.* **63**: 1256-1272.

Freedman, N.J., Liggett, S.B., Drachman, D.E., Pei, G., Caron, M.G. and Lefkowitz, R.J. (1995). Phosphorylation and Desensitization of the Human β-Adrenergic Receptor. *J. Biol. Chem.* **270**: 17953-17961.

Fueller, M., Wang, D.A., Tigyi, G. and Siess, W. (2003). Activation of human monocytic cells by lysophosphatidic acid and sphingosine-1-phosphate. Cellular Signalling. **15:** 367-375.

Fuertes, G., Martin De Llano, J.J., Villarroya, A., Rivett, A.J. and Knecht, E. (2003). Changes in the proteolytic activities of proteasomes and lysosomes in human fibroblasts produced by serum withdrawal, amino-acid deprivation and confluent conditions. *Biochem J.* **375**: 75-86.

Fukata, Y., Amano, M. and Kaibuchi, K. (2001). Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganisation of non-muscle cells. *Trends Pharmacol. Sci.* **22**: 32-38.

Fukui, K., Sasaki, T., Imazumi, K., Matsuura, Y., Nakanishi, H. and Takai, Y. (1997). Isolation and characterisation of a GTPase activating protein specific for the Rab3 subfamily of small G proteins. *J. Biol. Chem.* **272**: 4655-4658.

Fukushima, N., Ishii, I., Contos, J.J.A., Weiner, J.A. and Chun, J. (2001). Lysophospholipid receptors. *Annu. Rev. Pharmacol. Toxicol.* **41**: 507-34.

Fukushima, Y., Asano, T., Takata, K., Kunaki, M., Ogihara, T., Anai, M., Tsukuda, K., Saitoh, T., Katagiri, H., Aihara, M., Matsuhashi, N., Oka, Y., Yazaki, Y. and Sugano, K. (1997). Role of the C Terminus in Histamine H2 Receptor Signaling, Desensitization, and Agonist-induced Internalization. *J. Biol. Chem.* **272**: 19464-19470.

Furui, T., LaPushin, R., Mao, M., Khan, H., Watt, S.R., Watt, M.V., Lu, Y., Fang, X., Tsutsio, S., Siddick, Z.H., Bast, R.C. and Mills, G.B. (1999). Overexpression of Edg-2/vzg-1 Induces Apoptosis and Anoikisin Ovarian Cancer Cells in a Lysophosphatidic Acid-independent Manner. *Clin. Cancer Res.* **5**: 4308-4318.

Gagnon, A.W., Kallal, L and Benovic, J.L. (1998). Role of clathrin-mediated endocytosis in agonist-induced down-regulation of the ß2-adrenergic receptor. *J. Biol. Chem.* **273**: 6976-6981.

Gaidarov, I., Krupnick, J.G., Falck, J.R., Benovic, J.L. and Keen, J.H. (1999a). Arrestin function in G protein-coupled receptor endocytosis requires phosphoinositide binding. *EMBO J.* **18**: 871-881.

Gaidarov, I., Santini, F., Warren, R.A. and Keen, J.H. (1999b). Spatial control of coated pit dynamics in living cells. *Nature Cell Biol.* **1**: 1-7.

Gbahou, F., Rouleau, A., Morisset, S., Parmentier, R., Crochet, S., Lin, J-S., Ligneau, X., Tardivel-Lacombe, J., Stark, H., Schunack, W., Ganellin, C.R., Schwartz, J-C. and Arrang, J-M. (2003). Protean agonism at histamine H3 receptors in vitro and in vivo. *PNAS.* **100**: 11086–11091.

George, S.T., Ruoho, A.E. and Malbon, C.C. (1986). N-glycosylation in expression and function of ß-adrenergic receptors. *J. Biol. Chem.* **261**: 16559-16564.

Gether, U. (2000). Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocr. Rev.* **21**: 90-113.

Gether, U. and Kobilka, B.K. (1998). Minireview: G-protein coupled Receptors, II. *J Biol Chem.* **273**: 17979-17982.

Ghosh, S. and Karin, M. (2002). Missing pieces in the NF-kB puzzle. *Cell.* **109**: 81-96.

Glomset, J.A. and Farnsworth, C.C. (1994). Role of protein modification reactions in programming interactions between ras-related GTPases and cell membranes. *Annu. Rev. Cell. Biol.* **10**: 181-205.

Goetzl, E. J. and An, S. (1998). Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J.* **12**: 1589-1598.

Goetzl, E.J., Dolezalova, H., Kong, Y., Hu, Y-L., Jaffe, R.B., Kalli, K.R. and Conover, C.A. (1999). Distinctive Expression and Functions of the Type 4 Endothelial Differentiation Gene-encoded G Protein-coupled Receptor for Lysophosphatidic Acid in Ovarian Cancer. *Cancer Res.* **59**: 5370-5375.

Gonda, K., Okamoto, H, Takuwa, N., Yatomi, Y., Okazaki, H., Sakurai, T., Kimura, S., Sillard, R., Harii, K. and Takuwa, Y. (1999). The novel sphingosine 1-phosphate receptor AGR16 is coupled via pertussis toxin-sensitive and -insensitive G-proteins to multiple signalling pathways. *Biochem J.* **337**: 67-75.

Goodman, O.B., Krupnick, J.G., Gurevich, V.V., Benovic, J.L. and Keen, J.H. (1997). Arrestin/clathrin interaction. Localisation of the arrestin binding locus to the clathrin terminal domain. *J. Biol. Chem.* **272**: 15017-15022.

Graeler, M.H., Bernhardt, G. and Lipp, M. (1998). EDG6, a novel human G protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. *Genomics*. **53**: 164-169.

Graeler, M.H. and Goetzl, E.J. (2002). Activation-regulated expression and chemotactic function of sphingosine 1-phosphate receptors in mouse splenic T cells. *FASEB J.* **16**: 1874-8.

Graeler, M.H. and Goetzl, E.J. (2004). The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J.* **18**: 551-3.

Graeler, M.H., Grosse, R., Kusch, A., Kremmer, E., Gudermann, T. and Lipp, M. (2003). The sphingosine 1-phosphate receptor S1P4 regulates cell shape and motility via coupling to Gi and G12/13. *J Cell Biochem.* **89**: 507-19.

Granzin, J., Wilden, U., Choe, H.W., Labahn, J., Krafft, B. and Buldt, G. (1998). X-ray crystal structure of arrestin from bovine rod outer segments. *Nature*. **391**: 918-921.

Griffioen, A.W. and Molema, G. (2000). Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases and chronic inflammation. *Pharmacol. Rev.* **52**: 237-268.

Gruenberg, J. and Maxfield, F.R. (1995). Membrane transport in the endocytic pathway. *Curr. Opin. Cell Biol.* **7**: 552-563.

Gurevich, V.V. and Benovic, J.L. (1992). Cell-free expression of visual arrestin. Truncation mutagenesis identifies multiple domains involved in rhodopsin interaction. *J. Biol. Chem.* **267**: 21919-21923.

Gurevich, V.V., Dion, S.B., Onorato, J.J., Ptasienski, J., Kim, C.M., Sterne-Marr, R., Hosey, M.M. and Benovic, J.L. (1995). Arrestin interactions with G protein-coupled receptors. Direct binding studies of wild type and mutant arrestins with rhodopsin, ß2-adrenergic and m2 muscarinic cholinergic receptors. *J. Biol. Chem.* **270**: 720-731.

Gurevich, V.V., Richardson, R.M., Kim, C.M., Hosey, M.M. and Benovic, J.L. (1993). Binding of wild type and chimeric arrestins to the m2 muscarinic cholinergic receptor. *J. Biol. Chem.* **268**: 11628-11638.

Haasemann, M., Cartlaud, J., Muller-Esterl, W. and Dunia, I. (1998). Agonist-induced redistribution of bradykinin B2 receptor in caveolae. *J. Cell Sci.* **111**: 917-928.

Hall, A. (1990). The cellular functions of small GTP-binding proteins. *Science.* **249**: 635-640.

Hamm, H.E. (1998). The many faces of G protein signalling. *J. Biol. Chem.* **273**: 669-672.

Hannun, Y. (1996). Functions of ceramide in coordinating cellular responses to stress. *Science.* **274**: 1855-1859.

Hannun, Y.A. (1994). The sphingomyelin cycle and the second messenger function of ceramide. *J Biol Chem.* **269**: 3125-3128.

Hannun, Y.A., Luberto, C. and Argraves, K.M. (2001). Enzymes of sphingolipid metabolism: from modular to integrative signalling. *Biochemistry.* **40**: 4893-4903.

Hanyaloglu, A. C., Vrecl, M., Kroeger, K. M., Miles, L. E. C., Qian, H., Thomas, W. G. and Eidne, K. A. (2001). Casein Kinase II Sites in the Intracellular C-terminal Domain of the Thyrotropin-releasing Hormone Receptor and Chimeric Gonadotropin-releasing Hormone Receptors Contribute to beta -Arrestin-dependent Internalization. *J. Biol. Chem.* **276**: 18066-18074.

Harada, J., Foley, M., Moskowitz, M.A. and Waeber, C. (2004). Sphingosine-1-phosphate induces proliferation and morphological changes of neural progenitor cells. *J Neurochem.* **88**: 1026-39.

Harden, T.K., Cotton, C.U., Waldo, G.L., Lutton, J.K. and Perkins, J.P. (1980). Catecholamine-induced alteration in the sedimentation behavior of membrane bound ß-adrenergic receptors. *Science.* **210**: 441-443.

Hart, M.J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W.D., Gilman, A.G., Sternweis, P.C. and Bollag, G. (1998). Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13. *Science.* **280**: 2112-4.

Hatae, N., Sugimoto, Y. and Ichikawa, A. (2002). Prostaglandin receptors: advances in the study of EP3 receptor signaling. *J Biochem.* **131**: 781-4.

Hausdorff, W.P., Bouvier, M., O'Dowd, B.F., Irons, G.P., Caron, M.G. and Lefkowitz, R.J. (1989). Phosphorylation sites on two domains of the beta 2-adrenergic receptor are involved in distinct pathways of receptor desensitisation. *J. Biol. Chem.* **264**: 12657-12665.

Heck, D.A. and Bylund, D.B. (1998). Differential down-regulation of alpha-2 adrenergic receptor subtypes. *Life Sci.* **62**: 1467-1472.

Hedemann, J., Fetscher, C. and Michel, M.C. (2004). Comparison of noradrenaline and lysosphingolipid-induced vasoconstriction in mouse and rat small mesenteric arteries. *Auton Autacoid Pharmacol.* **24**: 77-85.

Hepler, J.R. (1999). Emerging roles for RGS proteins in cell signalling. *Trends Pharmacol. Sci.* **20**: 376-382.

Hirschberg, C. B., Kisic, A. and Schroepfer, G. J., Jr. (1970). Enzymatic Formation of Dihydrosphingosine 1-Phosphate. *J. Biol. Chem.* **245**: 3084-3090.

Hla, T., Lee, M-J., Ancellin, N., Paik, J.H. and Kluk, M.J. (2001). Lysophospholipids-receptor revelations. *Science.* **294**: 1875-1878.

Hla, T. and Maciag, T. (1990). An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. *J. Biol. Chem.* **265**: 9308-9313.

Hobson, J.P., Rosenfeldt, H.M., Barak, L.S., Olivera, A., Poulton, S., Caron, M.G., Milstien, S. and Spiegel, S. (2001). Role of the sphingosine-1-phosphate receptor EDG1 in PDGF-induced cell motility. *Science.* **291**: 1800-1803.

Hoekstra, D., Maier, O., van der Wouden, J. M., Slimane, T. A. and van IJzendoorn, S. C. D. (2003). Membrane dynamics and cell polarity: the role of sphingolipids. *J. Lipid Res.* **44**: 869-877.

Holdsworth, G., Osborne, D., Pham, T., Fells, J., Hutchinson, G., Milligan, G. and Parrill, A.L. (2004). A single amino acid determines preference between phospholipids and reveals length restriction for activation of the S1P4 receptor. *BMC Biochemistry.* **5**: 12.

Holtmann, M.H., Roettger, B.F., Pinon, D.I. and Miller, L.J. (1996). Role of receptor phosphorylation in desensitization and internalization of the secretin receptor. *J Biol Chem.* **271**: 23566-71.

Hong, G., Baudhuin, L.M. and Xu, Y. (1999). Sphingosine-1-phosphate modulates growth and adhesion of ovarian cancer cells. *FEBS Lett.*,. **460**: 513-518.

Howard, A.D., McAllister, Feighner, S.D., Liu, Q., Nargund, R.P., Van der Ploeg, L.H.T. and Patchett, A.A. (2001). Orphan G-protein-coupled receptors and natural ligand discovery. *Trends Pharmacol. Sci.* **22**: 132-140.

Hur, E-M. and Kim, K-T. (2002). G protein-coupled receptor signalling and cross-talk achieving rapidity and specificity. *Cellular Signalling.* **14**: 397-405.

Iacovelli, L., Franchetti, R., Grisolia, D. and De Blasi, A. (1999a). Selective regulation of G protein-coupled receptor kinase 2 in FRTL-5 cells: analysis of thyrotrophin, $\alpha 1_b$-adrenergic, and A1 adenosine receptor-mediated responses. *Mol. Pharmacol.* **56**: 316-324.

Iacovelli, L., Sallese, M., Mariggio, S. and De Blasi, A. (1999b). Regulation of G-protein-coupled receptor kinase subtypes by calcium sensor proteins. *FASEB J.* **13**: 1-8.

Igarashi, J. and Michel, T. (2000). Agonist-modulated targeting of the EDG-1 receptor to plasmalemma caveolae - eNOS activation by sphingosine 1-phosphate and the role of caveolin-1 in sphingolipid signal transduction. *J. Biol. Chem.* **275**: 32363-32370.

Igarashi, J., Michel, T. (2001). Sphingosine 1-phosphate and isoform-specific activation of phosphoinositide 3-kinase ß - Evidence for divergence and convergence of receptor-regulated endothelial nitric-oxide synthase signalling pathways. *J. Biol. Chem.* **276**: 36281-36288.

Igarashi, Y. and Yatomi, Y. (1998). Sphingosine 1-phosphate is a blood constituent released from activated platelets, possibly playing a variety of physiological and pathophysiological roles. *Acta Biochim. Pol.* **45**: 299-309.

Ignatov, A., Lintzel, J., Kreienkamp, H.-J. and Chica Schaller, H. (2003). Sphingosine-1-phosphate is a high-affinity ligand for the G protein-coupled receptor GPR6 from mouse and induces intracellular Ca2+ release by activating the sphingosine-kinase pathway. Biochem. and Biophys. Res. Comms. **311:** 329-336.

Ikeda, W., Kakunaga, S., Takekuni, K., Shingai, T., Satoh, K., Morimoto, K., Takeuchi, M., Imai, T. and Takai, Y. (2004). Nectin-like molecule-5/Tage4 enhances cell migration in an integrin-dependent, Nectin-3-independent manner. *J Biol Chem.* **279**: 18015-25.

Im, D-S, Fujioka, T., Katada, T., Kondo, Y., Ui, M. and Okajima, F. (1997). Characterisation of sphingosine 1-phosphate-induced actions and its signalling pathways in rat hepatocytes. *Am. J. Physiol.* **272**: G1091-G1099.

Im, D-S. (2004). Discovery of new G protein-coupled receptors for lipid mediators. *J. Lipid Res.* **45**: 410-418.

Im, D-S., Clemens, J., MacDonald, T.L. and Lynch, K.R. (2001). Characterisation of the human and mouse sphingosine 1-phosphate receptor, S1P5 (EDG-8): Structure-activity relationship of sphingosine 1-phosphate receptors. *Biochem.* **40**: 14053-14060.

Im, D-S., Heise, C.E., Ancellin, N., O'Dowd, B.F., Shei, G-J., Heavens, R.P., Rigby, M.R., Hla, T., Mandala, S., McAllister, G., George, S.R. and Lynch, K.R. (2000). Characterisation of a novel sphingosine 1-phosphate receptor, Edg-8. *J. Biol. Chem.* **275**: 14281-14286.

Innamorati, G., Le Gouill, C., Balamotis, M. and Birnbaumer, M. (2001). The Long and the Short Cycle. Alternative Intracellular Routes for trafficking of G-Protein-coupled receptors. *J. Biol. Chem.* **276**: 13096-13103.

Inouye, S. and Tsuji, F.I. (1994). Aequorea green fluorescent protein expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS Lett.* **341**: 277-280.

Ishii, I., Friedman, B., Ye, X., Kawamura, S., McGiffert, C., Contos, J.J.A., Kingsbury, M.A., Zhang, G., Brown, J.H. and Chun, J. (2001). Selective loss of sphingosine 1-phosphate signalling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LPB3/EDG-3. *J. Biol. Chem.* **276**: 33697-33704.

Itokawa, M., Toru, M., Ito, K., Tsuga, H., Kameyama, K., Haga, T., Arinami, T. and Hamaguchi, H. (1996). Sequestration of the short and long isoforms of dopamine D2 receptors expressed in chinese hamster ovary cells. *Mol. Pharmacol.* **49**: 560-566.

Jahn, T., Fuglsang, A.T., Olsson, A., Brüntrup, M., Collinge, D.B., Volkmann, D., Sommarin, M., Palmgren M.G. and Larsson, C. (1997). 14-3-3 protein interacts directly with the C- terminal region of the plant plasma membrane H+ATPase. *The Plant Cell.* **9**: 1805-1814.

January, B., Seibold, A., Whaley, B., Hipkin, R.W., Lin, D., Schonbrunn, A., Barber, R. and Clark, R.B. (1997). ß2-adrenergic receptor desensitisation, internalisation and phosphorylation in response to full and partial agonists. *J. Biol. Chem.* **272**: 23871-23879.

Jensen, A.A. and Spalding, T.A (2004). Allosteric modulation of G-protein coupled receptors. *Eur. J. Pharmaceut. Sci.* **21**: 407-420.

Jewell-Motz, E.A. and Liggett, SB. (1996). G protein-coupled receptor kinase specificity for phosphorylation and desensitization of alpha2-adrenergic receptor subtypes. *J. Biol. Chem.* **271**: 18082-18087.

Jin, Y., Knudsen, E., Wang, L., Bryceson, Y., Damaj, B., Gessani, S. and Maghazachi, A.A. (2003). Sphingosine 1-phosphate is a novel inhibitor of T-cell proliferation. *Blood.* **101**: 4909-15.

Jockers, R., Angers, S., Da Silva, A., Benaroch, P., Strosberg, A.D., Bouvier, M. and Marullo, S. (1999). b2-Adrenergic Receptor Down-regulation. Evidence for a pathway that does not require endocytosis. *J. Biol. Chem.* **274**: 28900-28908.

Jockers, R., Da Silva, A., Strosberg, A.D., Bouvier, M. and Marullo, S. (1996). New molecular and structural determinants involved in ß2-adrenergic receptor desensitisation and sequestration. *J. Biol. Chem.* **271**: 9355-9362.

Jolly, P. S., Bektas, M., Olivera, A., Gonzalez-Espinosa, C., Proia, R.L., Rivera, J., Milstien, S. and Spiegel, S. (2004). Transactivation of Sphingosine-1-Phosphate Receptors by Fc{varepsilon}RI Triggering Is Required for Normal Mast Cell Degranulation and Chemotaxis. *J. Exp. Med.* **199**: 959-970.

Jolly, P.S., Rosenfeldt, H.M., Milstien, S. and Spiegel, S. (2002). The roles of sphingosine-1-phosphate in asthma. *Mol Immunol.* **38**: 1239-45.

Jones, N., Iljin, K., Dumont, D.J. and Alitalo, K. (2001). Tie receptors: new modulators of angiogenic and lymphangiogenic responses. *Nature Rev. Mol. Cell Biol.* **2**: 257-267.

Jong, Y.J., Dalemar, L.R., Wilhelm, B. and Baenziger, N.L. (1993). Human bradykinin B2 receptors isolated by receptor-specific monoclonal antibodies are tyrosine phosphorylated. *Proc. Natl. Acad. Sci. USA.* **90**: 10994-10998.

Joost, P. and Methner, A. (2002). Phylogenetic analysis of 277 human G-protein-coupled receptors as a tool for the prediction of orphan receptor ligands. *Genome Biology.* **3**: 1-16.

Kabarowski, J.H.S., Zhu, K., Le, L.Q., Witte, O.N. and Yan Xu, Y. (2001). Lysophosphatidylcholine as a Ligand for the Immunoregulatory Receptor G2A. *Science.* **293**: 702-705.

Kallal, L. and Benovic, J.L. (2000). Using green fluorescent protein to study G-protein -coupled receptor localisation and trafficking. *Trends Pharmacol. Sci.* **21**: 175-180.

Karnik, S.S., C., Gogonea, S., Patil, Saad, Y. and Takezako, T. (2003). Activation of G-protein-coupled receptors: a common molecular mechanism. *Trends Endocrin. and Metab.* **14**: 431-437.

Kawasawa, Y., Kume, K., Izumi, T. and Shimizu, T. (2000). Mammalian PSP24s ([alpha] and [beta] Isoforms) Are Not Responsive to Lysophosphatidic Acid in Mammalian Expression Systems. *Biochemical and Biophysical Research Communications.* **276**: 957-964.

Kenakin, T. (2004). Principles: receptor theory in pharmacology. *Trends Pharmacol Sci.* **25**: 186-92.

Kimura, T., Sato, K., Malchinkhuu, E., Tomura, H., Tamama, K., Kuwabara, A., Murakami, M. and Okajima, F. (2003). High-density lipoprotein stimulates endothelial cell migration and survival through sphingosine 1-phosphate and its receptors. *Arterioscler Thromb Vasc Biol.* **23**: 1283-8.

Kimura, T., Watanabe, T., Sato, K., Kon, J., Tomura, H., Tamama, K-I., Kuwabara, A., Kanda, T., Kobayashi, I., Ohta, H., Ui, M. and Okajima, F. (2000). Sphingosine 1-phosphate stimulates proliferation and migration of human endothelial cells possibly through the lipid receptors, Edg-1 and Edg-3. *Biochem. J.* **348**: 71-76.

Kitazawa, H., Yagi, T., Miyakawa, T., Niki, H. and Kawai, N. (1998). Abnormal Synaptic Transmission in the Olfactory Bulb of Fyn-Kinase-Deficient Mice. *J Neurophysiol.* **79**: 137-142.

Kluk, M.J., Colmont, C., Wu, M.-T. and Hla, T. (2003). Platelet-derived growth factor (PDGF)-induced chemotaxis does not require the G protein-coupled receptor S1P1 in murine embryonic fibroblasts and vascular smooth muscle cells. FEBS Letters. **533:** 25-28.

Koenig, J.A. and Edwardson, J.M. (1997). Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol. Sci.* **18**: 276-287.

Kogo, H., Fujimoto, T. (2000). Caveolin-1 isoforms are encoded by distinct mRNAs. Identification of mouse caveolin-1 mRNA variants caused by alternative transcription initiation and splicing. *FEBS lett.* **465**: 119-123.

Kolesnick, R.N., Kronke, M. (1998). Regulation of ceramide production and apoptosis. *Annu. Rev. Physiol.* **60**: 643-665.

Kon, J., Sato, K., Watanabe, T., Tomura, H., Kuwabara, A., Kimura, T., Tamama, K-I., Ishizuka, T., Murata N., Kanda, T., Kobayashi, I., Ohta, H., Ui, M. and Okajima, F. (1999). Comparison of intrinsic activities of the putative sphingosine 1-phosphate receptor subtypes to regulate several signalling pathways in their cDNA-transfected chinese hamster ovary cells. *J. Biol. Chem.* **274**: 23940-23947.

Kono, M., Mi, Y., Liu, Y., Sasaki, T., Allende, M.L., Wu, Y.P., Yamashita, T. and Proia, R.L. (2004). The sphingosine-1-phosphate receptors S1P1, S1P2, and S1P3 function coordinately during embryonic angiogenesis.
J Biol Chem. **279**: 29367-73.

Kostenis, E. (2004). Novel clusters of receptors for sphingosine-1-phosphate, sphingosylphosphorylcholine, and (lyso)-phosphatidic acid: New receptors for Old ligands. *J Cell Biochem.* **92**: 923-936.

Kothapalli, R., Kusmartseva, I., Loughran Jr, T.P. (2002). Characterization of a human sphingosine-1-phosphate receptor gene
(S1P5) and its differential expression in LGL leukemia. *Biochim. et Biophys. Acta.* **1579**: 117–123.

Koyasu, S. (2003). The role of PI3K in immune cells. *Nature.* **4**: 313-319.

Kozasa, T., Jiang, X., Hart, M.J., Sternweis, P.M., Singer, W.D., Gilman, A.G., Bollag, G. and Sternweis, P.C. (1998). p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. *Science.* **280**: 2109-11.

Krueger, K. M., Daaka, Y., Pitcher, J. A. and Lefkowitz, R. J. (1997). The Role of Sequestration in G Protein-coupled Receptor Resensitization. Regulation of β 2-adrenergic receptor dephosphorylation by vesicular acidification. *J. Biol. Chem.* **272**: 5-8.

Krump-Konvalinkova, V., Yasuda, S., Rubic, T., Makarova, N., Mages, J., Erl, W., Vosseler, C., Kirkpatrick, C. J., Tigyi, G. and Siess, W. (2005). Stable Knock-Down of the Sphingosine 1-Phosphate Receptor S1P1 Influences Multiple Functions of Human Endothelial Cells. *Arterioscler Thromb Vasc Biol.* **25**: 546-552.

Krupnick, J. G. and Benovic, J. L. (1998). The role of receptor kinases and and arrestins in G protein-coupled receptor regulation. *Annual Review of Pharmacology and Toxicology.* **38**: 289-319.

Krupnick, J.G., Goodman, O.B., Keen, J.H., Benovic, J.L. (1994). Arrestin/clathrin interaction. Localisation of the clathrin binding domain of nonvisual arrestins to the carboxy terminus. *J. Biol. Chem.* **272**: 15011-15016.

Langlois, S., Gingras, D. and Beliveau, R. (2004). Membrane type 1-matrix metalloproteinase (MT1-MMP) cooperates with sphingosine 1-phosphate to induce endothelial cell migration and morphogenic differentiation. *Blood.* **103**: 3020-8.

Laporte, S.A., Miller, W.E., Kim, K.M. and Caron, M.G. (2002). beta-Arrestin/AP-2 interaction in G protein-coupled receptor internalization: identification of a beta-arrestin binding site in beta 2-adaptin. *J Biol Chem.* **277**: 9247-54.

Laporte, S.A., Oakley, R.H., Holt, J.A., Barak, L.S. and Caron, M.G. (2000). The interaction of ßarrestin with the AP-2 adaptor, rather than clathrin, is required for the clustering of ß2-adrenergic receptor in clathrin-coated pits. *J. Biol. Chem.* **275**: 23120-23126.

Le Stunff, H., Galve-Roperh, I., Peterson, C., Milstien, S. and Spiegel, S. (2002). Sphingosine-1-phosphate phosphohydrolase in regulation of sphingolipid metabolism and apoptosis. *J. Cell Biol.* **158**: 1039-1049.

Lee, H., Goetzl, E.J., An, S. (2000). Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. *Am. J. Physiol.* **278**: C612-C618.

Lee, M-J., Thangada, S., Claffey, K.P., Ancellin, N., Liu, C.H., Kluk, M., Volpi, M., Sha'afi, R.I. and Hla, T. (1999). Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell.* **99**: 301-312.

Lee, M-J., Thangada, S., Paik, J-H., Sapkota, G.P., Ancellin, N., Chae, S-S, Wu, M., Morales-Ruiz, M., Sessa, W.C., Alessi, D.R. and T., Hla (2001). Akt-mediated phosphorylation of the G protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis. *Mol. Cell.* **8**: 693-704.

Lee, M-J., Thangada, S., Liu, C.H., Thompson, B.D., Hla, T. (1998a). Lysophosphatidic acid stimulates the G-protein-coupled receptor EDG-1 as a low affinity agonist. *J. Biol. Chem.* **273**: 22105-22112.

Lee, M-J., Van Brockyln, J.R., Thangada, S., Liu, C.H., Hand, A.R., Menzelev, R., Spiegel, S., Hla, T. (1998b). Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science.* **279**: 1552-1555.

Lee, S. Y., Wolff, S. C., Nicholas, R. A. and O'Grady, S. M. (2003). P2Y Receptors Modulate Ion Channel Function through Interactions Involving The C-Terminal Domain. *Mol Pharmacol.* **63**: 878-885.

Lefkowitz, R.J. (1998). G Protein-coupled Receptors. III. New roles for receptor kinases and beta-arrestins in receptor signalling and desensitization. *J. Biol. Chem.* **273**: 18677-18680.

Lefkowitz, R.J. (2004). Historical review:A brief history and personal retrospective of seven-transmembrane receptors. *Trends Pharamacol. Sci.* **25**: 413-422.

Lefkowitz, R.J. and Whalen, E.J. (2004). [beta]-arrestins: traffic cops of cell signaling. *Current Opinion in Cell Biology.* **16**: 162-168.

Levade, T., Auge, N., Veldman, R.J., Cuvillier, O., Negre-Salvayre, A. and Salvayre, R. (2001). Sphingolipid mediators in cardiovascular cell biology and pathology. *Circ Res.* **89**: 957-68.

Levkau, B., Hermann, S., Theilmeier, G., van der Giet, M., Chun, J., Schober, O. and Schafers, M. (2004). High-density lipoprotein stimulates myocardial perfusion in vivo. *Circulation.* **110**: 3355-9.

Li, G. and Zhang, X.C. (2004). GTP Hydrolysis Mechanism of Ras-like GTPases. *J. Mol. Biol.* **340**: 921–932.

Li, X.-A., Guo, L., Dressman, J. L., Asmis, R. and Smart, E. J. (2005). A novel ligand-independent apoptotic pathway induced by SR-BI and suppressed by eNOS and HDL. *J. Biol. Chem.*: M500944200.

Liang, M., Eason, M.G., Jewell-Motz E.A., Williams, M.A., Theiss, C.T., Dorn, G.W. and S.B., Liggett (1998). Phosphorylation and functional desensitisation of the alpha 2 a adrenergic receptor by protein kinase C. *Mol. Pharmacol.* **54**: 44-49.

Licht, T., Tsirulnikov, L., Reuveni, H., Yarnitzky, T. and Ben-Sasson, S.A. (2003). Induction of pro-angiogenic signaling by a synthetic peptide derived from the second intracellular loop of S1P3 (EDG3). *Blood.* **102**: 2099-107.

Lin, P., Polverini, P., Dewhirst, M., Shan, S., Rao, P.S., Peters, K. (1997). Inhibition of tumour angiogenesis using a soluble receptor establishes a role for Tie2 in pathologic vascular growth. *J. Clin. Invest.* **100**: 2072-2078.

Liu, C.H., Thangada, S., Lee, M-J., Van Brockyln, J.R., Spiegel, S. and Hla, T. (1999). Ligand-induced trafficking of the sphingosine-1-phosphate receptor EDG-1. *Mol. Biol. Cell.* **10**: 1179-1190.

Liu, J., Liao, Z., Camden, J., Griffin, K. D., Garrad, R. C., Santiago-Perez, L. I., Gonzalez, F. A., Seye, C. I., Weisman, G. A. and Erb, L. (2004). Src Homology 3 Binding Sites in the P2Y2 Nucleotide Receptor Interact with Src and Regulate Activities of Src, Proline-rich Tyrosine Kinase 2, and Growth Factor Receptors. *J. Biol. Chem.* **279**: 8212-8218.

Liu, Y., Wada, R, . , Yamashita, T., Mi, Y., Deng, C-X., Hobson, J.P., Rosenfeldt, H.M., Nava, V.E., Chae, S-S., Lee, M-J., Liu, C.H., Hla, T., Spiegel, S. and Proia, R.L. (2000). Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J. Clin. Invest.* **106**: 951-961.

Luttrell, L.M., Ferguson, S.S., Daaka, Y., Miller, W.E., Maudsley, S., Della Rocca, G.J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D.K., Caron, M.G. and Lefkowitz, R.J. (1999). ß-arrestin-dependent formation of ß2-adrenergic receptor Src protein kinase complexes. *Science.* **283**: 655-661.

Luttrell, L.M. and Lefkowitz, R.J. (2002). The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J. Cell Sci.* **115**: 455-65.

Lynch, K.R. and Im, D.S. (1999). Life on the edg. *Trends Pharmacol. Sci.* **20**: 473-475.

Maceyka, M., Milstien, S. and Spiegel, S. (2005). Sphingosine kinases, sphingosine-1-phosphate and sphingolipidomics. *Prostaglandins & Other Lipid Mediators.* **In Press, Corrected Proof**.

MacLennan, A. J and Browe, C.S. (1994). Cloning and characterisation of a putative G-protein coupled receptor potentially involved in development. *Mol Cell Neurosci.* **5**: 201-209.

Madziva, M.T., Bai, J., Bhalla, A., Chapman, E.R. and Edwardson, J.M. (2005). Effects of synaptotagmin reveal two distinct mechanisms of agonist-stimulated internalization of the M4 muscarinic acetylcholine receptor. *Br J Pharmacol.* **144**: 761-71.

Maisonpierre, P.C., Suri, C., Jones, P.F., Bartunkova, S., Wiegand, S.J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T.H., Papadopoulos, N., Daly, T.J., Davis, S., Sato, T.N. and Yancopoulos, G.D. (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science.* **277**: 55-60.

Malek, R.L., Toman, R.E., Edsall, L.C., Wong, S., Chiu, J., Letterle, C.A., Van Brocklyn, J.R., Milstien, S., Spiegel, S. and Lee, N.H. (2001). Nrg-1 Belongs to the Endothelial Differentiation Gene Family of G Protein-coupled Sphingosine-1-phosphate Receptors. *J. Biol. Chem.* **276**: 5692-5699.

Marchese, A., Chen, C., Kim, Y-M. and Benovic, J.L. (2003). The ins and outs of G protein-coupled receptor trafficking. *Trends Biochem. Sci.* **28**: 369-376.

Marchese, A., Sawzdargo, M., Nguyen, T., Cheng, R., Heng, H. H. Q., Nowak, T., Im, D-S., Lynch, K. R., George, S. R. and O'Dowd, B. F. (1999). Discovery of Three Novel Orphan G-Protein-Coupled Receptors. *Genomics.* **56**: 12-21.

Marinissen, M.J. and Gutkind, J.S. (2001). G-protein-coupled receptors and signalling networks: emerging paradigms. *Trends Pharmacol. Sci.* **22**: 368-375.

Mariotti, A., Kedeshian, P. A., Dans, M., Curatola, A. M., Gagnoux-Palacios, L. and Giancotti, F. G. (2001). EGF-R signaling through Fyn kinase disrupts the function of integrin {alpha}6{beta}4 at hemidesmosomes: role in epithelial cell migration and carcinoma invasion. *J. Cell Biol.* **155**: 447-458.

Mayer, B.J. (2001). SH3 domains: complexity in moderation. *J Cell Sci.* **114**: 1253-1263.

Mayor Jr., F., Penela, P. and Ruiz-Gomez, A. (1998). Role of G Protein-Coupled Receptor Kinase 2 and Arrestins in [beta]-Adrenergic Receptor Internalization. *Trends in Cardiovascular Medicine.* **8**: 234-240.

Mazurais, D., Robert, P., Gout, B., Berrebi-Bertrand, I., Laville, M.P. and Calmels, T. (2002). Cell type-specific localization of human cardiac S1P receptors. *J Histochem Cytochem.* **50**: 661-70.

McLean, A.J., Bevan, N., Rees, S. and G., Milligan. (1999). Visualising differences in ligand regulation of wild-type and constitutively active mutant b2-adrenoceptor-green fluorescent protein fusion proteins. *Mol. Pharmacol.* **56**: 1182-1191.

Meacci, E., Becciolini, L., Nuti, F., Donati, C., Cencetti, F., Farnararo, M. and Bruni, P. (2002). A role for calcium in sphingosine 1-phosphate-induced phospholipase D activity in C2C12 myoblasts. FEBS Letters. **521:** 200-204.

Meacci, E., Cencetti, F., Donati, C., Nuti, F., Farnararo, M., Kohno, T., Igarashi, Y. and Brunia, P. (2003a). Down-regulation of EDG5/S1P2 during myogenic differentiation results in the specific uncoupling of sphingosine 1-phosphate signalling to phospholipase D. *Biochimica et Biophysica Acta.* **1633**: 133– 142.

Meacci, E., Donati, C., Farnararo, M. and Bruni, P. (2003b). Sphingosine 1-phosphate signal transduction in muscle cells. *Ital J Biochem.* **52**: 25-7.

Meacci, E., Vasta, V., Donati, C., Farnararo, M. and Bruni, P. (1999). Receptor-mediated activation of phospholipase D by sphingosine 1-phosphate in skeletal muscle C2C12 cells; a role for protein kinase C. *FEBS Lett.* **457**: 184-188.

Menard, L., Ferguson, S.S.G., Zhang, J., Lin, F-T., Lefkowitz, R.J., Caron, M.G. and Barak, L.S. (1997). Synergistic regulation of ß2-adrenergic receptor sequestration: intracellular complement of ß-adrenergic receptor kinase and ß-arrestin determine kinetics of internalisation. *Mol. Pharmacol.* **51**: 800-808.

Miller, W.E. and Lefkowitz, R.J. (2001). Expanding roles for ß-arrestins as scaffolds and adapters in GPCR signalling and trafficking. *Curr. Op. Cell Biol.* **13**: 139-145.

Miller, W.E., Maudsley, S., Ahn, S., Khan, K.D., Luttrell, L.M. and Lefkowitz, R.J. (2000). ß-arrestin1 interacts with the catalytic domain of the tyrosine kinase c-SRC. Role of ß-arrestin1-dependent targeting of c-SRC in receptor endocytosis. *J. Biol. Chem.* **275**: 11312-11319.

Milligan, G. (1995). Signal sorting by G-protein-linked receptors. *Adv Pharmacol.* **32**: 1-29.

Milligan, G. (1998). New Aspects of G-Protein-coupled Receptor Signalling and Regulation. *Trends Endocrin. and Metab.* **9**: 13-19.

Milligan, G. (1999). Exploring the dynamics of regulation of G protein-coupled receptors using green fluorescent protein. *Br. J. Pharmacol.* **128**: 501-510.

Milligan, G. (2000). Insights in to ligand pharmacology using receptor-G-protein fusion proteins. *Trends Pharamacol. Sci.* **21**: 24-28.

Milligan, G. and Bond, R.A. (1997). Inverse agonism and the regulation of receptor number. *Trends Pharmacol. Sci.* **18**: 468-474.

Milligan, G. and White, J.H. (2001). Protein-protein interactions at G-protein-coupled receptors. *Trends Pharmacol. Sci.* **22**: 513-518.

Mineo, C., James, G.J., Smart, E.J. and Anderson, R.G.W. (1996). Localisation of epidermal growth factor-stimulated Ras/Raf-1 interaction to caveolae membrane. *J. Biol. Chem.* **271**: 11930-11935.

Moffett, S., Mouillac, B., Bonin, H. and Bouvier, M. (1993). Altered phosphorylation and desensitisation patterns of a human b2-adrenergic receptor lacking the palmitoylated cys341. *EMBO J.* **12**: 349-356.

Moolenaar, W.H. (1999). Bioactive lysophospholipids and their G protein-coupled receptors. *Expt. Cell Res.* **253**: 230-238.

Morales-Ruiz, M., Lee, M-J., Zollner, S., Gratton, J-P., Scotland, R., Shiojima, I., Walsh, K., Hla, T. and Sessa, W.C. (2001). Sphingosine 1-phosphate activates Akt, nitric oxide production and chemotaxis through a Gi protein/phosphoinositide 3-kinase pathway in endothelial cells. *J. Biol. Chem.* **276**: 19672-19677.

Moro, O., Lameh, J. and Sadee, W. (1993). Serine- and threonine-rich domain regulates internalisation of muscarinic cholinergic receptors. *J. Biol. Chem.* **271**: 21490-21497.

Mukherjee, S., Ghosh, R.N. and Maxfield, F. (1997). Endocytosis. *Physiol. Rev.* **77**: 759-803.

Murakami, N., Yokomizo, T., Okuno, T. and Shimizu, T. (2004). G2A Is a Proton-sensing G-protein-coupled Receptor Antagonized by Lysophosphatidylcholine. *J. Biol. Chem.* **279**: 42484-42491.

Nakata, H., Kameyama, K., Haga, K. and T., Haga (1994). Location of agonist-dependent-phosphorylation sites in the third intracellular loop of muscarinic acetylcholine receptors (m2) subtype. *Eur. J. Biochem.* **220**: 29-36.

Niedernberg, A., Blaukat, A., Schoneberg, T. and Kostenis, E. (2003a). Regulated and constitutive activation of specific signalling pathways by the human S1P5 receptor. *Br J Pharmacol.* **138**: 481-93.

Niedernberg, A., Scherer, C.R., Busch, A. E. and Kostenis, E. (2002). Comparative analysis of human and rat S1P5 (edg8): differential expression profiles and sensitivities to antagonists. *Biochemical Pharmacology.* **64**: 1243-1250.

Niedernberg, A., Tunaru, S., Blaukat, A., Ardati, A. and Kostenis, E. (2003b). Sphingosine 1-phosphate and dioleoylphosphatidic acid are low affinity agonists for the orphan receptor GPR63. Cellular Signalling. **15:** 435-446.

Nofer, J.R., van der Giet, M., Tolle, M., Wolinska, I., von Wnuck Lipinski K, Baba HA, Tietge UJ, Godecke A, Ishii I, Kleuser B, Schafers M, Fobker M, Zidek W, Assmann G, Chun J and B., Levkau (2004). HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest.* **113**: 569-81.

Noguchi, K., Ishii, S. and Shimizu, T. (2003). Identification of p2y9/GPR23 as a Novel G Protein-coupled Receptor for Lysophosphatidic Acid, Structurally Distant from the Edg Family. *J. Biol. Chem.* **278**: 25600-25606.

Oakley, R.H., Laporte, S.A., Holt, J.A., Barak, L.S. and Caron, M.G. (1999). Association of ß-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitisation. *J. Biol. Chem.* **274**: 32248-32257.

O'Connell, B. J. and Genest, J., Jr. (2001). High-Density Lipoproteins and Endothelial Function. *Circulation.* **104**: 1978-1983.

Offermanns, S. (2001). In vivo functions of heterotrimeric G-proteins: studies in Ga-deficient mice. *Oncogene.* **20**.

Oh, P., McIntosh, D.P. and Schnitzer, J.E. (1998). Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. *J. Cell Biol.* **141**: 101-114.

Okamoto, H., Takuwa, N., Gonda, K., Okazaki, H., Chang, K., Yatomi, Y., Shigematsu, H. and Takuwa, Y. (1998). EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to multiple signalling pathways, including phospholipase C activation, Ca2+ mobilization, Ras-Mitogen-activated protein kinase activation, and adenylate cyclase inhibition. *J. Biol. Chem.* **273**: 27104-27110.

Okamoto, H., Takuwa, N., Yokomizo, T., Sugimoto, N., Sakurada, S., Shigematsu, H. and Takuwa, Y. (2000a). Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. *Mol. Cell. Biol.* **20**: 9247-9261.

Okamoto, Y., Ninomiya, H., Miwa, S. and Masaki, T. (2000b). Cholesterol oxidation switches the internalisation pathway of endothelia receptor type A from caveolae to clathrin-coated pits in chinese hamster ovary cells. *J. Biol. Chem.* **275**: 6439-6446.

Okazaki, H. and Ishizaka, N. (1993). Molecular cloning of a novel putative G-protein coupled receptor expressed in the cardiovascular system. *Biochem Biophys Res Commun.* **190**: 1104-1109.

Oldenhof, J., Vickery, R., Anafi, M., Oak, J., Ray, A., Schoots, O., Pawson, T., von Zastrow, M. and Van Tol, H.H. (1998). SH3 binding domains in the dopamine D4 receptor. *Biochemistry.* **37**: 15726-36.

Oliveira, L., Hulsen, T., Lutje Hulsik, D., Paiva, A.C.M. and Vriend, G. (2004). Heavier-than-air flying machines are impossible. *FEBS Lett.* **564**: 269-273.

Olivera, A., Kohama, T., Edsall, L., Nava, V., Cuvillier, O. and Poulton, S. (1999). Sphingosine kinase expression increases intracellular sphingosine-1-phosphate and promotes cell growth and survival. *J Cell Biol.* **147**: 545-557.

Olivera, A. and Spiegel, S. (1993). Sphingosine 1-phosphate, a novel lipid as a second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature.* **365**: 557-560.

Onorato, J.J., Palczewski, K., Regan, J.W., Caron, M.G., Lefkowitz, R.J. and Benovic, J.L. (1991). Role of acidic amino acids in peptide substrates of the beta-adrenergic receptor kinase and rhodopsin kinase. *Biochemistry.* **30**: 5118-25.

Opperman, M., Freedman, NJ., Alexander, RW. and Lefkowitz, RJ. (1996). Phosphorylation of the type 1A angiotensin II receptor by G protein-coupled receptor kinases and protein kinase C. *J. Biol. Chem.* **271**: 13266-13272.

Ostrom, R.S. (2002). New Determinants of Receptor-Effector Coupling: Trafficking and Compartmentation in Membrane Microdomains. *Mol Pharmacol.* **61**: 473-476.

Paik, J.H., Chae, S-S., Lee M-J, Thangada S. and T., Hla (2001). Sphingosine 1-phosphate-induced endothelial cell migration requires the expression of EDG-1 and EDG-3 receptors and Rho-dependent activation of anb3- and b1-containing integrins. *J. Biol. Chem.* **276**: 11830-11837.

Pak, Y., O'Dowd, B.F., Wang, J.B. and George, S.R. (1999). Agonist-induced, G Protein-dependent and -independent down-regulation of the μ Opioid Receptor. The receptor isa direct substrate for protein-tyrosine kinase. *J. Biol. Chem.* **274**: 27610-27616.

Palczewski, K. (1994). Structure and functions of arrestins. *Protein Sci.* **3**: 1355-61.

Palczewski, K. , Buczylko, J., Ohguro, H., Annan, RS., Carr, SA., Crabb, JW. , Kaplan, MW. , Johnson, RS. and Walsh, KA. (1994). Characterization of a truncated form of arrestin isolated from bovine rod outer segments. *Protein Sci.* **3**: 314-324.

Palmer, T.M., Benovic, J.L. and Stiles, G.L. (1995). Agonist-dependent phosphorylation and desensitisation of the rat A3 adenosine receptor. *J. Biol. Chem.* **270**: 29607-29613.

Palmer, T.M., Benovic, J.L. and Stiles, G.L. (1996). Molecular basis for subtype-specific desensitisation of inhibitory adenosine receptors-analysis of a chimeric A1-A3 adenosine receptor. *J. Biol. Chem.* **271**: 15272-15278.

Panettieri, R. A., Jr. (2004). Effects of Corticosteroids on Structural Cells in Asthma and Chronic Obstructive Pulmonary Disease. *Proc Am Thorac Soc.* **1**: 231-234.

Parrill, A.L., Sardar V.M. and Yuan, H. (2004). Sphingosine 1-phosphate and lysophosphatidic acid receptors: agonist and antagonist binding and progress toward development of receptor-specific ligands. *Semin Cell Dev Biol.* **15**: 467-76.

Parrill, A.L., Wang, D-A., Bautista, D.L., Van Brocklyn, J.R., Lorincz, Z., Fischer, D.J., Baker, D.L., Liliom, K., Spiegel, S. and Tigyi, G. (2000). Identification of Edg1 receptor residues that recognise sphingosine 1-phosphate. *J. Biol. Chem.* **275**: 39379-39384.

Parruti, G. , Peracchia, F. , Sallese, M. , Ambrosini, G. , Masini, M. , Rotilio, D. and De Blasi, A. (1993). Molecular analysis of human beta-arrestin-1: cloning, tissue distribution, and regulation of expression. Identification of two isoforms generated by alternative splicing. *J. Biol. Chem.* **268**: 9753-9761.

Parton, R.G. (1996). Caveolae and caveolins. *Curr. Op. Cell Biol.* **8**: 542-548.

Patan, S. (1998). Tie1 and Tie2 receptor tyrosine kinase inversely regulate embryonic angiogenesis by the mechanism of intussusceptive microvascular growth. *Microvasc. Res.* **56**: 1-21.

Payne, S.G., Milstien, S., Barbour, S.E. and Sarah Spiegel, S. (2004). Modulation of adaptive immune responses by sphingosine-1-phosphate. *Seminars in Cell & Developmental Biology.* **15**: 521–527.

Payne, S.G., Milstien, S. and Spiegel, S. (2002). Sphingosine 1-phosphate: dual messenger functions. *FEBS Lett.* **531**: 54-57.

Pebay-Peyroula, E., Rummel, G., Rosenbusch, JP. and Landau, EM. (1997). X-ray structure of bacteriorhodopsin at 2.5 angstroms from microcrystals grown in lipidic cubic phases. *Science.* **277**: 1676-1681.

Penela, P., Ribas, C. and Mayor, Jr., F. (2003). Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. *Cellular Signalling.* **15**: 973-981.

Pie, G., Samama, P., Lohse, M., Wang, M., Codina, J. and Lefkowitz, R.J. (1994). A constitutively active mutant ß2-adrenergic receptor is constitutively desensitised and phosphorylated. *Proc. Natl. Acad. Sci. USA.* **91**: 2699-2702.

Pierce, K.L. and Lefkowitz, R.J. (2001). Classical and new roles for ßarrestins in the regulation of G-protein-coupled receptors. *Nat. Rev. Neurosci.* **2**: 727.

Pippig, S., Andexinger, S. , Daniel, K., Puzicha, M. , Caron, MG., Lefkowitz, RJ. and Lohse, MJ. (1993). Overexpression of beta-arrestin and beta-adrenergic receptor kinase augment desensitization of beta 2-adrenergic receptors. *J. Biol. Chem.* **268**: 3201-3208.

Pippig, S., Andexinger, S. and Lohse, MJ. (1995). Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization. *Mol. Pharmacol.* **47**: 666-676.

Pizard, A., Blaukat, A., Muller-Esterl, W., Alhenc-Gelas, F. and Rajerison, R. M. (1999). Bradykinin-induced Internalization of the Human B2 Receptor Requires Phosphorylation of Three Serine and Two Threonine Residues at Its Carboxyl Tail. *J. Biol. Chem.* **274**: 12738-12747.

Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G. and Cromier, M.J. (1992). Primary structure of the Aequorea victoria green fluorescent protein. *Gene.* **111**: 229-233.

Pronin, A.N. and Benovic, J.L. (1997). Regulation of the G-protein-coupled receptor kinase GRK5 by protein kinase C. *J. Biol. Chem.* **272**: 3806-3812.

Pronin, A.N., Satpaev, D.K., Slepak, V.Z. and Benovic, J.L. (1997). Regulation of G protein-coupled receptor kinases by calmodulin and localisation of the calmodulin binding domain. *J. Biol. Chem.* **272**: 18273-18280.

Prossnitz, E.R., Kim, C.M., Benovic, J.L. and Ye, R.D. (1995). Phosphorylation of the N-formyl peptide receptor carboxyl terminus by the G protein-coupled receptor kinase, GRK2. *J Biol Chem.* **270**: 1130-7.

Pyne, N., Waters, C., Moughal, N.A., Sambi, B.S. and Pyne, S. (2003). Receptor tyrosine kinase–GPCR signal complexes. *Biochem. Soc. Trans.* **31**: 1220–1225.

Pyne, S. and Pyne, N.J. (2000a). Sphingosine 1-phosphate signalling in mammalian cells. *Biochem. J.* **349**: 385-402.

Pyne, S. and Pyne, N.J. (2000b). Sphingosine 1-phosphate signalling via the endothelial differentiation gene family of G-protein-coupled receptors. *Pharmacol. Ther.* **88**: 115-131.

Qiu , Y., Law, P.-Y. and Loh, H.H. (2003). μ-Opioid Receptor Desensitization role of receptor phosphorylation, internalisation, and resensitization. *J. Biol. Chem.* **278**: 36733-36739.

Radeff-Huang, J., Seasholtz, T.M., Matteo, R.G. and Brown, J.H. (2004). G protein mediated signaling pathways in lysophospholipid induced cell proliferation and survival. *J Cell Biochem.* **92**: 949-66.

Radhika, V. and Dhanasekaran, N. (2001). Transforming G proteins. *Oncogene.* **20**: 1607-1614.

Rakhit, S., Pyne, S. and Pyne, N.J. (2000). The platelet-derived growth factor receptor stimulation of p42/p44 mitogen-activated protein kinase in airway smooth muscle involves a G-protein-mediated tyrosine phosphorylation of Gab1. *Mol. Pharmacol.* **58**: 413-420.

Rao, T.S., Lariosa-Willingham, K.D., Lin, F.F., Palfreyman, E.L., Yu, N., Chun, J. and Webb, M. (2003). Pharmacological characterization of lysophospholipid receptor signal transduction pathways in rat cerebrocortical astrocytes. *Brain Res.* **990**: 182-94.

Raposo, G., Dunia, I., Delavier-Klutchko, C., Kaveri, S., Strosberg, AD. and Benedetti, EL. (1989). Internalization of beta-adrenergic receptor in A431 cells involves non-coated vesicles. *Eur. J. Cell Biol.* **50**: 340-352.

Richard, D.E., Vouret-Craviari, V. and Pouyssegur, J. (2001). Angiogenesis and G-protein-receptors: signals that bridge the gap. *Oncogene.* **20**: 1556-1562.

Rim, J. and Oprian, D.D. (1995). Constitutive activation of opsin: Interaction of mutants with rhodopsin kinase and arrestin. *Biochemistry.* **34**: 11938-11941.

Romiti, E., Meacci, E., Tani, M., Nuti, F., Farnararo, M., Ito, M. and Bruni, P. (2000). Neutral/alkaline and acid ceramidase activities are actively released by murine endothelial cells. *Biochem. Biophys. Res. Commun.* **275**: 746-751.

Rosen, H. and Liao, J. (2003). Sphingosine 1-phosphate pathway therapeutics: a lipid ligand-receptor paradigm. Current Opinion in Chemical Biology. **7:** 461-468.

Rosenfeldt, H.M., Amrani, Y., Watterson, K.R., Murthy, K.S., Panettieri, R. A. and Spiegel, S. (2003). Sphingosine-1-phosphate stimulates contraction of human airway smooth muscle cells. *FASEB J.* **17**: 1789-1799.

Rosenfeldt, H.M., Hobson, J.P., Maceyka, M., Olivera, A., Nava, V.E., Milstien, S. and Spiegel, S. (2001). EDG-1 links the PDGF receptor to Src and focal adhesion kinase activation leading to lamellipodia formation and cell migration. *FASEB J.* **15**: 2649-2659.

Rosenkilde, M.M., Waldhoer, M., Luttichau, H.R. and Schwartz, T.W. (2001). Virally encoded 7TM receptors. *Oncogene.* **20**: 1582-1593.

Roviezzo, F., Del Galdo, F., Abbate, G., Bucci, M., D'Agostino, B., Antunes, E., De Dominicis, G., Parente, L., Rossi, F., Cirino, G. and De Palma, R. (2004). Human eosinophil chemotaxis and selective in vivo recruitment by sphingosine 1-phosphate. *Nat. Acad. Sci.* **101**: 11170-11175.

Rutherford, C., Ord-Shrimpton, F.U., Sands, W.A., Pediani, J.D., Jeffrey L. Benovic, J.L., McGrath, J.C. and Palmer, T.M. (2005). Phosphorylation-independent internalisation and desensitisation of the human sphingosine-1-phosphate receptor S1P3. *Cell Signalling.*

Saaristo, A., Karpanen, T. and Alitalo, K. (2000). Mechanisms of angiogenesis and their use in the inhibition of tumour growth and metastasis. *Oncogene.* **19**: 6122-6129.

Saba, J.D. and Hla, T. (2004). Point-Counterpoint of Sphingosine 1-Phosphate Metabolism. *Circ Res.* **94**: 724-734.

Sachinidis, A., Kettenhofen, R., Seewald, S., Gouni-Berthold, I., Schmitz, U., Seul, C., Ko, Y. and Vetter, H. (1999). Evidence that lipoproteins are carriers of bioactive factors. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **19**: 2412-2421.

Sah, V.P., Seasholtz, T.M., Sagi, S.A. and Brown, J.H. (2000). The role of Rho in G protein-coupled receptor signal transduction. *Annu. Rev. Pharmacol. Toxicol.* **40**: 459-489.

Sakmar, T.P. (1998). Rhodopsin: a prototypical G protein-coupled receptor. *Prog. Nucleic Acid Res. Mol. Biol.* **59**: 1-34.

Salim, K., Fenton, T., Bacha, J., Urien-Rodriguez, H., Bonnert, T., Skynner, H.A., Watts, E., Kerby, J., Heald, A., Beer, M., McAllister, G. and Guest, P.C. (2002). Oligomerization of G-protein-coupled receptors shown by selective co-immunoprecipitation. *J Biol Chem.*

Salomone, S., Yoshimura, S., Reuter, U., Foley, M., Thomas, S.S., Moskowitz, M.A. and Waeber, C. (2003). S1P3 receptors mediate the potent constriction of cerebral arteries by sphingosine-1-phosphate. *Eur J Pharmacol.* **469**: 125-34.

Sanna, M. G., Liao, J., Jo, E., Alfonso, C., Ahn, M.-Y., Peterson, M.S., Webb, B., Lefebvre, S., Chun, J., Gray, N. and Rosen, H. (2004). Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. *J. Biol. Chem.* **279**: 13839–13848.

Sarvazyan, N.A., Lim, W.K. and Neubig, R.R. (2002). Fluorescence analysis of receptor-G protein interactions in cell membranes. *Biochemistry.*

Sato, K. and Murata, N. (1998). Downregulation of mRNA expression of Edg-3, a putative sphingosine 1-phosphate receptor coupled to Ca2+ signalling, during differentiation of HL60 Leukemia cells. *Biochem Biophys Res Commun.* **253**: 253-256.

Sato, K., Tomura, H., Igarashi, Y., Ui, M., Okajima, F. (1999). Possible involvement of cell surface receptors in sphingosine 1-phosphate-induced activation of extracellular signal-regulated kinase in C6 glioma cells. *Mol. Pharmacol.* **55**: 126-133.

Schlador, M.L. and Nathanson, N.M. (1997). Synergistic regulation of m2 muscarinic acetylcholine receptor desensitisation and sequestration by G-protein coupled receptor kinase-2 and ß-arrestin-1. *J. Biol. Chem.* **272**: 18882-18890.

Schmid, S.L. (1997). Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.* **66**: 511-548.

Schulein, R., Lorenz, D., Oksche, A., Wiesner, B., Hermosilla, R., Ebert, J. and Rosenthal, W. (1998). Polarized cell surface expression of the green fluorescent protein-tagged vasopressin V2 receptor in Madin Darby canine kidney cells. *FEBS Lett.* **441**: 170-176.

Seachrist, J., Anborgh, P.H. and Ferguson, S.S.G. (2000). ß2-adrenergic receptor internalisation, endosomal sorting and plasma membrane recycling are regulated by Rab GTPases. *J. Biol. Chem.* **273**: 7637-7642.

Shapiro, M.J., Trejo, J., Zeng, D. and Coughlin, S.R. (1996). Role of the Thrombin Receptor's Cytoplasmic Tail in Intracellular Trafficking. Distinct determinants for agonist-triggered versus tonic internalisation and intracellular localisation. *J. Biol. Chem.* **271**: 32874 - 32880.

Shaul, P. W. and Mineo, C. (2004). HDL action on the vascular wall: is the answer NO? *J. Clin. Invest.* **113**: 509-513.

Shayo, C., Fernandez, N. , Legnazzi, BL. , Monczor, F , Mladovan, A. , Baldi, A. and Davio, C. (2001). Histamine H2 receptor desensitization: involvement of a select array of G protein-coupled receptor kinases. *Mol. Pharmacol.* **60**: 1049-1056.

Shenoy, S.K. and Lefkowitz, R.J. (2003a). Multifaceted roles of b-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. *Biochem. J.* **375**: 503–515.

Shenoy, S.K. and Lefkowitz, R.J. (2003b). Trafficking Patterns of β-Arrestin and G Protein-couple Receptors Determined by the Kinetics of β-Arrestin Deubiquitination. *J Biol Chem.* **278**: 14498-14506.

Shenoy, S.K., McDonald, P.H., Kohout, T.A. and Lefkowitz, R.J. (2001). Regulation of receptor fate by ubiquitination of activated ß2-adrenergic receptor and ß-arrestin. *Science.* **294**: 1307-1313.

Shimizu, H., Takahashi, M., Kaneko, T., Murakami, T. , Hakamata, Y. , Kudou, S., Kishi, T. , Fukuchi, K I., Wanami, S., Kuriyama, K. , Yasue, T., Enosawa, S. , Matsumoto, K. , Takeyoshi, I., Morishita, Y. and Kobayashi, E. (2005). KRP-203, a novel synthetic immunosuppressant, prolongs graft survival and attenuates chronic rejection in rat skin and heart allografts. *Circulation.* **111**: 222-229.

Sibley, D.R. and Lefkowitz, R.J. (1985). Molecular mechanisms of receptor desensitization using the beta-adrenergic receptor-coupled adenylate cyclase system as a model. *Nature.* **317**: 124-129.

Siehler, S. and Manning, D.R. (2002). Pathways of transduction engaged by sphingosine 1-phosphate through G protein-coupled receptors. *Biochim Biophys Acta.* **1582**: 94-9.

Siehler, S., Wang, Y., Fan, X., Windh, R.T. and Manning, D.R. (2001). Sphingosine 1-phosphate activates nuclear factor-kappa B through Edg receptors. Activation through Edg-3 and Edg-5, but not Edg-1, in human embryonic kidney 293 cells. *J. Biol. Chem.* **276**: 48733-9.

Simons, K. and Ikonen, E. (1997). Functional rafts in cell membranes. *Nature.* **387**: 569-573.

Simpson, F., Hussain, N.K., Qualmann, B., Kelly, R.B., Kay, B.K., McPherson, P.S. and Schmid, S.L. (1999). SH3-domain-containing proteins function at distinct steps in clathrin-coated vesicle formation. *Nature Cell Biol.* **1**: 119-124.

Singer, S.J. and Nicolson, G.L. (1972). The fluid mosaic model of the structure of cell membranes. *Science.* **175**: 720-31.

Small, K.M., Brown, K.M., Forbes, S.L. and Liggett, S.B. (2001). Polymorphic deletion of three intracellular acidic residues of the alpha 2B-adrenergic receptor decreases G protein-coupled receptor kinase-mediated phosphorylation and desensitization. *J Biol Chem.* **276**: 4917-22.

Smit, M.J., Leurs, R., Alewijnse, A.E., Blauw, J., Van Niew Amerongen, G.P., Van De Vrede, Y., Roovers, E. and Timmerman, H. (1996). Inverse agonism of histamine H2 antagonists accounts for upregulation of spontaneously active histamine H2 receptors. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 6802-6807.

Smith, E. R. and Merrill, A. H. Jr. (1995). Differential Roles of de Novo Sphingolipid Biosynthesis and Turnover in the ``Burst'' of Free Sphingosine and Sphinganine, and Their 1-Phosphates and N-Acyl-Derivatives, That Occurs upon Changing the Medium of Cells in Culture. *J. Biol. Chem.* **270**: 18749-18758.

Smith, R.D., Hunyady, L., Olivares-Reyes, J.A., Mihalik, B., Jayadev, S. and Catt, K.J. (1998). Agonist-induced phosphorylation of the angiotensin AT1A receptor is localised to a serine/threonine-rich region of its cytoplasmic tail. *Mol. Pharmacol.* **54**: 935-941.

Smith, W.C., Milam, A.H., Dugger, D., Arendt, A., Hargrave, P.A. and Palczewski, K. (1994). A splice variant of arrestin. Molecular cloning and localisation in bovine retina. *J. Biol. Chem.* **269**: 15407-15410.

Soldi, R., Mitola, S., Strasly, M., Defilippi, P., Tarone, G. and Bussolino, F. (1999). Role of $\alpha v\beta 3$ integrin in the activation of vascular endothelial growth factor receptor-2. *EMBO J.* **18**: 882-892.

Sorkin, A., Eriksson, A., Heldin, C.H., Westermark, B. and Claesson-Welsh, L. (1993). Pool of ligand-bound platelet-derived growth factor beta-receptors remain activated and tyrosine phosphorylated after internalization. *J Cell Physiol.* **156**: 373-82.

Spiegel, S. (1999). Sphingosine 1-phosphate: a prototype of a new class of second messengers. *J. Leukoc. Biol.* **65**: 341-344.

Spiegel, S., English, D. and Milstien, S. (2002). Sphingosine 1-phosphate signalling: providing cells with a sense of direction. *Trends Cell Biol.* **12**: 236-242.

Spiegel, S. and Merrill Jr, A.H. (1996). Sphingolipid metabolism and cell growth regulation. *FASEB J.* **10**: 1388-1397.

Spiegel, S. and Milstien, S. (2000a). Functions of a new family of sphingosine-1-phosphate receptors. *Biochim. Biophys. Acta.* **1484**: 107-116.

Spiegel, S. and Milstien, S. (2000b). Sphingosine-1-phosphate: signalling inside and out. *FEBS Lett.* **476**: 55-57.

Spiegel, S. and Milstien, S. (2002). Sphingosine 1-Phosphate, a Key Cell Signaling Molecule. *J. BIOL. CHEM.* **277**: 25851–25854.

Spiegel, S. and Milstien, S. (2003). Sphingosine 1-Phosphate: An enigmatic signalling lipid. *Nature.* **4**: 397-406.

Stadel, J.M., Wilson, S. and Bergsma, D.J. (1997). Orphan G protein-coupled receptors: a neglected opportunity for pioneer drug discovery. *Trends Pharmacol Sci.* **18**: 430-7.

Sterne-Marr, R. , Gurevich, VV. , Goldsmith, P. , Bodine, RC. , Sanders, C. , Donoso, LA. and Benovic, JL. (1993). Polypeptide variants of beta-arrestin and arrestin3. *J. Biol. Chem.* **268**: 15640-15648.

Stoffel, R.H., Inglese, J., Macrae, A.D., Lefkowitz, R.J., Premont, R.T. (1998). Palmitoylation increases the kinase activity of the G protein-coupled receptor kinase, GRK6. *Biochemistry.* **37**: 16053-16059.

Stoffel, R.H., Randall, R.R., Premount, R.T., Lefkowitz, R.J. and Inglese, J. (1994). Palmitoylation of G protein-coupled receptor kinase, GRK6. Lipid modification diversity in the GRK family. *J. Biol. Chem.* **269**: 27791-27794.

Strassheim, D. and Williams, C.L. (2000). P2Y2 purinergic and M3 muscarinic acetylcholine receptors activate different phospholipase C-beta isoforms that are uniquely susceptible to protein kinase C-dependent phosphorylation and inactivation. *J Biol Chem.* **275**: 39767-92.

Stratmann, A., Risau, W. and Plate, K.H. (1998). Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. *Am. J. Pathol.* **153**: 1459-1466.

Sugimoto, N., Takuwa, N., Okamoto, H., Sakurada, S. and Takuwa, Y. (2003). Inhibitory and stimulatory regulation of Rac and cell motility by the G12/13-Rho and Gi pathways integrated downstream of a single G protein-coupled sphingosine-1-phosphate receptor isoform. *Mol Cell Biol.* **23**: 1534-45.

Summers, Scott A. and Nelson, Don H. (2005). A Role for Sphingolipids in Producing the Common Features of Type 2 Diabetes, Metabolic Syndrome X, and Cushing's Syndrome. *Diabetes.* **54**: 591-602.

Sumpio, B.E., Riley, J.T. and Dardik, A. (2002). Cells in focus: Endothelial cell. *Int J Biochem & Cell Biol.* **1328**: 1-5.

Sun, X.-M. and Soutar, A. K. (2003). The Transmembrane Domain and PXXP Motifs of ApoE Receptor 2 Exclude It from Carrying out Clathrin-mediated Endocytosis. *J. Biol. Chem.* **278**: 19926-19932.

Suri, C., Jones, P.F., Patan, S., Bartunkova, S., Maisonpierre, P.C., Davis, S., Sato, T.N. and Yancopoulos, G.D. (1996). Requisite role of angiopiotin-1, a ligand for the Tie2 receptor, during embryonic angiogenesis. *Cell.* **87**: 1171-1180.

Tabas, I. (1999). Secretory sphingomyelinase. *Chem. Phys. Lipids.* **102**: 123-130.

Taha, T. A., Argraves, K. M. and Obeid, L. M. (2004). Sphingosine-1-phosphate receptors: receptor specificity versus functional redundancy. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids.* **1682**: 48-55.

Takai, Y., Kaibuchi, K., Kikuchi, A. and Kawata, M. (1992). Small GTP-binding proteins. *Int. Rev. Cytol.* **133**: 187-230.

Takai, Y., Sasaki, T. and Matozaki, T. (2001). Small GTP-binding proteins. *Physiol Rev.* **81**: 153-208.

Takei, K. and Haucke, V. (2001). Clathrin-mediated endocytosis: membrane factors pull the trigger. *Trends Cell Biol.* **11**: 385-391.

Takuwa, Y., Okamoto, H., Takuwa, N., Gonda, K., Sugimoto, N. and Sakurada, S. (2001). Subtype-specific, differential activities of the EDG family receptors for sphingosine-1-phosphate, a novel lysophospholipid mediator. *Mol. Cell. Endocrin.* **177**: 3-11.

Tang, H., Guo, D.F., Porter, J.P., Wanaka, Y. and Inagami, T. (1998). Role of cytoplasmic tail of the type 1A angiotensin II receptor in agonist- and phorbol ester-induced desensitisation. *Circ. Res.* **82**: 523-531.

Tang, Y., Hu, L.A., Miller, W.E., Ringstad, N., Hall, R.A., Pitcher, J.A., DeCamilli, P. and Lefkowitz, Robert J. (1999). Identification of the endophilins (SH3p4/p8/p13) as novel binding partners for the ß1-adrenergic receptor. *Proc Natl Acad Sci U S A.* **96**: 12559–12564.

Tanimoto, T., Lunga, A.O. and Berk, B.C. (2004). Sphingosine transactivates the platelet-derived growth factor β receptor and epidermal growth factor receptor in vascular smooth muscle. *Circ Res.*: 1050-1058.

Tarasova, N.I., Stauber, R.H., Choi, J.K., Hudson, E.A., Czerwinski, G., Miller, J.L., Pavlakis, G.N., Michejda, C.J. and Wank, S.A. (1997). Visualisation of G protein-coupled receptor trafficking with the aid of the green fluorescent protein. Endocytosis and recycling of cholecystokinin receptor type A. *J. Biol. Chem.* **272**: 14817-14824.

Tham, C.-S., Lin, F.-F., Rao, T. S., Yu, N. and Webb, M. (2003). Microglial activation state and lysophospholipid acid receptor expression. *International Journal of Developmental Neuroscience.* **21**: 431-443.

Thudichum, J.L.W. (1884). A Treatise on the Chemical Constitution of the Brain. London.

Tobin, A. B. (2002). Are we β-ARKing up the wrong tree? Casein kinase 1α provides an additional pathway for GPCR phosphorylation. *Trends in Pharmacol. Sci.* **23**: 337-343.

Tohgo, A., Choy, E.W., Gesty-Palmer, D., Pierce, K.L., Laporte, S., Oakley, R.H., Caron, M.G., Lefkowitz, R.J. and Luttrell, L.M. (2003). The stability of the G protein-coupled receptor-beta-arrestin interaction determines the mechanism and functional consequence of ERK activation. *J Biol Chem.* **278**: 6258-67.

Tolle, M., Levkau, B., Keul, P., Brinkmann, V., Giebing, G., Schonfelder, G., Schafers, M., von Wnuck Lipinski, K., Jankowski, J., Jankowski, V., Chun J, Zidek, W., and van der Giet, M. (2005). Immunomodulator FTY720 Induces eNOS-Dependent Arterial Vasodilatation via the Lysophospholipid Receptor S1P3. *Circ Res.* **Ahead of print.**

Toman, R.E. and Spiegel, S. (2002). Lysophospholipid receptors in the nervous system. *Neurochem Res.* **27**: 619-27.

Tsao, P., Cao, T. and von Zastrow, M. (2001). Role of endocytosis in mediating downregulation of G-protein-coupled receptors. *Trends Pharmacol. Sci.* **22**: 91-96.

Tsien, R.Y. (1998). The green fluorescent protein. *Annu. Rev. Biochem.* **67**: 509-544.

Tsuga, H., Kameyama, K., Haga, T., Kurose, H. and Nagao, T. (1994). Sequestration of muscarinic acetylcholine receptor m2 receptor subtypes. Facilitation by G-protein-coupled receptor kinase (GRK2) and attenuation by a dominant-negative mutant of GRK2. *J. Biol. Chem.* **269**: 32522-32527.

Ueda, T., Kikuchi, A., Ohga, N., Yamamoto, J. and Takai, Y. (1990). Purification and characterisation from bovine brain cytosol of a novel regulatory protein inhibiting the dissociation of GDP from and the subsequent binding of GTP to RhoB p20, a ras p21-like GTP-binding protein. *J. Biol. Chem.* **265**: 9373-9380.

Uhlenbrock, K., Gassenhuber, H and Evi Kostenisa, E. (2002). Sphingosine 1-phosphate is a ligand of the human gpr3, gpr6 and gpr12 family of constitutively active G protein-coupled receptors. *Cellular Signalling.* **14**: 941-953.

Ulloa-Aguirre, A., Stanislaus, D., Janovick, J.A. and Conn, P.M. (1999). Structure-activity relationships of G protein-coupled receptors. *Arch Med Res.* **30**: 420-35.

Unger, VM., Hargrave, PA., Baldwin, JM. and Schertler, GF. (1997). Arrangement of rhodopsin transmembrane alpha-helices. *Nature.* **389**: 203-206.

Usui, S., Sugimoto, N., Takuwa, N., Sakagami, S., Takata, S., Kaneko, S. and Takuwa, Y. (2004). Blood Lipid Mediator Sphingosine 1-Phosphate Potently Stimulates Platelet-derived Growth Factor-A and -B Chain Expression through S1P1-Gi-Ras-MAPK-dependent Induction of Kruppel-like Factor 5. *J. Biol. Chem.* **279**: 12300-12311.

Vaidehi, N., Floriano, W.B., Trabanino, R., Hall, S.E., Freddolino, P., Choi, E.J., Zamanakos, G. and Goddard III, W.A. (2002). Prediction of structure and function of G protein-coupled receptors. *PNAS.* **99**: 12622–12627.

Van Brocklyn, J.R., Behbahani, B. and Lee, N.H. (2002). Homodimerization and Heterodimerization of S1P/EDG sphingosine-1-phosphate receptors. *Biochim Biophys Acta.* **1582**: 89-93.

Van Brocklyn, J.R., Tu, Z., Edsall, L.C., Schmidt, R.R. and Spiegel, S. (1999). Sphingosine-1-phosphate-induced cell rounding and neurite retraction are mediated by the G protein-coupled receptor H218. *J. Biol. Chem.* **274**: 4626-4632.

Van Brockyln, J.R., Lee, M-J., Menzeleev, R., Olivera, A., Edsall, L., Cuvillier, O., Thomas, D.M., Coopman, P.J.P., Thangada, S., Liu, C.H. and Hla, T. (1998). Dual actions of Sphingosine 1-phosphate: extracellular through the Gi-coupled receptor Edg1 and intracellular to regulate proliferation and survival. *J. Cell Biol.* **142**: 229-240.

van der Giet, M and Tolle, M. (2004). Why HDL cholesterol is 'good cholesterol'. *J Clin Invest.* **34**: 247-248.

Vassilatis, D.K. and Hohmann, J.G. (2003). The G protein-coupled receptor repertoires of human and mouse. *PNAS.* **100**: 4903-4908.

Vesper, H., Schmelz, E-M, Nikolova-Karakashian, M.N., Dillehay, D.L., Lynch, D.V and Merril Jr., A.H. (1999). Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *J. Nutr.* **129**: 1239-1250.

Vincenti, F. (2002). What's in the pipeline? New immunosuppressive drugs in transplantation. *Am J Transplant.* **2**: 898-903.

Vogler, Rudiger, Sauer, Bettina, Kim, Dong-Seok, Schafer-Korting, Monika and Kleuser, Burkhard (2003). Sphingosine-1-Phosphate and Its Potentially Paradoxical Effects on Critical Parameters of Cutaneous Wound Healing. *J Invest Dermatol.* **120**: 693-700.

Volchuk, A., Narine, S., Foster, L.J., Grabs, D., De Camilli, P. and Klip, A. (1998). Perturbation of dynamin II with an amphiphysin SH3 domain increases GLUT4 glucose transporters at the plasma membrane in 3T3-L1 adipocytes. Dynamin II participates in GLUT4 endocytosis. *J Biol Chem.* **273**: 8169-76.

von Zastrow, M. and Kobilka, B.K. (1992). Ligand-regulated internalization and recycling of human beta 2- adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J. Biol. Chem.* **267**: 3530-3538.

Wada, M., Nakanishi, H., Satoh, A., Hirano, H., Obaishi, H., Matsuura, Y. and Takai, Y. (1997). Isolation and characterisation of a GDP/GTP exchange protein specific for the Rab3 subfamily small G proteins. *J. Biol. Chem.* **272**: 3875-3878.

Walker, J.K., Premont, R.T., Barak, L.S., Caron, M.G. and Shetzline, M.A. (1999). Properties of secretin receptor internalization differ from those of the beta(2)-adrenergic receptor. *J Biol Chem.* **274**: 31515-23.

Wang, F., Van Brocklyn, J.R., Hobson, J.P., Movafagh, S., Zukowska-Grojec, Z., Milstien, S. and Spiegel, S. (1999). Sphingosine 1-phosphate stimulates cell migration through a Gi-coupled cell surface receptor. *J. Biol. Chem.* **274**: 35343-35350.

Waters, C., Pyne, S. and Pyne, N. (2004). The role of G-protein coupled receptors and associated proteins in receptor tyrosine kinase signal transduction. *Seminars in Cell & Developmental Biology.* **15**: 309-323.

Waters, C., Sambi, B. S., Kong, K. C., Thompson, D., Pitson, S. M., Pyne, S. and Pyne, N.J. (2002). Sphingosine 1-phosphate and platelet-derived growth factor act via platelet-derived growth factorbeta receptor-sphingosine 1-phosphate receptor complexes in airway smooth muscle cells. *J Biol Chem.* **278**: 6282-6290.

Waters, C., Sambi, B. S., Kong, K. C., Thompson, D., Pitson, S. M., Pyne, S. and Pyne, N.J. (2003). Sphingosine 1-phosphate and platelet-derived growth factor act via platelet-derived growth factorbeta receptor-sphingosine 1-phosphate receptor complexes in airway smooth muscle cells. *J Biol Chem.* **278**: 6282-6290.

Waters, C.M., Connell, M.C., Pyne, S. and Pyne, N. J. (2005). c-Src is involved in regulating signal transmission from PDGFß receptor–GPCR(s) complexes in mammalian cells. *Cellular Signalling.* **17**: 263-277.

Watterson, K., Johnston, E., Chalmers, C., Pronin, A.N., Cook, S.J., Benovic, J.L. and T.M., Palmer (2002). Dual Regulation of EDG1/$S1P_1$ Receptor Phosphorylation and Internalisation by Protein Kinase C and G-protein-coupled Receptor Kinase 2. *J Biol Chem.* **277**: 5767-5777.

Weiner, J.A. and Chun, J. (1999). Schwann cell survival mediated by the signalling phospholipid lysophosphatidic acid. *Proc. Natl. Acad. Sci. USA.* **96**: 5233-5238.

Wenk, M.R. and De Camilli, P. (2004). Protein-lipid interactions and phosphoinositide metabolism in membrane traffic: insights from vesicle recycling in nerve terminals. *Proc Natl Acad Sci U S A.* **101**: 8262-9.

Wess, J. (1998). Molecular basis of receptor/G-protein-coupling selectivity. *Pharmacol Ther.* **80**: 231-64.

Whistler, J.L. and von Zastrow, M. (1998). Morphine-activated opioid receptors elude desensitisation by ß-arrestin. *Proc. Natl. Acad. Sci. USA.* **95**: 9914-9919.

Willard, F.S. and Crouch, M.F. (2000). Nuclear and cytoskeletal translocation and localisation of heterotrimeric G-proteins. *Immun. Cell Biol.* **78**: 387-394.

Willets, J.M., Challiss, R.A.J., Kelly, E. and Nahorski, S.R. (2001). G protein-coupled receptor kinases 3 and 6 use different pathways to desensitize the endogenous M3 muscarinic acetylcholine receptor in human SH-SY5Y cells. *Mol. Pharmacol.* **60**: 321-330.

Windh, R.T., Lee, M-J., Hla, T., An, S., Barr, A.J., Manning, D.R. (1999). Differential coupling of the sphingosine 1-phosphate receptors Edg-1, Edg-3, and H218/Edg-5 to the G_i, G_q, and G_{12} families of heterotrimeric G proteins. *J. Biol. Chem.* **274**: 27351-27358.

Winstel, R., Freund, S., Krasel, C., Hoppe, E., Lohse, M.J. (1996). Protein kinase cross-talk: membrane targeting of the ß-adrenergic receptor kinase by protein kinase C. *Proc. Natl. Acad. Sci. USA.* **93**: 2105-2109.

Wong, A.L., Haroon, Z.A., Werner, S., Dewhirst, M.W., Greenberg, C.S. and Peters, K.G. (1997). Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues. *Circ. Res.* **81**: 567-574.

Xiao, Z., Yao, Y., Long, Y. and Devreotes, P. (1999). Desensitisation of G-protein-coupled receptors. Agonist-induced phosphorylation of the chemoattractant receptor cAR1 lowers its intrinsic affinity for cAMP. *J. Biol. Chem.* **274**: 1440-1448.

Xu, Y., Zhu, K., Hong, G., Wu, W., Baudhuin, L.M., Xiao, Y.-J. and Damron, D.S. (2000). Sphingosylphosphorylcholine is a ligand for ovarian cancer G-protein-coupled receptor 1. *Nature Cell Biology.* **2**: 261-267.

Yaku, H., Sasaki, T. and Takai, Y. (1994). The Dbl oncogene product as a GDP/GTP exchange protein for the Rho family: its properties compared with those of Smg GDS. *Biochem. Biophys. Res. Commun.* **198**: 811-817.

Yamada, M., Banno, Y., Takuwa, Y., Koda, M., Hara, A. and Nozawa, Y. (2004). Overexpression of phospholipase D prevents actinomycin D-induced apoptosis through potentiation of phosphoinositide 3-kinase signalling pathways in Chinese-hamster ovary cells. *Biochem J.* **378**: 649-56.

Yamaguchi, F., Tokuda, M., Hatase, O. and Brenner, S. (1996). Molecular cloning of the novel human G-protein coupled receptor (GPCR) gene mapped on chromosome 9. *Biochem Biophys Res Commun.* **227**: 608-614.

Yamanaka, M., Shegogue, D., Pei, H., Bu, S., Bielawska, A., Bielawski, J., Pettus, B., Hannun, Y. A., Obeid, L. and Trojanowska, M. (2004). Sphingosine Kinase 1 (SPHK1) Is Induced by Transforming Growth Factor-{beta} and Mediates TIMP-1 Up-regulation. *J. Biol. Chem.* **279**: 53994-54001.

Yang, L., Yatomi, Y., Miura, Y., Satoh, K. and Ozaki, Y. (1999). Metabolism and functional effects of sphingolipids in blood cells. *Br. J. Haematol.* **107**: 282-293.

Yatomi, Y., Ruan, F., Hakomori, S. and Igarashi, Y. (1995). Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. *Blood.* **86**: 193-202.

Yuan, N., Friedman, J., Whaley, B.S. and Clark, R.B. (1994). cAMP-dependent protein kinase and protein kinase C consensus site mutations of the ß-adrenergic receptor. Effect on desensitisation and stimulation of adenylylcyclase. *J. Biol. Chem.* **269**: 23032-23038.

Yuhanna, I.S., Zhu, Y., Cox, B.E., Hahner, L.D., Osborne-Lawrence, S., Lu, P., Marcel, Y.L., Anderson, R.G.W., Mendelsohn, M.E., Hobbs, H.H. and Shaul, P.W. (2001). High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. *Nature*, 7: 853 - 857.

Zhang, G., Contos, J.J.A., Weiner, J.A., Fukushima, N. and Chun, J. (1999). Comparitive analysis of three murine G-protein coupled receptors activated by sphingosine-1-phosphate. *Gene*. **227**: 89-99.

Zhang, H., Buckley, N.E., Gibson, K. and Spiegel, S. (1990). Sphingosine stimulates cellular proliferation via a protein kinase C- independent pathway. *J. Biol. Chem.* **265**: 76-81.

Zhang, H., Desai, N.N., Olivera, A., Seki, T., Brooker, G. and Spiegel, S. (1991). Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. *J. Cell Biol.* **114**: 155-167.

Zhang, J., Barak, L.S., Winkler, K.E., Caron, M.G. and Ferguson, S.S.G. (1997). A central role for ß-arrestins and clathrin-coated vesicle-mediated endocytosis in ß2-adrenergic receptor resensitisation. *J. Biol. Chem.* **272**: 27005-27014.

Zhang, J., Ferguson, S.S.G., Barak, L.S., Menard, L. and Caron, M.G. (1996). Dynamin and ß-arrestin reveal distinct mechanisms for G protein-coupled receptor internalisation. *J. Biol. Chem.* **271**: 18302-18305.

Zhu, K., Baudhuin, L. M., Hong, G., Williams, F. S., Cristina, K. L., Kabarowski, J. H. S., Witte, O. N. and Xu, Y. (2001). Sphingosylphosphorylcholine and Lysophosphatidylcholine Are Ligands for the G Protein-coupled Receptor GPR4. *J. Biol. Chem.* **276**: 41325-41335.

Zondag, G.C.M, Postma, F.R., Etten, I.V., Verlaan, I. and Moolenaar, W.H. (1998). Sphingosine 1-phosphate signalling through the G-protein-coupled receptor Edg-1. *Biochem. J.* **330**: 605-609.