



<https://theses.gla.ac.uk/>

Theses Digitisation:

<https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/>

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses

<https://theses.gla.ac.uk/>
research-enlighten@glasgow.ac.uk



**UNIVERSITY
of
GLASGOW**

**Characterisation of Small G protein Regulation and
Survival Signalling by the Human Sphingosine-1-
phosphate Receptor S1P₁**

Shona Childs BSc. (Hons)

This thesis is presented for the degree of

Doctor of Philosophy

September 2006

Institute of Biomedical and Life Sciences

University of Glasgow

© Shona Childs



ProQuest Number: 10390695

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10390695

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

GLASGOW
UNIVERSITY
LIBRARY:

Abstract

The lipid mediator sphingosine-1-phosphate (S1P) is involved in many growth-related processes including migration, angiogenesis and survival. S1P exerts its effects through the G protein-coupled receptors named S1P₁₋₅. S1P₁, the first receptor to be identified is associated with regulation of cytoskeletal dynamics by differential regulation of small G proteins such as Rac, Rho and Cdc42 which are crucial mediators of migration. S1P-mediated signalling has long been associated with cell survival but the mechanisms are not completely understood.

However, using the model system of Chinese hamster lung fibroblasts (CCL39) stably expressing the S1P₁ receptor, S1P-mediated Rac and Cdc42 activation was not detected. S1P₂ which inhibits Rac activation was not detected and the selective inhibitor which binds all S1P receptors except S1P₂ did not rescue this effect.

Using CCL39mycS1P₁ cells it was demonstrated that the presence of this receptor has a protective effect against apoptosis caused by the removal of trophic factors. Control CCL39 cells showed increased levels of apoptotic initiator protein, Bim and the executioner protein active caspase-3. This effect is not ERK-dependent nor is it dependent on total levels of the pro-apoptotic mediator Bim. This also occurred in the absence of agonist or endogenous S1P production.

Inhibition of S1P₁-mediated signalling pathways such as PI 3-kinase/PKB pathway and PKC completely attenuated this protective effect. Furthermore, blocking protein synthesis by emetine treatment is also reversed the protective which strongly suggests that S1P₁ receptor signalling is the driving force behind the up-regulation of one or more anti-apoptotic proteins which bind and sequester Bim. Understanding the mechanisms by which control growth patterns are regulated is important in the development of new drugs to combat growth-related diseases such as cancer.

Contents

Abstract	II
List of Experimental Figures	VII
Acknowledgements	IX
Abbreviations	XI
Chapter 1 Introduction	1
1.1 G protein Coupled Receptors	2
1.1.1 GPCR classification	2
1.1.2 Structural features of GPCRs	3
1.1.3 Receptor Activation	4
1.1.4 Heterotrimeric G proteins	4
1.1.5 Heterotrimeric G protein Activation and Regulation	5
Figure 1.1 Crystal Structure of Bovine Rhodopsin	7
Figure 1.2 Illustration of the Seven TM Domains Typical of GPCRs	9
Figure 1.3 GPCRs Exist in Equilibrium between Inactive and Active States	11
Table 1.1 Amino Acid Identity of G α protein subunits	13
Table 1.2 G $\beta\gamma$ Effector Targets	14
1.2 Regulation of GPCRs	15
1.2.1 Regulation of GPCRs by Phosphorylation	15
1.2.2 Regulation of GPCRs by Internalisation	17
Figure 1.4 Model for GPCR Internalisation <i>via</i> Clathrin-coated pits	19
1.3 Lysophospholipids	21
1.3.1 Sphingosine-1-phosphate	21
1.3.2 S1P Metabolism and Secretion	22
1.3.3 SphK Regulation	22

Figure 1.5	The Chemical Structures of Sphingosine-1-phosphate (S1P) and Lysophosphatidic Acid (LPA)	25
Figure 1.6	The Metabolism of Sphingosine-1-phosphate (S1P)	27
Figure 1.7	The Structural Composition of Sphingosine kinases 1 and 2	29
1.4	Identification of S1P Receptors	31
1.4.1	S1P Receptor Signalling	32
1.4.2	Orphan GPCRs (oGPCRs) with homology S1P and LPA Receptors	33
Figure 1.8	The EDG Receptor Family and Related Receptors	34
Figure 1.9	The EDG Family Amino Acid Alignment	36
Figure 1.10	S1P Receptor Signalling	38
1.5	The Small G protein Superfamily	40
1.5.1	Small G protein Regulation	40
1.5.2	The Rho GTPases	41
1.5.3	Rho, Rac, Cdc42 and Migration	41
1.5.4	Regulation of endocytic traffic by Rho-like GTPases	43
1.5.5	Integrin Signalling	44
1.5.6	S1P ₁ and Rac Activation	45
1.5.7	The Role of S1P in Migration	46
1.5.8	The Role of S1P in Angiogenesis	47
Figure 1.11	The Rho-like GTPase Cycle	51
Figure 1.12	The Rho-like GTPase Family	53
Figure 1.13	The Rho-like GTPases Regulate Cell Migration	55
Figure 1.14	Signalling Pathways of Cytoskeletal Dynamics	57
Figure 1.15	S1P ₁ Activates Rac in a PKB-Dependent Manner	59
Figure 1.16	The Compound FTY720P is Structurally Similar to S1P	61
1.6	Morphological Features of Apoptosis	63

1.6.1	The Bcl-2 Family	63
1.6.2	The BH3-only protein Bim	64
1.6.3	The Caspase Family	65
1.6.4	Inhibitors of Caspases	66
1.6.5	The Extrinsic Pathway of Apoptosis	67
1.6.6	The Intrinsic Pathway of Apoptosis	68
1.6.7	Apaf-1 and the Apoptosome	69
1.6.8	The Role of SphK in Cell Survival	70
1.6.9	The Role of S1P Receptors in Cell Survival	72
Figure 1.17	The Bcl-2 Family	75
Figure 1.18	The Caspase Family	77
Figure 1.19	Apaf-1 and the Apoptosome	79
Table 1.3	Caspase Substrates	81
Aims		82
Chapter 2	Materials and Methods	
2.1	Materials	83
2.2	Cell Culture	88
2.2.1	Transfection of cells with Lipofectamine	88
2.3	Molecular Biology Techniques	
2.3.1	Preparation of Antibiotic Agar Plates	89
2.3.2	Preparation of Plasmid DNA	89
2.3.3	Preparation of Competent BL21 <i>E.coli</i> cells	89
2.3.4	Transformation of Competent BL21 <i>E.coli</i> cells	89
2.3.5	GST Fusion Protein Preparation	90

2.4	Laboratory Techniques	
2.4.1	BCA Assay to Determine Protein Concentration	91
2.4.2	Bradford's Assay to Determine Protein Concentration	91
2.4.3	SDS PAGE and Immunoblotting	92
2.4.4	Preparation of Stock S1P	93
2.4.5	Preparation of Stock FTY720P	93
2.4.6	Cell Surface Labelling of S1P ₁ with Biotin-LC-hydrazide	93
2.4.7	Whole Cell Phosphorylation Assay	95
2.4.8	GST Pull Down Assay to Detect Small G protein Activation	96
2.4.9	Assay of Caspase-3 Activity	97
2.4.10	Detection of S1P ₁ by Immunofluorescence	98
2.4.11	Detection of Stress Fibres with Alexa Fluor 594-conjugated Phalloidin	99
2.4.12	Statistical Analysis	100
Chapter 3	S1P₁ and Small G protein Activation Status	101
3.1	Introduction	102
3.2	Results	105
3.3	Discussion	108
Chapter 4	Regulation of Apoptosis by S1P₁	152
4.1	Introduction	153
4.2	Results	155
4.3	Discussion	157
Chapter 5		187
Summary		188
References		192

List of Experimental Figures

Figure 3.1	Confocal Image of CCL39 Cells Stably Expressing the Myc Epitope Tagged Receptor S1P ₁	110
Figure 3.2	The Effect of the Cytoskeletal Inhibitor Colchicine on Levels of S1P ₁ Receptor Phosphorylation	112
Figure 3.3	The Effect of the Cytoskeletal Inhibitor Cytochalasin B on Levels of S1P ₁ Receptor Phosphorylation	114
Figure 3.4	The Effect of Pertussis Toxin on Levels of S1P ₁ Receptor Phosphorylation	116
Figure 3.5	The Effect of Cytoskeletal Inhibitors on Agonist-induced S1P ₁ Receptor Internalisation	118
Figure 3.6	Detection of Rac in CCL39 Cells Over-expressing MycS1P ₁	120
Figure 3.7	The Effect of S1P Stimulation on Rac Activation Status in Parental CCL39 Cells	122
Figure 3.8	The Effect of S1P Stimulation on Cdc42 Activation Status in Parental CCL39 Cells	124
Figure 3.9	Rac Activation Status in S1P-stimulated CCL39mycS1P ₁ Cells	126
Figure 3.10	Cdc42 Activation Status in S1P-stimulated CCL39mycS1P ₁ Cells	128
Figure 3.11	The Effect of S1P Stimulation on Rac Activation Status in CCL39mycS1P ₁ Cells Cultured on Fibronectin	130
Figure 3.12	The Effect of S1P Stimulation on Cdc42 Activation Status in CCL39mycS1P ₁ Cells Cultured on Fibronectin	132
Figure 3.13	The Effect of S1P Stimulation on Rac Activation Status in CCL39mycS1P ₁ Cells Cultured on Vitronectin	134
Figure 3.14	The Effect of S1P Stimulation on Cdc42 Activation Status in CCL39mycS1P ₁ Cells Cultured on Vitronectin	136

Figure 3.15	Detection of Dose-Dependent ERK Activation in CCL39mycS1P ₁ cells stimulated with FTY720P	138
Figure 3.16	Detection of ERK Activation in CCL39mycS1P ₁ Cells Stimulated with S1P	140
Figure 3.17	Detection of ERK Activation in CCL39mycS1P ₁ Cells Stimulated with FTY720P	142
Figure 3.18	The Effect of FTY720P Stimulation on the Rac Activation Status in CCL39mycS1P ₁ Cells	144
Figure 3.19	The Effect of FTY720P Stimulation on the Cdc42 Activation Status in CCL39mycS1P ₁ Cells	146
Figure 3.20	Detection of Stress Fibres in Control CCL39 Cells Treated with S1P	148
Figure 3.21	Detection of Stress Fibres in CCL39mycS1P ₁ Cells Treated with S1P	150
Figure 4.1	Control CCL39 Cell Morphology in Response to Serum Deprivation	161
Figure 4.2	CCL39mycS1P ₁ Cell Morphology in Response to Serum Deprivation	163
Figure 4.3	Serum Deprivation of Control CCL39 cells and CCL39mycS1P ₁ Cells and its Effect on Caspase-3 Cleavage	165
Figure 4.4	Colorimetric Determination of Caspase-3 Activity in Control CCL39 Cells and CCL39mycS1P ₁ Cells	167
Figure 4.5	The Effect of Serum Deprivation of Control CCL39 Cells and CCL39mycS1P ₁ Cells on the Level of Bim Expression	169
Figure 4.6	The Effect of Agonist Stimulation on Caspase-3 Cleavage in Control CCL39 Cells and CCL39mycS1P ₁ Cells	171

Figure 4.7	The Effect of Agonist Stimulation on Bim Expression in Control CCL39 Cells and CCL39mycS1P ₁ Cells	173
Figure 4.8	The Effect of the Sphingosine kinase Inhibitor DHS on Caspase-3 Cleavage	175
Figure 4.9	The Effect of the Sphingosine kinase Inhibitor DHS on Bim Expression	177
Figure 4.10	The Effect of Pertussis Toxin and U0126 on Caspase-3 Cleavage and Bim Expression	179
Figure 4.11	The Effect of the Inhibitors LY294002 and GF109203X on Caspase-3 Cleavage	181
Figure 4.12	The Effect of the Inhibitors LY294002 and GF109203X on Bim Expression Levels	183
Figure 4.13	The Effect of Emetine Treatment on Caspase-3 Cleavage and Bim Expression	185

Acknowledgements

I would like to thank my supervisor Dr. Tim Palmer for all his help and guidance throughout this project. I would also like to acknowledge the National Heart Foundation and the University of Glasgow for funding this project. A big thank you also goes to all the people in the department who have assisted me in various ways over the years-there are far too many of you to mention!

I would like to say thanks to all the people who have worked in Tim's empire over the years. Special thanks must go to Claire for answering all my silly questions and to all the girls- Kirsty, Hayley, Laura, Gillian, Elaine, Vicky and Sarah for making me laugh even when things go drastically wrong! I would like to thank Jenni also for her encouragement and advice. I don't want to leave the boys out, so I would also like to express thanks to Mohammed and the old-timers Kenneth and Tony.

Grateful thanks must go to my parents who have supported me all throughout my university career. Finally, a gynormous thank you to my dearest Ally for all of his emotional (and financial!) support. I could not have done this without you.

Abbreviations

AC	Adenylyl cyclase
Apaf-1	Apoptotic protease-activating factor
ATP	Adenosine 5' triphosphate
β_2 AR	Beta 2-adrenergic receptor
bFGF	Basic fibroblastic growth factor
BCA	Bichinchonic acid
BH3	Bcl-2 homology domain
Bim	Bcl-2 interacting mediator of cell death
BIR	Baculovirus IAP repeats
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CCL39	Chinese hamster lung fibroblast
CD	Cluster of differentiation
c-FLIP	FLICE-like inhibitory protein
cGMP	Guanosine 3', 5' cyclic monophosphate
CHOs	Chinese hamster ovary cells
DD	Death domain
DED	Death effector domain
DH	Dbl-homology domain
DHS	<i>L-threo</i> -dihydrosphingosine
DIABLO	Direct IAP binding protein with low pI
DLC1	Dynein light chain
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide

DR	Death receptor
DTT	Dithiothreitol
EC ₅₀	Concentration of drug required to produce a half-maximal response
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDG	Endothelial differentiation gene
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated protein kinase
FADD	Fas associated protein with death domain
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FLICE	Fas-associated death domain protein-like interleukin-1 β -converting enzyme
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDI	GDP dissociation protein
GDP	Guanine 5' diphosphate
GEF	Guanine nucleotide exchange factor
GF109203FX	Bisindolylmaleimide I
GITs	G protein-coupled receptor kinase-interacting proteins
GPCR	G protein-coupled receptor
GPI	Glycosyl-phosphatidylinositol

GRK	G protein-coupled receptor kinase
GSK-3 β	Glycogen synthase kinase-3 β
GST	Glutathione S-transferase
GTP	Guanosine 5' triphosphate
HDL	High density lipoprotein
HEKs	Human embryonic kidney cells
HEPES	4-2-hydroxyethyl-1- piperazineethanesulphonic acid
HUVECs	Human umbilical vein endothelial cells
HRP	Horseradish peroxidase
IAP	Inhibitors of apoptosis
IgG	Immunoglobulin G
IPTG	Isopropyl- β -D-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
K_d	Concentration of ligand that binds to half of the receptor population at equilibrium
K_i	equilibrium binding constant
KDa	Kilo Daltons
LDL	Low density lipoprotein
LIMK	LIM kinase
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MEF	Murine embryonic fibroblast
MEK	Mitogen-activated protein kinase kinase
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase

NF κ B	Nuclear factor kappa B
NGF	Nerve growth factor
NO	Nitric oxide
OD	Optical density
oGPCR	Orphan G protein-coupled receptor
Ox-LDL	Oxidised low density lipoprotein
PBS	Phosphate-buffered saline
PBS-CM	PBS supplemented with CaCl ₂ and MgCl ₂
PC12	Rat pheochromocytoma cells
PDGF	Platelet-derived growth factor
PDK	Phosphoinositide-dependent kinase
PH	Pleckstrin homology domain
PI 3K	Phosphatidylinositol 3-kinase
PIP ₂	Phosphatidylinositol (4,5)-bisphosphate
PIP ₃	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenyl methyl sulphonyl fluoride
pNA	p-nitroaniline
PS	Phosphatidyl serine
PTX	Pertussis toxin
RIPA	Radioimmunoprecipitation buffer

RGS	Regulator of G protein signalling
RNA	Ribonucleic acid
ROCK	Rho kinase
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
S1P	D- <i>ethryo</i> -sphingosine-1-phosphate
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
Smac	mitochondria-derived activator of caspases
Sos	Son of sevenless
SphK	Sphingosine kinase
SPC	sphingosylphosphocholine
SR-B1	Scavenger receptor, class B, type 1
TBS	TRIS-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
Tiam-1	T-lymphoma invasion and metastasis
TM	Transmembrane domain
TNF α	Tumour necrosis factor α
TRAF	TNF α receptor-associated factors
TRIS	Hydroxymethyl-aminomethane
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor

VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cell
VZ	Ventricular zone
WASp	Wiskott-Aldrich Syndrome protein
WAVE	WASP family verpolin homology

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

Chapter 1
Introduction

1.1 G Protein Coupled Receptors

G protein coupled receptors (GPCRs) comprise the largest and most functionally diverse superfamily of transmembrane proteins involved in cell signalling. It is estimated that the human genome contains approximately eight hundred GPCR sequences [1]. GPCRs respond to a diverse range of stimuli such as lipids, light, odorants, peptide hormones, neurotransmitters and chemokines each activating specific intracellular signalling cascades *via* interaction with heterotrimeric G proteins [2]. GPCRs are involved in many physiological and pathophysiological processes and thereby constitute major therapeutic targets. In humans there are approximately 367 receptors for which the endogenous ligand is known, but there remains a rich source of potential drug targets within orphan receptors (oGPCRs) i.e. receptors whose ligands are unknown and whose physiological relevance remains undetermined [3, 4].

1.1.1 GPCR Classification

GPCRs can be categorised in reference to the type of stimulus that activates them: those that are activated in response to exogenous stimuli such as pheromones, tastes and odours (csGPCRs) or those that respond to endogenous molecules (endoGPCRs) [4]. GPCRs have been grouped in various ways over the years. Recent phylogenetic analyses divide the GPCR superfamily into 5 groups (the GRAFS classification) which only include receptors from the human genome [1]:

- **Family G** for glutamate-like receptors with 15 members
- **Family R** for rhodopsin-like receptors with 701 members (241 of which are non-olfactory)
- **Family A** for adhesion with 24 members
- **Family F** for frizzled/taste2 consisting of 10 frizzled receptors and 13 group-2 taste receptors
- **Family S** for secretin with 15 members [5].

The rhodopsin-like family (family R) are the best characterised receptors both structurally and functionally and in relevance to this thesis, are the family to which the sphingosine-1-phosphate (S1P) receptors belong (Figure 1.1).

1.1.2 Structural Features of GPCRs

The prerequisites for classification as a GPCR are seven hydrophobic sequence stretches (25-35 amino acids in length) and the ability to associate with a G protein [3]. Typically, each GPCR consists of a single polypeptide chain variable in length with seven hydrophobic regions spanning the plasma membrane, with an extracellular N-terminal domain and an intracellular C-terminal domain. These transmembrane (TM) domains are believed to form a barrel shape in the membrane with three extracellular and three intracellular connecting loops. This barrel-shaped receptor is orientated in the membrane to provide a pocket in which small ligands can bind [6, 7].

In the majority of receptors belonging to the rhodopsin-like family there are a number of conserved structural features. For example, the Asp residue in TM-II and Asn in TMs-I and VII are highly conserved [8-10]. These residues function to keep the TM-II and -VII regions in close proximity to allow receptor activation [6]. Another conserved structural feature is the presence of a disulphide bond linking TM-III to the second extracellular loop [5] (Figure 1.2).

The C-terminal domains of GPCRs vary in length and typically contain amino acid residues that can be covalently modified by phosphorylation like serine and threonine. There are also cysteine residues within the C-terminal domain that can be modified by the addition of palmitic acid which can insert into the membrane forming a fourth intracellular loop, also known as 'helix 8' [11, 12]. The N-terminal domains also vary in length and most contain an N-linked glycosylation motif (N-X-T/X).

The ligand binding site of these GPCRs depends on the structure of the ligand which varies greatly for the rhodopsin-like family of receptors. If the agonist is small like an amine or lipid then the binding site is believed to be within the TM pocket, whereas larger activators tend to interact with residues in the extracellular loop and N-terminal domain [6]. For example, the S1P₁ receptor contains two Arg residues at positions 120 and 292 respectively which form ion pairs with the phosphate group on S1P and a Glu residue at position 121 which interacts with the quaternary amine group of S1P. In fact, a corresponding Glu residue has been identified in the other S1P receptors [13].

1.1.3 Receptor Activation

Although related, ligand binding and the subsequent signal transduction are distinguishable events. The consequences of ligand binding depend on the specific regions of the receptor bound, the type of receptor, and the structure and size of the ligand [6]. Tradition has placed receptors in either an inactive (quiescent) state or active (functional) state [14]. It is now widely accepted that there are varying degrees of receptor activation. GPCRs exist in an equilibrium between two states of inactive (R) and active (R*) [6] (Figure 1.3).

Efficacy is a term used to describe the level of change that occurs upon agonist binding to the receptor. Affinity describes the ligand's capacity for binding the receptor, which is expressed as its dissociation constant (K_d). An agonist displays positive efficacy as it evokes a functional response after receptor binding and a partial agonist displays relative efficacy which is expressed as a percentage of the effect accomplished by a full agonist (i.e. 100%) [15]. Whereas an antagonist exhibits zero efficacy as it produces no activity when bound to the receptor, it also prevents the binding of agonists [16]. Recent evidence suggests that GPCRs have the ability exist in an active conformation without being ligand occupied. Ligands that bind and block this constitutive activity were previously described as antagonists but are now termed inverse agonists [17].

1.1.4 Heterotrimeric G proteins

In contrast to variety of ligands a GPCR can bind, there is a limited number of heterotrimeric G proteins (consisting of α , β and γ subunits) with which they can associate. G proteins act as mediators of receptor activated signal transduction pathways. The pathways stimulated are determined by the type of G protein coupled to each receptor, which can associate with one or more type(s) of G protein [18]. There are 16 G_α subunits that have been grouped into 4 families: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$, 5 G_β and 12 G_γ subunits [19] (Table 1.1).

1.1.5 Heterotrimeric G protein Activation and Regulation

The association between GPCR and G protein is usually *via* second or third intracellular loop of the receptor [20, 21]. G proteins are activated by the binding of an agonist to its receptor which causes a conformational change that promotes the exchange of GDP for GTP in the guanine nucleotide binding site of the G_{α} subunit [18, 22]. This interaction induces the dissociation of G_{α} from the $G\beta\gamma$ subunits which remain bound to one another. The active (GTP-bound) G_{α} subunit and the liberated $\beta\gamma$ subunits then act as mediators of receptor-stimulated effector activation [18].

G_{α} subunits contain two domains: a GTP binding domain that exhibits GTPase activity and a unique helical domain concealed within the interior of the protein. G proteins are classified according to the organisation and properties of their α subunit. $G_{\alpha i}$ and $G_{\alpha s}$ differentially regulate adenylyl cyclase (AC). $G_{\alpha i}$ inhibits AC activity whereas $G_{\alpha s}$ stimulates it. $G_{\alpha q}$ activates phospholipase C (PLC) mediating calcium mobilisation and $G_{\alpha 12/13}$ which are implicated in small G protein activation and cytoskeletal remodelling [6].

The $G\beta$ subunit contains seven so-called WD40 repeats within what is described as a 7-blade propeller structure. This is comprised of seven β -sheets containing four anti-parallel β -strands that form the "blades". The N-terminal helix of the $G\gamma$ subunit forms a coiled coil with the N-terminal helix of the $G\beta$ subunit. The structure of the $G\beta\gamma$ complex remains the same regardless of interaction with the α subunit and is thereby regarded as a negative regulator of G_{α} activity [11, 18]. The $G\beta\gamma$ itself subunit activates a vast array of proteins including PLC β , AC, PI 3-kinase and G protein-coupled receptor kinases (GRKs) [23] (Table 1.2).

G protein signalling is also subject to regulation by a family of proteins call regulators of G-protein signalling (RGS) that are characterised by a 130 residue homologous region that binds preferentially to active G_{α} subunits [24]. They function to attenuate GPCR signalling by inactivation of G proteins by acting as GTPase-activating proteins (GAPs) i.e. they accelerate GTP hydrolysis, so G_{α} returns to its inactive GDP-bound state which is followed by the re-association of the heterotrimer. RGS proteins also exert

an antagonistic effect by physically preventing G proteins from activating their target. In addition to this, RGS proteins possess the ability to alter the number of free $\beta\gamma$ subunits available to downstream effectors by increasing the affinity between G_α and $G\beta\gamma$ [16].

G_α proteins are also regulated by covalent modifications such as *N*-myristoylation and palmitoylation while G_γ subunits are subject to prenylation. These alterations serve to target the subunits to the membrane but are also relevant in the interaction between subunits and with other proteins. Regulation of G_α and G_γ subunits by phosphorylation also occurs and this has implications for signal amplification [25]. The addition of ADP-ribose on a cysteine residue within the C-terminal domain of G_{oi} proteins which causes the inhibition of G_α activity by pertussis toxin is a useful tool for studying receptors coupled to this class of G_α proteins [19, 26].

Figure 1.1 Crystal Structure of Bovine Rhodopsin

Comparison of the predicted structure for bovine rhodopsin (green) and the x-ray crystal structure (blue) (taken from Vaidehi *et al*) [27].

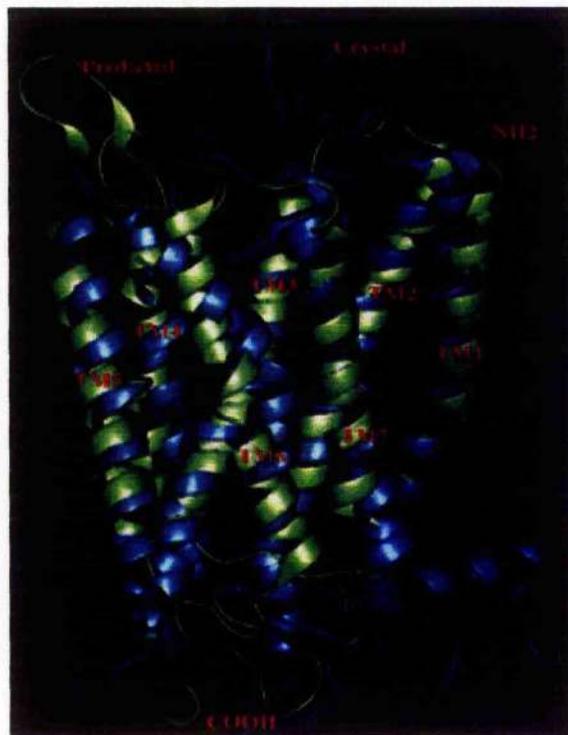


Figure 1.2 Illustration of the Seven Transmembrane Domains Typical of GPCRs

Counter clockwise orientation of the seven transmembrane (TMI-VII) domains typical of GPCRs. In this arrangement the core is composed of TMII, III, V and VI with TMI and VII in the periphery [6].

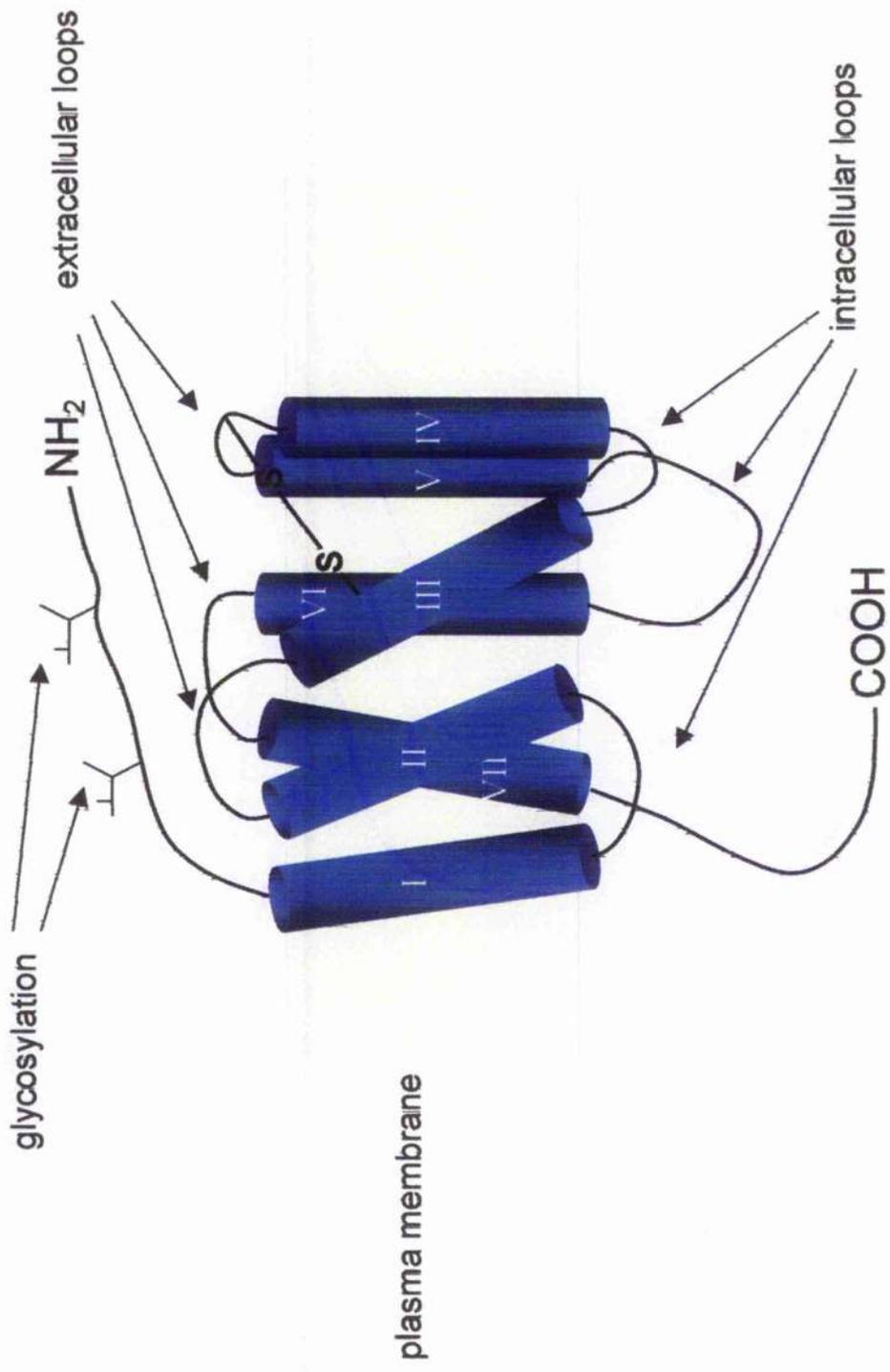
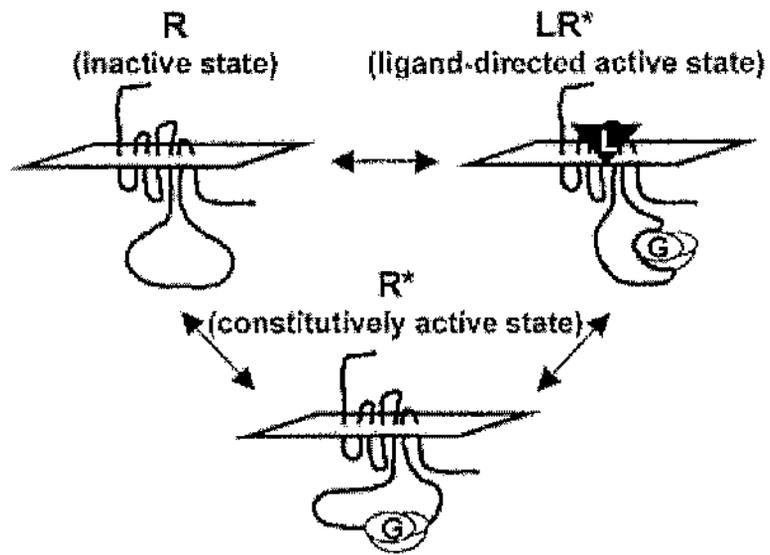


Figure 1.3 GPCRs Exist in Equilibrium between Inactive and Active States

GPCRs can adopt various conformations in equilibrium. The active state (R^*) receptor can associate with G proteins whereas the inactive state (R) receptor cannot and an active conformation that forms spontaneously or through ligand binding (LR^*). The relationship between LR^* and R^* for G protein results in receptor agonism, antagonism or inverse agonism (taken from Gbahou *et al*) [28].



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

Table 1.1 Amino acid identity of G α protein subunits

Numbers indicate the % amino acid identity between subtypes.

Subfamily	α of	α i1	α i2	α i3	α oa	α t1	α t2	α gus	α z	α q	α 11	α 14	α 15	α 12	α 13
α s	77	40	40	40	42	39	40	41	37	39	40	39	35	36	36
α of		41	41	40	42	42	42	42	38	39	39	38	35	36	37
α i			87	93	72	67	69	67	67	50	51	51	43	41	39
α i1															
α i2				85	66	65	69	66	66	50	50	50	43	41	40
α i3					66	61	65	62	65	50	48	48	41	39	36
α oa						61	61	61	59	50	49	50	41	43	42
α t1							78	75	53	52	51	48	44	41	38
α t2								79	57	51	50	49	43	41	38
α gus									57	51	50	50	41	41	40
α z										49	48	48	41	41	39
α q										84	84	76	54	41	43
α 11												82	56	43	44
α 14													54	42	44
α 15														40	38
α 12															67

Table 1.2 Gβγ Effector Targets

Effector	Gβγ	Gα
Ion Channels	+	
I _{KG}	+	
GIRK1, 2 & 4	+	
Phospholipase A ₂	+	
PLC β 1-3	+	+
Adenylyl cyclase Type II & IV	+	+
GRKs	+	
PI 3 kinase	+	
Shc phosphorylation	+	
Raf-1	+	
Ras-GEF CDC25	+	
Plasma membrane Ca ²⁺	+	

1.2 Regulation of GPCRs

The continued stimulation of a receptor results in a rapid decrease of responsiveness of the receptor to the same agonist. This is described as desensitisation and its purpose is to protect the cell from over-stimulation. Early desensitisation occurs seconds after receptor exposure to agonist and involves the covalent modification of the intracellular loops and/or C-terminal tail by phosphorylation [6, 12]. Long term desensitisation entails receptor sequestration from the cell surface (internalisation) followed by eventual down-regulation (a reduction of total receptors in the cell) [29]. This is also associated with reduced mRNA and protein synthesis in addition to lysosomal and proteosomal targeted degradation of pre-existing receptors [30, 31].

1.2.1 Regulation of GPCRs by Phosphorylation

Covalent modification of the receptors by phosphorylation by intracellular kinases causes a rapid dissociation of the heterotrimeric G-protein from the GPCR. Agonist occupation of the receptor produces a conformational change that facilitates its phosphorylation by a family of proteins called G protein-coupled receptor kinases (GRKs). This results in the attenuation of GPCR signalling which is described as homologous desensitisation. The targets for phosphorylation by these kinases are clusters of serine and threonine residues localised within either the C terminal tail or the third intracellular loop [32, 33].

The GRK family of kinases has seven members that share significant sequence homology. GRK1 (rhodopsin kinase) and GRK7 (cone opsin kinase) expression is limited to retinal cells and these are described as the visual GRKs [34]. The non-visual GRKs 2, 3, 4, 5 and 6 are expressed in many cell types [35]. Each GRK has a similar structural organisation: a core catalytic domain, and N-terminal containing an RGS-like domain thought important in substrate recognition, a catalytic domain and a C-terminal domain of variable length involved in plasma membrane targeting (in the case of GRK2, 3 and 5) [35]. GRK2 is known to phosphorylate many GPCRs and also contains a clathrin-binding domain within its C-terminus. The available evidence suggests that specific sites of phosphorylation have

functional consequences for the regulation of a GPCR by different GRKs [36].

GRKs phosphorylate target GPCRs in an agonist-dependent manner which promotes the binding of cytoplasmic adaptor proteins called β -arrestins. Binding of β -arrestin sterically blocks receptor association with the heterotrimeric G protein complex allowing β -arrestin-mediated association with clathrin [37] thereby attenuating the signal to effector proteins and targeting the receptor for internalisation (which is to be addressed in the next section). β -arrestins are also emerging as scaffolding proteins [38] that facilitate the transactivation of activation of mitogen-activated protein kinases (MAPK) such as the extracellular signal-related kinases (ERK1 and ERK2) [39].

Other MAPK pathways identified are p38 protein kinases (α , β , γ and δ) and c-Jun N-terminal kinases (JNKs) of which there are three encoded by separate genes JNK1, 2 and 3. Activation of JNK occurs when threonine and tyrosine residues are phosphorylated by MAPK kinases like MKK4 and MKK7 which in turn are phosphorylated by MAPKK kinases like MEKK1 and ASK1 [40, 41]. β -arrestin 2 acts as a scaffolding protein and has been shown to interact with JNK3, thereby bringing the MAPKs under the control of GPCRs [42].

Stimulation of GPCRs leads to an increase in intracellular second messengers such as cAMP and Ca^{2+} , and hence the activation of second messenger-dependent kinases such as cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), which respond by phosphorylating GPCRs thereby functioning as a feedback mechanism [12]. These kinases phosphorylate GPCRs on a serine or threonine present within the intracellular loops and C-terminal tail of GPCRs [32] in an agonist-independent manner, this is termed heterologous desensitisation. PKC and PKA have also been shown to modulate the activity of GRKs [43]. PKC-induced phosphorylation of GRK2 results in increased phosphorylation of receptor but not soluble proteins, indicating that PKC-mediated phosphorylation of GRK2 induces its translocation to the plasma membrane [44, 45]. Conversely, GRK5 activity toward receptors and cytosolic

substrates is inhibited upon PKC phosphorylation [46]. PKA activity which is induced by G_s -coupled receptors also leads to a direct phosphorylation of GRK2 which also enhances its activity [47].

1.2.2 Regulation of GPCRs by Internalisation

GPCR signalling is attenuated by receptor phosphorylation, which is sufficient to interfere with the coupling of the heterotrimeric G-protein to the receptor. It is a rapid process occurring in the order of seconds, allowing interaction with the arrestins, permitting sequestration of the receptor from the cell surface to intracellular vesicles. Resensitisation eventually occurs due to the subsequent recycling of the receptor back to the plasma membrane following agonist removal [48, 49]

The arrestin family is well conserved in eukaryotes and consists of at least four members two visual arrestins (rod and cone) expression of which is mainly restricted to the retina and β -arrestins1 and 2 that are expressed ubiquitously [37]. GPCRs are able to bind different arrestin isoforms with different affinities, thus it may be the presence of various β -arrestin isoforms expressed in the cell that controls the capacity of a specific GPCR to internalise [50].

β -arrestins act as adaptor molecules that facilitate the clathrin-mediated endocytosis of GPCRs. β -arrestin1 has been shown to interact with the tyrosine kinase c-Src via its Src homology domain (SH1) and using a kinase dead mutant, blocked receptor internalisation and Src-mediated phosphorylation of dynamin. Src activity on other substrates was unaffected, thus implicating β -arrestin in receptor internalisation [51]. It was experiments involving the β_2 -adrenergic receptor (β_2 AR) interaction with β -arrestin and dynamin I mutants which blocked endocytosis, that first indicated their involvement in the targeting of GPCRs to clathrin-coated pits [52]. Dynamin I is a large GTPase distributed in the surface of a newly formed clathrin-coated pits and is involved in vesicle closure. β -arrestin binds to clathrin with a high affinity but also binds adaptor proteins that mediate vesicle recruitment, particularly the β_2 -subunit of the heterotetrameric AP2 adaptor complex [53]. Resensitisation occurs in the presence of a GPCR-specific phosphatase

within the endocytic compartment which causes the dephosphorylation of the receptor and subsequent recycling back to the cell surface or degradation [39] (Figure 1.4).

Repeated or prolonged agonist exposure results in down-regulation which is induced after a period of hours to days [54]. This negatively regulates GPCR signalling by lowering the number of receptors present in the cell either by lysosomal or proteosomal-mediated proteolysis [29, 55]. β_2 AR has been studied extensively and these studies indicate that ligand-induced activation can alter receptor transcription [56] and RNA stability [57].

The mechanism for endocytosis is receptor- and cell type-specific but many GPCRs undergo internalisation *via* clathrin-coated pits. For example, the β_2 AR will internalise *via* the clathrin-coated vesicle route but will also endocytose into distinct membrane domains called caveolae depending on the cell type in which it is expressed [58, 59]. Caveolae are omega-shaped, specialised domains of the plasma membrane containing a protein called caveolin. These domains are Triton X-100 insoluble due to a high concentration of cholesterol, sphingomyelin and glycosphingolipids. Caveolae are extremely common in certain cell types such as endothelial and smooth muscle cells [60]. These specialised membrane domains have been implicated in signal transduction as well as endocytosis. A number of signalling molecules have been shown to aggregate there including G proteins [61], AC [59], eNOS [62], PKC [63], GRK2 and 3 [61].

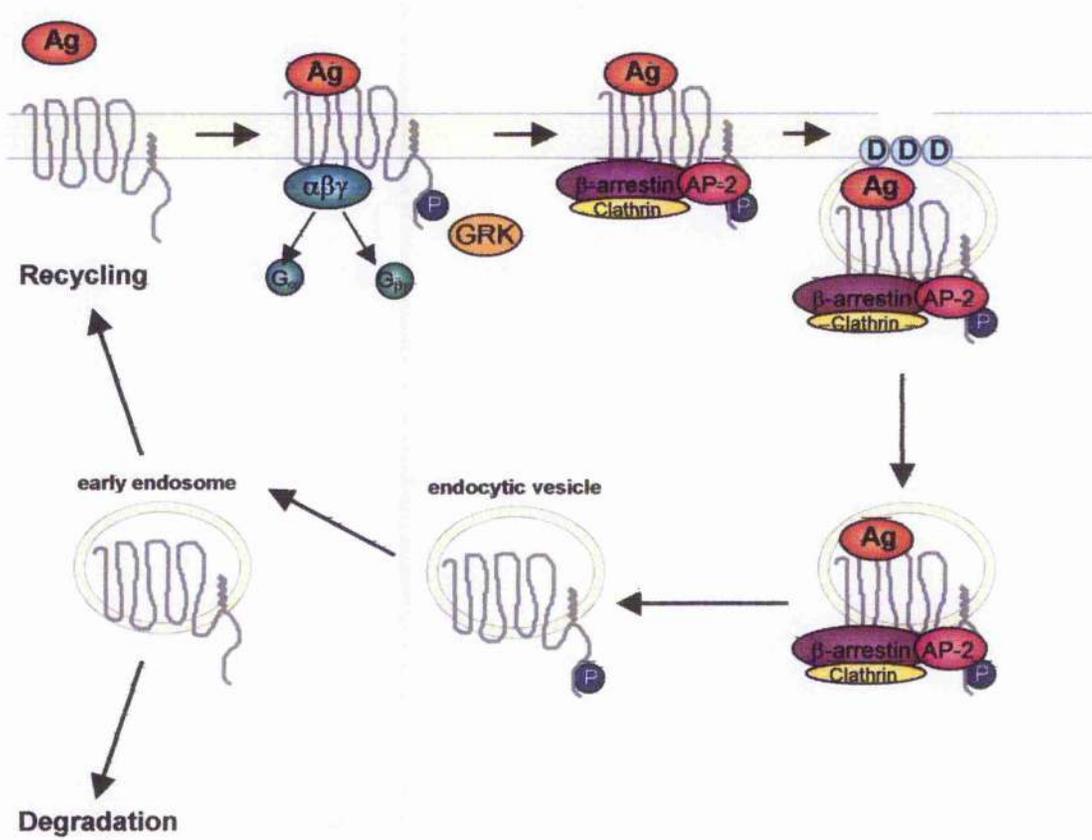
Figure 1.4 Model for GPCR Internalisation via Clathrin-coated pits

Agonist (Ag) binding causes a conformational change which allows the dissociation of the heterotrimeric G protein. The agonist occupied receptor is then a substrate for intracellular kinases to phosphorylate the C- terminus. Phosphorylation promotes the binding of adaptor molecules such as arrestins and AP-2, preventing receptor association with G proteins and targeting the receptor to clathrin-coated pits for internalisation into an endocytic vesicle where the receptor is de-phosphorylated.

The receptor can be recycled to cell surface or if agonist exposure continues targeted to the lysosome for degradation. If agonist exposure is prolonged, this results in down-regulation, which is a decrease in the total number of receptors present in the cell.

GRK= G protein coupled receptor kinase

D= Dynamin I



1.3 Lysophospholipids

Lysophospholipids are not only metabolites of phospholipid synthesis but are potent activators of widely expressed GPCRs. The best characterised are lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) (Figure 1.5). LPA is a lipid with a glycerol backbone and a long saturated or unsaturated fatty acid chain. LPA can be generated either intracellularly or extracellularly and this is mediated by various enzymes including phospholipases. S1P is composed of a sphingoid backbone and a phosphate group. Both S1P and LPA function as autocrine and paracrine mediators of cell function and are capable of binding to multiple receptors eliciting among predominantly growth-related responses. However, the focus of this thesis will be on the actions of S1P [64, 65].

1.3.1 Sphingosine-1-Phosphate (S1P)

Sphingosine was named by its discoverer J.L.W. Thudichum in 1884 after the Greek mythological creature the Sphinx due to its mysterious qualities [66]. High levels of sphingosine are toxic to cells and so low levels are maintained by specific enzymes that metabolise sphingosine into non-toxic derivatives. One such derivative, *D-erythro*-sphingosine-1-phosphate (S1P) is produced by the phosphorylation of sphingosine which is then subsequently degraded and recycled. It was first thought that S1P was only an intermediate in the detoxification of sphingosine but over the past few decades S1P has emerged as a crucial modulator of lipid signalling [67, 68].

In addition to mammals, S1P and related sphingoid lipids have been detected in plants [69, 70], worms, flies, slime moulds and yeast [71]. The discovery that S1P regulates proliferation [72], suppresses programmed cell death [73] and the subsequent cloning and identification of cell surface receptors to which S1P binds [74-76] has led to numerous studies linking S1P to physiological and pathophysiological processes such as cancer, angiogenesis, vascular permeability and transplant rejection. The ubiquity of S1P and its receptors and the evolutionary conservation of S1P metabolism demonstrates its importance as a signalling molecule [67].

1.3.2 S1P Metabolism and Secretion

Sphingolipids are ubiquitous constituents of eukaryotic membranes. Sphingomyelin (SM) is metabolised by sphingomyelinases to produce an acylated sphingoid base called ceramide. Deacylation of ceramide forms sphingosine which can be phosphorylated by sphingosine kinases (SphKs) to yield S1P [68, 77]. S1P is irreversibly degraded in the endoplasmic reticulum by S1P lyase to ethanolamine and hexadecenal [78]. Alternatively, S1P can be converted back to sphingosine by specific phosphatases (Figure 1.6) [79, 80].

All the enzymes involved in S1P metabolism are conserved from yeast to man [68]. The evidence to date suggests that S1P levels are predominantly regulated by its synthesis from sphingosine by SphKs rather than its degradation by S1P lyase [81] and S1P phosphatase [82]. The basal level of S1P in cells is usually low and an increase in S1P levels consistently corresponds with an increase in SphK activity [83].

1.3.3 SphK Regulation

The first sphingosine kinases discovered were cloned from *S.cerevisiae*. The genes *Lcb4* and *Lcb5* encode a sphingolipid long chain base (LCB) kinases, which were found to be unrelated to any known protein thus revealing a new class of lipid kinase [84]. This led to the search for mammalian homologues, of which the murine was the first isolated. Subsequently the human homologue SphK1 (hSphK1) was cloned [83]. Both have similar tissue distributions and are highly expressed in the lung and spleen. SphK1 contains no transmembrane domains or signal sequence and is found primarily in the cytosol [85].

Based on sequence homology to SphK1 another isoform termed SphK2 was cloned from both humans and mice. SphK2 expression is detected in most adult tissues with the heart, kidney and testis showing the highest levels of expression. Both isoforms have five evolutionary conserved domains (named C1-C5); regions C1-C3 are a unique catalytic domain with an ATP binding site within C2 (Figure 1.7). In addition, the 2 isoforms of SphK differ in developmental and tissue expression which implies they may have distinct functions [86].

SphK is activated by a number of agonists including epidermal growth factor (EGF) [87] platelet-derived growth factor (PDGF) [88], nerve growth factor (NGF) [89-91], tumour necrosis factor α (TNF α) [92], basic fibroblast growth factor (bFGF) [90], serum [88], phorbol 12-myristate 13-acetate (PMA), S1P itself [93] and other activators such as protein B cell lymphoma gene-2 (Bcl-2) [94]. SphK2 contains four transmembrane domains as predicted by hydropathy studies, though like SphK1 most of its activity is detected in the cytosol despite the fact their substrate is membrane-localised [86]. However, a more recent study reveals that SphK2 has a functional nuclear localisation signal (NLS) and its activity is predominantly localised in the nucleus [95]. However, these differences in SphK2 localisation were observed in different cell types where the NLS-mediated translocation of SphK may be prevented by interaction with another protein.

It has been shown that PDGF causes the translocation of SphK1 to the leading edge of the lamellipodia in murine embryonic fibroblasts (MEFs) [96]. It has also been demonstrated in human embryonic kidney cells (HEKs) that treatment with PMA triggers the translocation of SphK1 to the cell membrane which corresponds with an accumulation of extracellular S1P. This indicates that SphK1 action on membrane-localised sphingosine allows the crossing of S1P through the membrane, despite the polar nature of its head group [93].

Studies using *S.cerevisiae* identified an ATP-binding cassette protein called yeast oligomycin resistance gene (YOR1) that rendered the cells resistant to the apoptotic effect of sphingosine. The accumulation of sphingosine is prevented by pumping S1P out of cells [97]. In mammals, haematopoietic cells such as activated platelets, monocytic and mast cells are known to actively secrete S1P [98]. Interestingly, platelets are the only mammalian cells that lack S1P lyase activity [99].

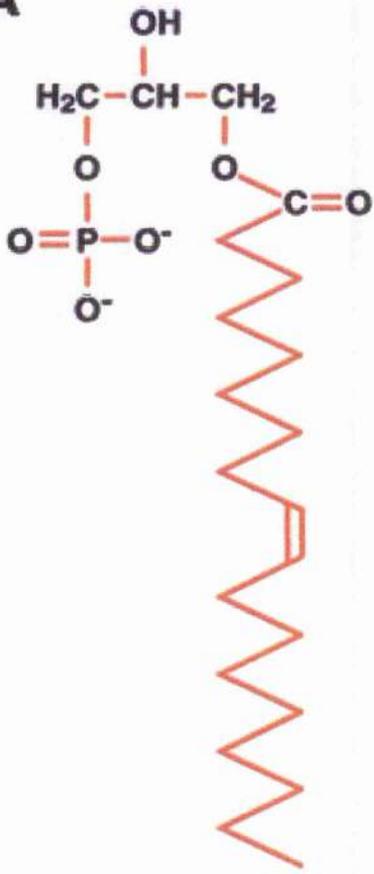
It has been shown recently that permeabilised platelets activated by thrombin release S1P. This requires the presence of bovine serum albumin (BSA) and indicates that S1P release may be a carrier-mediated process [100]. Circulating S1P is primarily bound to high and low density lipoproteins though it can also bind albumin [101, 102]. The concentration of S1P in plasma ranges between 0.2-0.5 μ M. However the concentration in serum is

much higher (1-5 μ M) and has been attributed to its secretion from activated platelets [103, 104]. This is much higher than the K_d for binding of S1P to the extracellular surface receptors identified for this ligand which is in the order of 8-25nM [105, 106]. Specific lipoproteins are associated with cardiovascular diseases such as atherosclerosis, so measurement of S1P content in lipoproteins in addition to the total plasma and serum content is important in characterisation of these diseases [107].

Figure 1.5 The Chemical Structures of Sphingosine-1-phosphate (S1P) and Lysophosphatidic Acid (LPA)

Both S1P and LPA are synthesised by cells *de novo* or through intermediate metabolism.

LPA



S1P

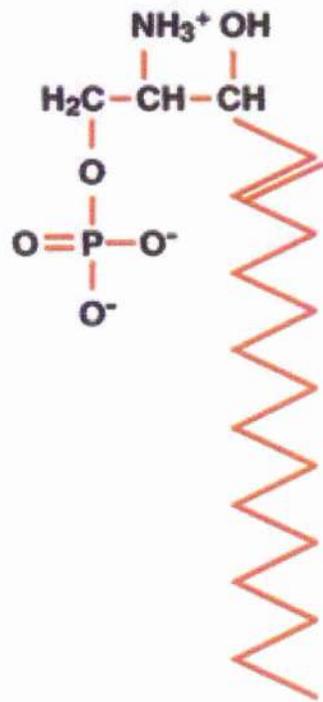


Figure 1.6 The Metabolism of Sphingosine-1-phosphate (S1P)

Pathways of S1P metabolism. The de novo synthesis of S1P is initiated by the condensation of serine and palmitate to 3-ketosphinganine which is rapidly reduced to dihydrosphingosine (DHS). The enzyme ceramidase catalyses the N-acylation of DHS to dihydroceramide. Ceramide is generated by the introduction of a 4-5 *trans* double bond. Sphingosine is formed by the deacylation of ceramide or by the liberation from sphingomyelin. Ceramide can be converted into sphingosine by the addition of phosphocholine. S1P is produced by the phosphorylation of sphingosine by SphKs. S1P can be converted back to sphingosine by S1P phosphatase. Conversely, S1P can irreversibly degraded by S1P lyase [99].

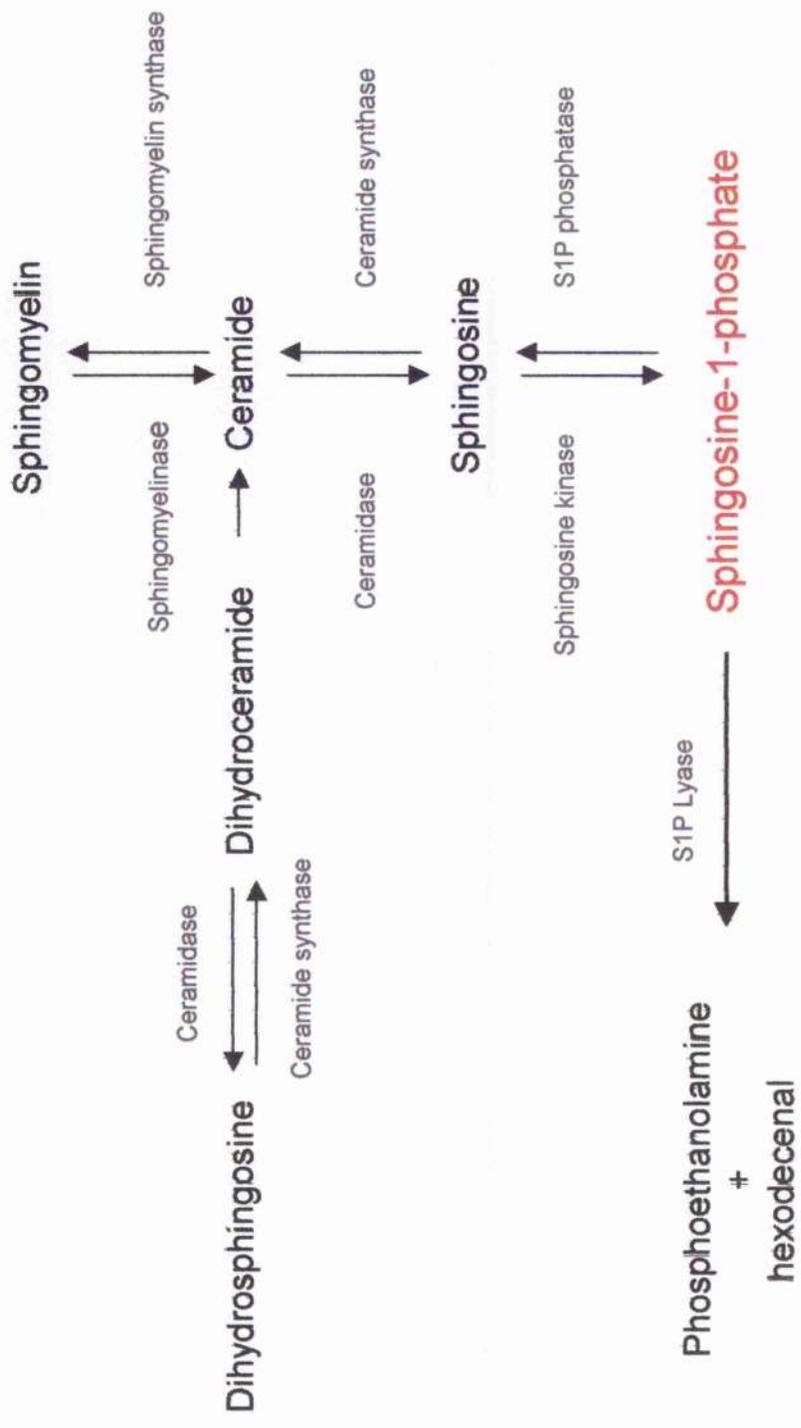


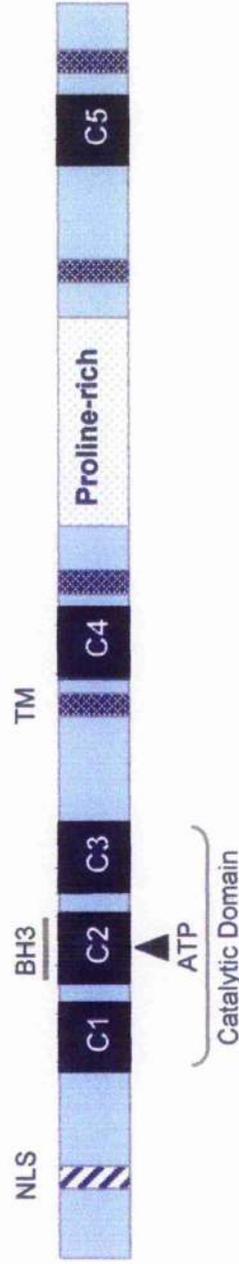
Figure 1.7 The Structural Composition of Sphingosine kinases 1 and 2

SphK1 and 2 have five conserved domains (C1-C5) with the unique catalytic domain contained within C1-C3. The ATP binding site (SGDXK 17-21 (R)) is present within C2. Unlike SphK1, SphK2 contains four transmembrane domains, a nuclear localisation sequence (NLS) and a Bcl-2 homology domain (BH3) [68].

SphK1



Sphk2



1.4 Identification of S1P Receptors

S1P receptors are involved in diverse cell signalling pathways controlling many growth-related or cytoskeletal cell processes such as apoptosis [78], angiogenesis [67], inflammation [108], proliferation [109] and migration [110, 111]. S1P receptors belong to a family termed the Endothelial Differentiation Gene (EDG) family. The first EDG receptor was identified in 1996 during a search for genes expressed predominantly in the ventricular zone (VZ) of cerebral cortex and was called ventricular zone gene 1 (VZG-1) [112]. This was shown to be a GPCR with high affinity to LPA [113]. The search for homologues ensued and it was realised that EDG-1 and VZG-1 (EDG-2) were related [114]. EDG-1 was discovered as an abundant protein expressed on endothelial cells, which was believed to have a potential role in endothelial differentiation. It was cloned as an immediate early gene termed *edg-1* (endothelial differentiation gene-1) and the resulting polypeptide contained seven transmembrane stretches indicating that it was a GPCR [115].

The exogenous agonist for the EDG-1 receptor remained a mystery until 1998, when two groups identified the lipid S1P as a high affinity ligand. HEKs over-expressing EDG-1 were observed to have similar morphology to endothelial cells, increased expression of cadherins and extensive adherens junctions. S1P promotes the formation of capillary-like networks in endothelial cell cultures. These responses were dependent on S1P and the small GTP binding protein Rho [76, 116].

The other receptors were subsequently cloned using degenerate PCR strategies and cDNA libraries [117, 118], and to date, there are eight EDG receptors identified that have been re-named according to their ligand specificity. There are five EDG receptors that bind S1P with high affinity: S1P₁ (EDG-1), S1P₂ (EDG-5), S1P₃ (EDG-3), S1P₄ (EDG-6) and S1P₅ (EDG-8). The other three EDG receptors are high affinity receptors for LPA called LPA₁ (EDG-2), LPA₂ (EDG-4) and LPA₃ (EDG-7) [119] (Figure 1.8).

The EDG family can be categorised into three groups according to their amino acid sequence similarity:

1. S1P₁, S1P₂, S1P₃ and S1P₅ which are approximately 50% identical,
2. LPA₁, LPA₂, and LPA₃ which are approximately 55% identical,

3. S1P₄ which is 35-42% identical to the other EDG receptors [120] (Figure 1.9).

1.4.1 S1P Receptor Signalling

S1P receptors are integral membrane proteins with seven transmembrane domains typical of GPCRs with an extracellular N-terminus and an intracellular C terminus. They share approximately 50% homology to each other. Each receptor couples to one or more signal transduction pathways *via* heterotrimeric G-proteins [119, 121].

S1P₁ is expressed in most mammalian tissues but is highly expressed in endothelial cells the heart, lungs and the brain. S1P₁ signalling is associated with the inhibition of AC, the mobilisation of calcium through PLC activation [122], the activation of Ras and the activation of PI 3 kinase [123, 124]. These effects are all mediated by the actions of the $\beta\gamma$ subunit of G_i except the inhibition of AC which is regulated by G _{α} [125] and are pertussis toxin sensitive. S1P₁ also exerts control of the small GTPase Rac [124, 126]. S1P₂ and S1P₃ couple to G_i, G_{12/13} and G_q [127]. S1P₃ is expressed in many tissues especially heart, lung, kidney and spleen [64]. S1P₃ activation results in Ras and Rac activation through G_q and its activity can be partially blocked by pertussis toxin suggesting an a G_i component [128]. Like S1P₃, S1P₂ is ubiquitously expressed showing higher expression levels in heart and lung tissues [64]. S1P₂ activates ERK and PLC *via* G_i but also activates AC [129]. S1P₂ also stimulates c-Jun amino terminal kinase (JNK) and p38 activity in a pertussis toxin insensitive manner [130].

S1P₄ and S1P₅ signalling pathways have not been extensively characterised. Both S1P₄ and S1P₅ are believed to couple to G_i and G₁₂ [131-133]. S1P₄ expression is mostly confined to lymphoid tissues and platelets [134] and S1P₄ has been shown to stimulate Cdc42, PLC and ERK in a pertussis toxin sensitive manner. S1P₄ also activates Rho *via* G_{12/13} [131, 135, 136]. S1P₅ expression is confined to the central nervous system and the skin [132] and is associated with AC inhibition and unusually, the inhibition of ERK. S1P₅ also promotes JNK activity through its interaction with G₁₂ [132, 133] (Figure 1.10).

Distinct receptor expression patterns and their ability to couple to multiple G proteins that signal to a range of effector proteins allows S1P receptors to regulate many cellular processes in a specific manner to give a co-ordinated cellular response to S1P [68]. S1P₁ receptor signalling has a particularly important role in development, angiogenesis and migration and these aspects of S1P₁ signal transduction will be addressed later.

1.4.2 Orphan GPCRs (oGPCRs) with homology to S1P and LPA Receptors

In addition to the more conventional members of the EDG family there are a number of receptors identified which are closely related. These include GPR3, GPR6, GPR12, GPR23 and GPR63. They share 57-61% sequence identity. The receptors GPR3, 6, 12 and 63 have been identified as S1P receptors [137, 138].

The oGPCR GPR45 is a mammalian homologue of a LPA receptor identified in *X. laevis* [139]. Also, PSP24 (cloned from *Xenopus*) has been identified as a LPA receptor but is not functional in mammals [140]. GPR68 which was cloned from an ovarian cancer cell line has been identified as a sphingosylphosphocholine (SPC) receptor [141]. The availability of information on the human genome has accelerated the identification of receptors. Pairing receptor with ligand and discovering the signalling pathways which regulate will aid the search for therapeutic targets [142].

Figure 1.8 The EDG Receptor Family and Related Receptors

Phylogenetic tree representing receptors with high and low affinities for the ligands: S1P, LPA and sphingosylphosphocholine (SPC). It also shows related receptors for which the agonist is undetermined (Taken from Kostensis) [143].

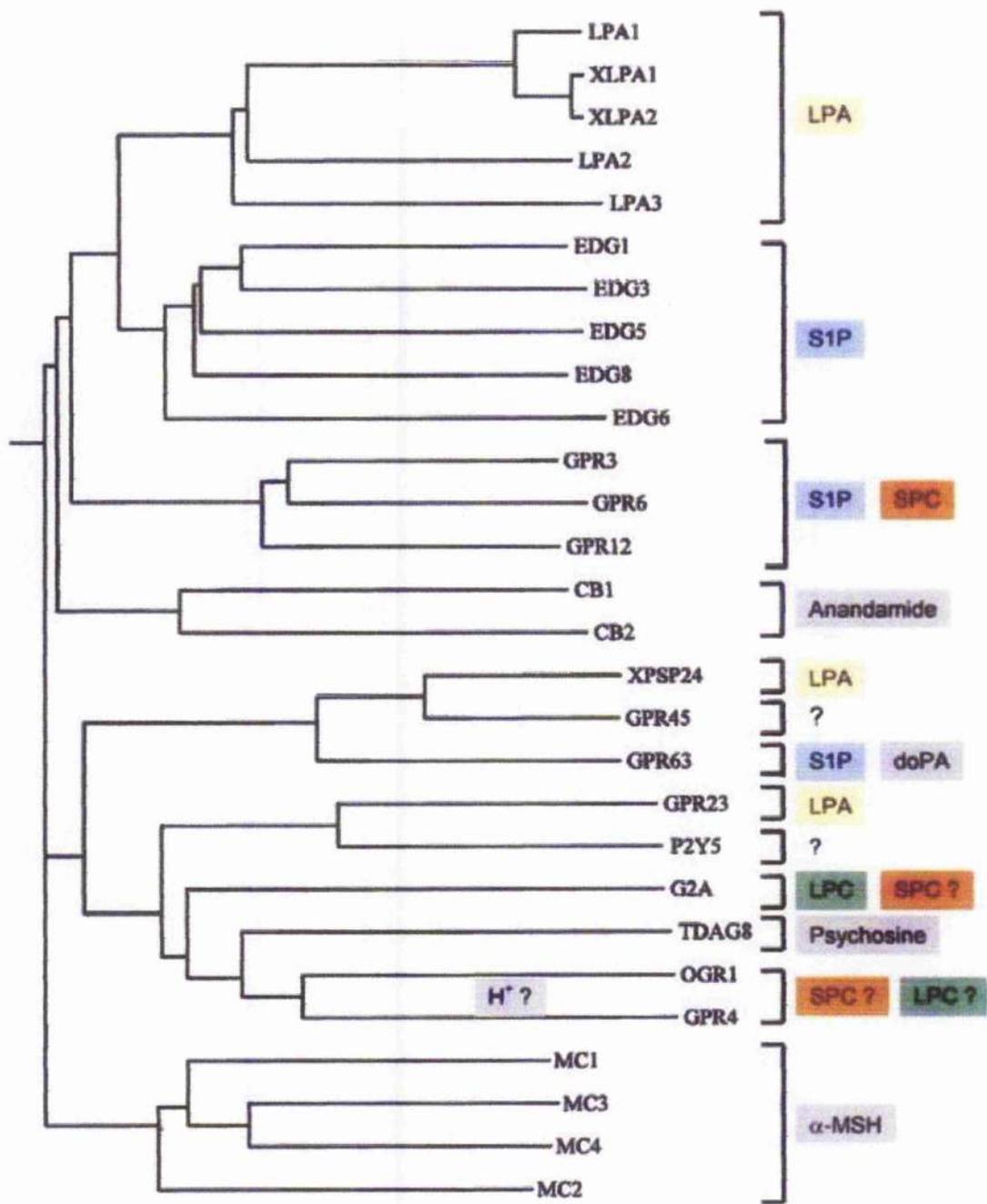
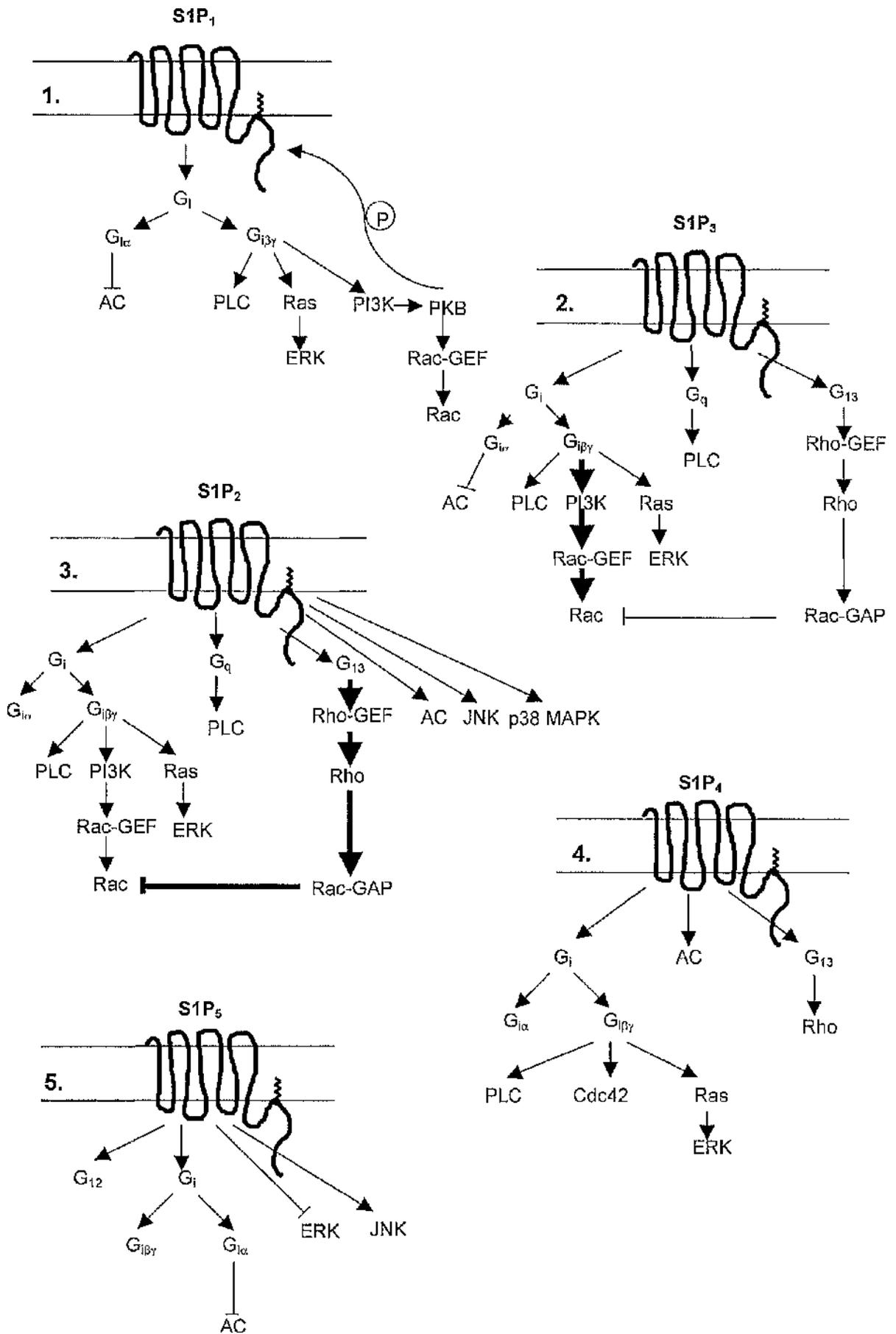


Figure 1.9 The EDG Family Amino Acid Alignment

The amino acid sequence of human EDG receptors 1-7. EDG-1,-3, -5, -6 and -8 are high affinity for S1P. EDG-2,-4 and -7 are high affinity for LPA. The black shading highlights the identical residues and the grey shading represents similar residues.(take from Pyne and Pyne, 2000) [77].

Figure 1.10 S1P Receptor Signalling

1. S1P₁ couples exclusively to G_i , it regulates Rac *via* PKB,
2. S1P₃ couples to G_i, G_q and G₁₃, it activates Rac,
3. S1P₂ couples to G_i, G_q and G₁₃ but unlike S1P₃ it inhibits Rac,
4. S1P₄ couples to G_i and G₁₃ , and activates adenylyl cyclase *via* an unknown mechanism,
5. S1P₅ couples to G_i and G₁₂, it inhibits ERK activation and promotes JNK activity, (adapted from Taha *et al*) [123].



1.5 The Small G protein Superfamily

Small GTP-binding proteins are monomeric G proteins with molecular masses in the order of 20-40kDa. The Ras proteins were first identified as viral oncogenes (v-Ha-Ras and v-Ki-Ras) [144, 145]. Their counterparts in humans were identified in carcinomas and were shown to stimulate transformation and proliferation [146-149]. To date there are more than a hundred identified small G proteins in eukaryotes from yeast to human [150]. The members of this superfamily are categorised into five families:

- **Ras Family** are involved in gene expression,
- **Rho Family** (Rho/Rac/Cdc42) regulate cytoskeletal reorganisation,
- **Rab and Sar1/Arf Family** regulate vesicular trafficking,
- **Ran Family** regulate nucleoplasmic transport and microtubule dynamics during cell cycle processes [150].

1.5.1 Small G protein Regulation

Small G proteins share similar structural organisation with other G proteins: a consensus amino acid sequence responsible for interaction with GDP and GTP and intrinsic GTPase activity. The Ras, Rab and Rho family have C-terminal tails that undergo post-translational modifications with isoprenoid and palmitate lipid groups [151].

By cycling between GDP- and GTP-bound states, small G proteins function as molecular switches. The exchange of GDP for GTP causes a conformational change that allows their interaction with downstream effectors. The rate limiting step in GDP/GTP exchange is the dissociation of GDP which is enhanced by guanine nucleotide exchange factors (GEFs). GEFs vary in substrate specificity, such as son of sevenless (Sos) which activates Ras and Rac. The GEFs Dbl and Vav1 show wider substrate activity by acting on Rho, Rac and Cdc42 [152]. GAPs, (GTPase-activating proteins) function to stimulate the small G protein's intrinsic GTPase activity thereby favouring the inactive state. The Rho GTPases in particular are subject to further regulation by GDP dissociation inhibitors (GDIs) which sequester the GDP-bound form so the G protein remains soluble despite any lipid modification [153] (Figure 1.11). The activation of this family of proteins is thereby tightly regulated by both positive and negative mechanisms.

1.5.2 The Rho GTPases

The mammalian Rho GTPase family consists of at least 20 members (Figure 1.12) and their activation stimulates signalling pathways leading to regulation of the cytoskeleton, cell cycle, gene transcription, cell polarity and vesicle transport. The role of Rho GTPases in regulation of the cytoskeleton was elucidated using C3 toxin which ADP-ribosylates an Asn residue within the effector region of RhoA [154] which lead to rapid changes in the morphology of the cell caused by re-organisation of the actin microfilaments. Rho proteins were subsequently identified as regulators of cytoskeletal operations [155, 156] such as stress fibre formation, focal adhesion dynamics and muscle contraction [157, 158], whereas Rac and Cdc42 were discovered to be involved in the regulation of other cytoskeletal processes such as the formation of lamellipodia and filopodia, respectively [159-161].

It has been estimated that there are as many as fifty GEFs, forty GAPs and forty effectors identified for Rho-like G proteins [162, 163]. The GEFs identified hitherto have a conserved motif called the Dbl-homology domain (DH) and a pleckstrin homology domain (PH) which is involved in cellular localisation with PIP₂ [152, 164]. In addition to activation of GTPases GEFs also participate in the selection of downstream targets by either binding with effectors or scaffold proteins that complex with components of the targeted pathway. For instance, the Rac GEF Tiam-1 (T-lymphoma invasion and metastasis) stimulated the accumulation of IB2 which is a scaffold protein for the p38 mitogen-activated protein kinase cascade but also enhances ERK signalling [165].

1.5.3 Rho, Rac, Cdc42 and Migration

The role of the small G proteins Rac, Rho and Cdc42 in migration is perhaps the best characterised of their functions. Rac activity is localised at the front of the cell (leading edge) to regulate localised actin polymerisation required for membrane extension. Rho is required to generate contractile force which results in the rounding of the cell body and microtubule polymerisation at the rear of the cell. Cdc42 is necessary to establish polarity of the cell and filopodial protrusions [162, 166]. All these actions must be co-ordinated spatially and temporally to produce a net forward movement of the

migrating cell (Figure 1.13). In addition to this, all three GTPases induce the assembly of integrin-based matrix-adhesion complexes ([161]).

PI 3-kinase and its product PIP₃ are known to influence cell polarity and migration [167]. The production of PIP₃ increases level of GTP-bound Rac in various cell types by interaction with the Rac GEFs Vav and Sos-1 [168, 169]. Expression of constitutive Rac in fibroblasts results in extensive lamellipodia and membrane ruffling but not activation of other Rac-mediated signals like the JNK pathway [170]. Rac and PI 3-kinase interact with each other directly and GTP-bound Rac stimulates PI 3-kinase promoting PIP₃ production, thus implying the existence of a positive feedback loop [171]. Another Rac/Cdc42 GEF called PIX is also regulated by PIP₃. At the leading edge of the cell, PIX promotes actin polymerisation and interacts with the Rac effector p65PAK which is believed to control focal adhesion turnover [172]. p65PAK phosphorylates and activates LIM kinase (LIMK) which then phosphorylates and inactivates cofilin [173]. Cofilin functions to promote actin filament treadmilling at the leading edge of migrating cells. Like p65PAK, p160Rho kinase (ROCK) a Rho substrate, can phosphorylate and activate LIMK which leads to the phosphorylation and inactivation of cofilin, thereby facilitating the stabilisation actin filaments within actin-myosin filament bundles [174]. ROCK phosphorylates and inactivates myosin light chain kinase (MLCK) [175] which further promotes the cross-linking of actin-myosin bundles, thus generating contractile force and also encouraging detachment at the rear of the cell [176]. Another downstream target of Rho is mDia which is a scaffold protein that cooperates with ROCK in the assembly of actin-myosin filaments [177].

Both Rac and Cdc42 regulate the actions of WASp or WAVE proteins. WASp (Wiskott-Aldrich Syndrome protein) is a downstream target of Cdc42 that interacts directly with the Arp2/3 complex, a ubiquitous activator of actin polymerisation in eukaryotic cells [178, 179]. Additionally, Rac interacts with WASP-like proteins WAVE (WASP family verpolin homology) indirectly and activates the Arp2/3 complex *via* interaction with an Nck-adaptor complex [180, 181] (Figure 1.14). It is at present unclear how Rac and Cd42-mediated activation of Arp2/3 results in morphologically different structures [166].

Rho GTPases also regulate microtubule dynamics which are important in continual migration which requires established cell polarity [162]. A role for Rho GTPases in microtubule regulation was realised when it was discovered that the chemical nocodazole, which interrupts microtubules, caused the activation of Rho. Furthermore, the removal of nocodazole from cells resulted in the activation of Rac [182]. Rho is also implicated in the stabilisation of microtubules by capping [183] whereas Rac stimulates microtubule elongation *via* p65PAK-mediated phosphorylation and subsequent inactivation of stathmin (a microtubule destabilising protein) [184]. Although most of the evidence available points to a role for the Rho-like GTPases in determining cytoskeletal dynamics with respect to the regulation of migration, it is becoming clear that they function in many facets of cell morphology and maintenance.

1.5.4 Regulation of Endocytic Traffic by Rho-like GTPases

The small G protein families of Rab and Arf have crucial functions in vesicle trafficking and budding respectively [150]. There is also evidence of a role for Rho-like GTPases in various aspects of endocytosis. Internalisation, the budding off of the plasma membrane represents the initial step in endocytosis. Clathrin-mediated internalisation is the most studied form of endocytosis where receptors are clustered into clathrin-rich patches which are pinched off to form coated vesicles. This process does not seem to require the presence of Rho GTPases but they contribute to the organisation and efficiency of the process [185].

Rac stimulates the production of PIP₂ through the activation of PI-4,5 kinase this mediates the dissociation of the capping protein from the barbed end of actin filaments [186]. Cdc42 is believed to bind directly with the coatamer binding domain, this interaction is important for golgi transport and the formation of vesicles, and if disrupted causes defective protein sorting and transport [187, 188].

Internalisation may also occur by mechanisms that are independent of clathrin. As stated in section 1.2.2, caveolae are associated with actin and many receptors and signalling molecules can localise there including activated forms of Rac and RhoA [189]. The most widely researched

example of Rho-like GTPase-mediated internalisation is phagocytosis which is coordinated by Rac and Cdc42 [190, 191]. Receptor internalisation can also be mediated by glycosyl-phosphatidylinositol (GPI) which is dependent entirely on Cdc42 function [192].

1.5.5 Integrin Signalling

Another important aspect of Rho GTPase function is cell adherence. The proliferation of most normal cells requires in addition to growth factors, adherence to the substratum [193]. Integrins are heterodimeric transmembrane receptors that control the adhesion of cells to extracellular matrix (ECM) proteins such as laminin, collagen, fibronectin and vitronectin. The extracellular regions of the α and β subunits are non-covalently linked to form a globular head which interacts with ECM components in a specific manner determined by the make up of the heterodimer of α and β subunits of which there are over 20 combinations identified [194, 195]. Integrin signalling mediates important cell fate decisions. The cell integrates information from both the ECM and growth factor receptors to regulate cell migration *via* signalling intermediates.

Fibroblastic, epithelial and endothelial cells have to be in contact with the appropriate ECM proteins to survive. Integrin receptors possess short cytoplasmic tails without enzymatic activity but are able to connect to signalling complexes *via* adaptor proteins and the cytoskeleton through structural proteins such as vinculin, talin and α -actinin which bind to F-actin [195, 196]. Integrin-mediated adhesion can influence calcium levels, activate Focal adhesion kinase (FAK), PKB, PKC, MAPK Kinase (MEK) and the Rho-like GTPases [193, 196-198].

Fibronectin mediates adhesion through $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrin receptors [199] and adhesion of fibroblasts to fibronectin induces the activation of Rac and Cdc42 leading to downstream PAK activity which causes cell spreading [200, 201]. The Rac GEF Vav1 has been implicated in integrin-mediated activation of Rac and Cdc42 in haematopoietic cells [202]. Sos can be recruited to adhesion complexes through FAKP130^{Cas} or Shc which also links integrin signalling to Rac activation [169]. Addition of S1P to

fibroblast cells was shown to increase fibronectin assembly and binding of the cell to the ECM and fibronectin assembly, suggesting a $G_{12/13}$ -coupled receptor is responsible for the observed effect, such as $S1P_2$ and $S1P_3$ [203].

Vitronectin mediates adhesion *via* the integrin receptors $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$ [199]. The integrin dimer $\alpha_v\beta_3$ has been demonstrated to promote migration through the PI 3-kinase/PKB pathway when activated by vitronectin [204]. In addition to this cells adhering to fibronectin *via* $\alpha_v\beta_1$ and to vitronectin through interaction with $\alpha_v\beta_3$ show increased levels of Bcl-2 transcription which promotes survival [205].

1.5.6 $S1P_1$ and Rac Activation

The link between $S1P_1$ signalling and Rac activation has been reported widely but the mechanism remained unknown till Lee and colleagues proposed a PKB-dependent mechanism. PKB phosphorylates substrates that contain the consensus sequence RXRXXS/T [206] including eNOS [207], GSK-3 β (glycogen synthase kinase) [208], Bad [209] and Rac [197]. The model proposed involves activation of the G_i pathway *via* $S1P$ binding to $S1P_1$ which results in PI 3-kinase activation and subsequent translocation of PKB to the plasma membrane [210]. PKB is activated by phosphorylation at Ser473 and Thr308. PKB is then believed to phosphorylate $S1P_1$ at Thr236 which is located in the third intracellular loop. It is this covalent modification of $S1P_1$ which is implicated in Rac activation and subsequent actin assembly (Figure 1.15) [126]. Further evidence that the PKB signalling is essential in the ability of $S1P$ to activate Rac is the finding that siRNA mediated reduction of endogenous Rac1 expression significantly reduces $S1P$ -mediated phosphorylation of PKB as well as downstream substrates eNOS and GSK3 β . Expression of the dominant negative form of the Rac GEF Tiam-1 significantly blocks $S1P$ -mediated Rac1 activation. This indicates that $S1P$ -induced Rac activation is dependent on Tiam-1 activation and implicates a role for $S1P$ -mediated activation of PI 3-kinase [124].

1.5.7 The Role of S1P in Migration

Migration is an essential process for all multicellular organisms not only in embryonic development and but also for the immune response, angiogenesis and wound healing. The Rho-like GTPase family have a crucial role in the signalling pathways that regulate cell migration [162]. S1P is a potent inducer of cell migration and can act alone or as a co-mitogen (e.g. with VEGF or bFGF). S1P stimulates migration of endothelial cells derived from vasculature over a broad range of concentrations from nanomolar to micromolar [211] and more recently LPA has also been shown to stimulate transmigration of endothelial cells on gelatine and collagen-coated filters but not through fibronectin or vitronectin-coated filters. This reveals how interaction with ECM proteins may influence the cell's migratory abilities which suggests a role for LPA in angiogenesis [212].

S1P₁ has been implicated in both endothelial and smooth muscle cell migration [211, 213]. However, S1P receptor signalling differentially regulates the small GTPases Rac and Rho which results in dynamic cycles of MLCK activation and inactivation leading to the formation of stress fibres and membrane ruffles [214]. S1P₁, S1P₂ and S1P₃ expression correlates with Rac activation status as shown in Chinese Hamster Ovary cells (CHOs). S1P₁ and S1P₃ activate Rac regulating the formation of lamellipodia thereby inducing cell migration. In addition to this, in vascular smooth muscle cells (VSMC) over-expression of S1P₁ enhances S1P-induced migration and proliferation [213]. It has been suggested that S1P₁ and S1P₃ -induced endothelial migration is mediated through Rho-dependent activation of $\alpha_v\beta_3$ and β_1 -containing integrins. Interestingly though, expression of both receptors is necessary for Rho activation, clustering of integrins and subsequent cell spreading and migration [215].

S1P₂ activity has a negative impact on cell migration, by inhibiting the activation of Rac and abolishing membrane ruffling [216]. This has also been demonstrated in VSMCs where S1P₂ is highly expressed. These cells proliferate in response to S1P but do not migrate [217, 218]. The S1P₂ receptor's inhibitory effect on cell migration was also demonstrated in a glioblastoma cell line over-expressing S1P₂. S1P₂ mediates inhibition of migration in response to S1P was found to occur in a ROCK-dependent

manner but, in contrast to other studies, this was not accompanied by a concurrent inhibition of Rac activity [219].

S1P₂ also utilises the tumour suppressor phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) as a signalling intermediary. PTEN controls signalling molecules critical in proliferation and apoptosis such as PKB. S1P₂ regulates PTEN *via* a Rho GTPase dependent mechanism since expression of a RhoA dominant negative restored S1P-induced migration [220]. S1P₂ activity has also been shown to diminish PDGF receptor function. Deletion of S1P₂ dramatically improved the cells' migratory capacity toward S1P and PDGF. S1P₂ null MEFs also showed enhanced Rac and SphK1 activity [221]. However, S1P₁ has a positive role in PDGF-induced migration as expression of the receptor appears to be critical for cell motility [222].

S1P also induces the rearrangement of focal adhesion complexes by stimulating the tyrosine phosphorylation and activation of FAK. S1P was also shown to promote a transient association of paxillin (a component of focal adhesions) with GITs (G protein-coupled receptor kinase-interacting proteins). Both FAK and paxillin co-localise to the cell cortical area suggesting role for S1P in barrier enhancement [223].

1.5.8 The Role of S1P in Angiogenesis

Angiogenesis is defined as the formation of new blood vessels from pre-existing ones. This is a crucial function in development, wound healing and pathological events such as cancer [224]. Angiogenesis involves several complex steps including ECM degradation, migration, proliferation of endothelial cells and their differentiation into capillary-like structures. These processes are regulated by growth factors like VEGF and angiopoietin. VEGF mediates the production of nitric oxide (NO) *via* PKB-induced phosphorylation of eNOS [207]. NO production has a crucial role in regulation of vascular tone, remodelling and angiogenesis [225]. S1P also induces NO-mediated angiogenesis through the same mechanism [226].

S1P was ascertained to be a regulator of angiogenesis when it was discovered that the receptors S1P₁ and S1P₃ were required for the endothelial cell morphogenesis into capillary-like tubes by regulating the

assembly of vascular endothelial adherens junctions [227, 228]. Endothelial cells treated with S1P display increased recruitment of adheren molecules such as VE-cadherin and catenin α, β and γ to the region of cell-cell contact [229].

S1P has been demonstrated to be an essential mediator of neovascularisation during embryonic development. In fact, deletion of the S1P₁ in mice by homologous recombination is lethal *in utero* at E12.5-13.5 caused by massive haemorrhaging due to incomplete vascular maturation. Histological data from the S1P₁ mutants show that the endothelial cells form vascular networks implying that it is not essential for differentiation, proliferation and migration of endothelial cells. The abnormalities can be attributed to the failure of VSMCs and pericytes to be recruited to nascent vessels. Without the stabilising support of the periendothelium the newly formed blood vessel is leaky [230]. As S1P₁ is expressed in both endothelial cells and VSMCs the question of which cell type mediated vascular maturation was still unanswered since VSMC were found in the locality. Mice lacking the S1P₁ gene in endothelial cells only were generated using the Cre/loxP system and the phenotype mimicked that of embryos that were globally deficient in S1P₁. These data indicate that endothelial cells direct VSMCs and expression of S1P₁ in these cells is not crucial in the process of vascular maturation [231]. In addition, local injection of S1P₁ siRNA into established tumours suppressed vascular stabilisation and angiogenesis. This resulted in a significant reduction in tumour growth *in vivo* implicating that siRNA technology may provide a valuable therapeutic tool in treating cancer [232]. Furthermore, PDGFR β -null mice show similar aberrations to S1P₁ null mice, i.e. a lack of pericyte recruitment and death at E9.5-12.5 [233, 234].

Surprisingly, S1P₃ knockout mice have no phenotypic aberrations and produce viable offspring although S1P-induced activation of PLC is defective in S1P₃-null MEFs [235]. The S1P₂ receptor knockouts also produce apparently normal mice but again, selective loss of S1P signal transduction is observed but in this instance it is S1P-induced Rho-activation that is impaired [236]. Signal transduction in S1P₁ knockout MEFs is also impaired as these cells showed an inability to activate Rac [230].

It has been established that S1P is an important mediator in vascular stabilisation. S1P signalling also enhances endothelial barrier integrity and it has been shown to regulate the assembly of cell-cell junctions. S1P₁ and S1P₃ signalling enhances endothelial cell barrier function [229]. In addition to this S1P₁ receptor activation has linked to the strengthening of cell-cell adhesion by promoting N-cadherin trafficking and activation [237].

S1P signalling has been demonstrated in endothelial remodelling through the association of the Arp2/3 complex and cortactin (cortical actin-associated protein) as disruption of this interaction impedes S1P-induced formation of capillary networks [238]. S1P has also been shown to be a potent regulator of lumen formation and morphogenesis on collagen and fibrin three-dimensional matrices. Matrix metalloproteinases (MMPs) mediate the degradation of ECM proteins and are regulated by tissue inhibitors of metalloproteinases (TIMPs) of which there are four isoforms (TIMP1-4). TIMP-3 was the only inhibitor that was able to attenuate the S1P-induced invasion of cells into three-dimensional matrices [239].

FTY720 is a fungal metabolite that has been demonstrated by clinical trials, to be a potent immunosuppressor in *de novo* renal transplantation [240]. It acts by sequestering circulating lymphocytes into secondary lymphoid organs, thereby preventing their redistribution to areas of inflammation [241]. FTY720 induces an immunosuppressed state that is not generalised, and is also reversible [242, 243]. It is phosphorylated *in vivo* by SphK and the resulting metabolite, FTY720 phosphate (FTY720P) bears structural similarity to S1P (Figure 1.16). FTY720P displays a high affinity for all the S1P receptors except S1P₂, with which it has an equilibrium binding constant (K_i) of over 1000 [244]. Furthermore, FTY720P has been shown to stimulate migration, differentiation and survival in a mode comparable to S1P [245].

However, FTY720 has been identified as an antagonist of S1P-mediated signalling *via* S1P₁, S1P₂ and S1P₅ at nanomolar concentrations. It induces S1P₁ internalisation without stimulating receptor signalling and targets the receptor for degradation rather than recycling back to the cell surface. However, at present, the mechanism for this phenomenon is unknown [246]. In addition to this, FTY720 has been demonstrated to inhibit

both S1P and VEGF-mediated angiogenesis and block tumour progression *in vivo*. Thus, functional antagonism of the S1P receptors by this molecule may provide a new therapeutic approach to dysfunctional regulation of angiogenesis in cancerous tumours [247].

Prior to establishing a vascular network in order to grow, the cancerous cell must bypass the intrinsic cell suicide mechanisms triggered by aberrant growth patterns. Understanding dysfunction of apoptosis, the body's method of removing damaged, infected and superfluous cells is another vital avenue for research into cancer therapeutics [248].

Figure 1.11 The Rho-like GTPase Cycle

Rho-like proteins such as Rac cycle between an inactive GDP-bound state and an active GTP-bound state. Transitions between the two are controlled by GEFs (guanine nucleotide exchange factors), GAPs (GTPase-activating proteins) and GDIs (GDP dissociation inhibitors). GEFs also select downstream effectors either directly or *via* interaction with scaffold proteins.

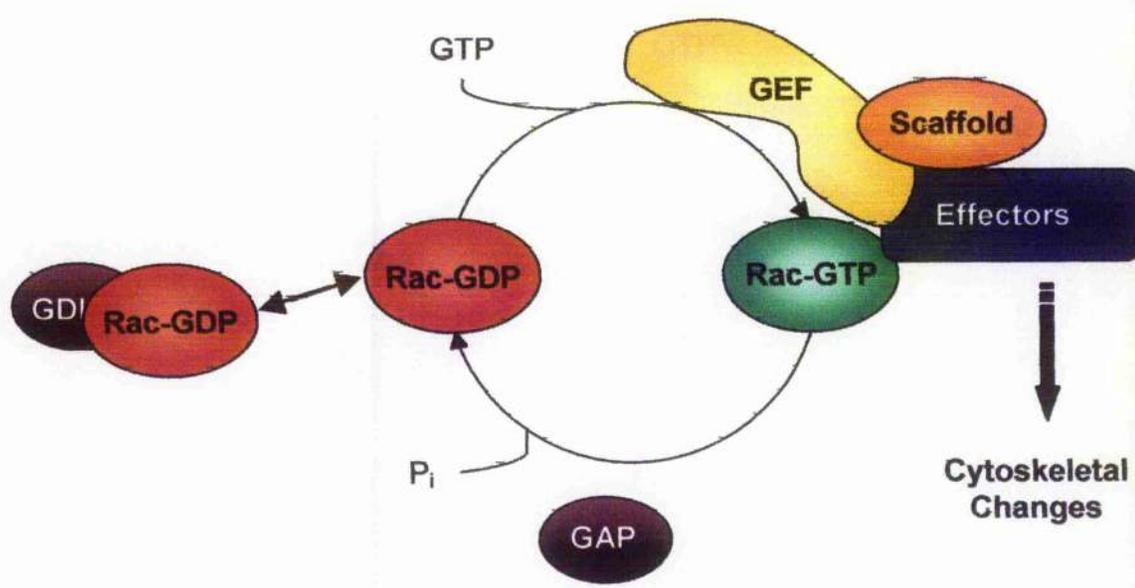


Figure 1.12 The-like Rho GTPase Family

Phylogenetic tree representing the twenty members of the Rho GTPase family (taken from Burrige *et al*) [249].

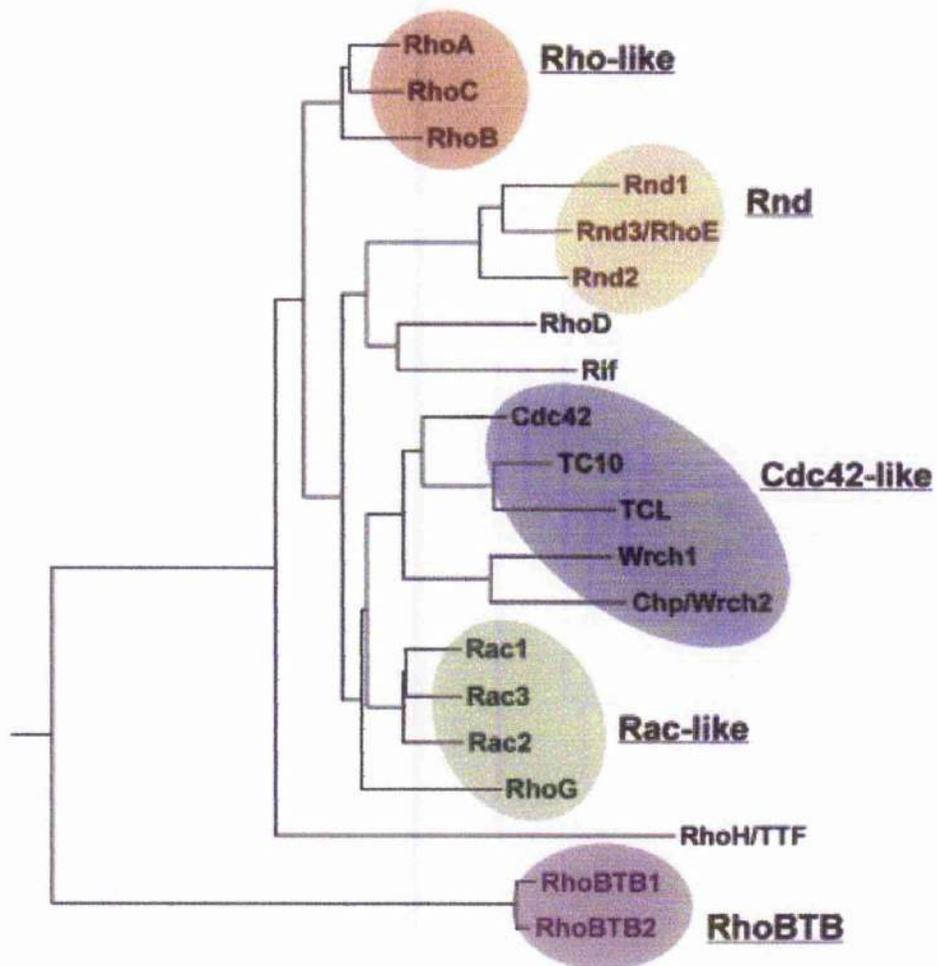


Figure 1.13 The Rho-like GTPases Regulate Cell Migration

An illustration of a migrating cell; Cdc42 regulates the direction of movement, Rac induces membrane protrusion at the leading edge through promotion of actin polymerisation and integrin adhesion complexes. Rho promotes actin-myosin mediated contraction and cell rounding. All these actions must be co-ordinated in space and time to result in a net forward movement [162].

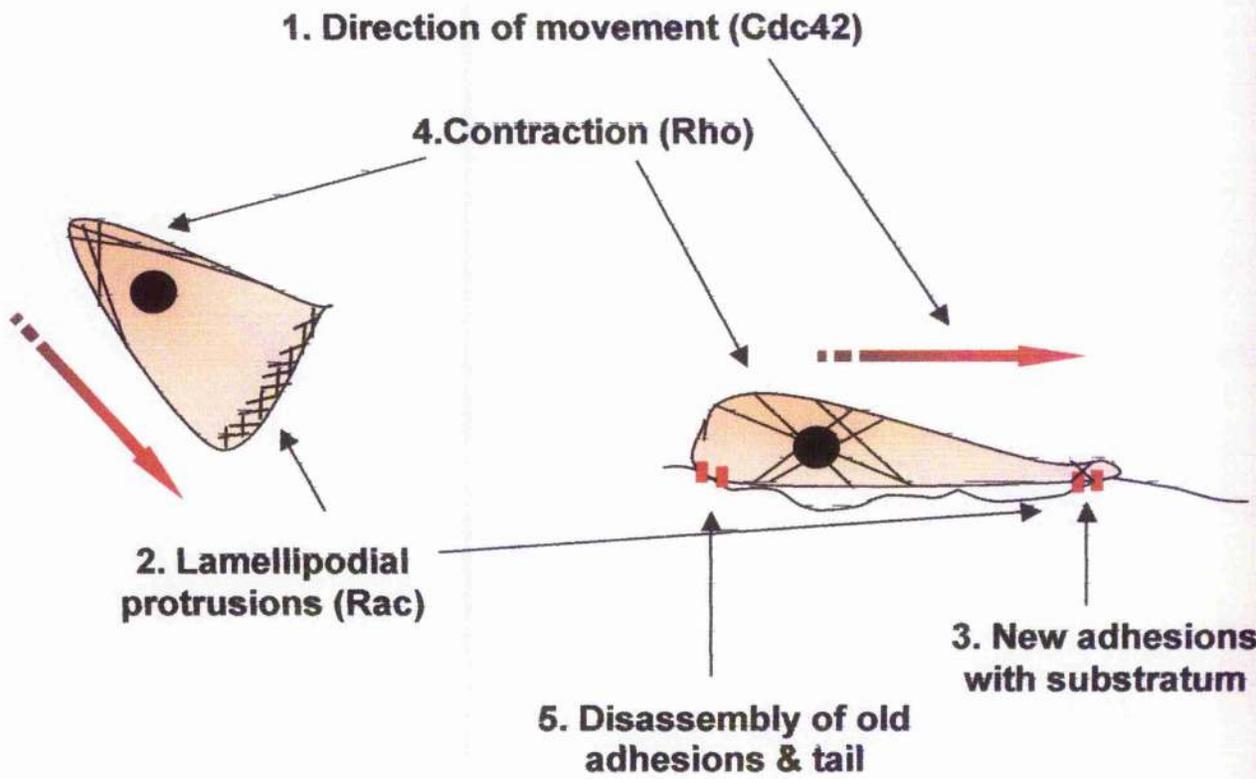


Figure 1.14 Signalling Pathways of Cytoskeletal Dynamics

Rho mediates actin-myosin assembly through mDia and ROCK. ROCK is known to phosphorylate LIMK which results in phosphorylation of cofilin and myosin light chain phosphatase encouraging MLC phosphorylation and actin-myosin crosslinking. Rac and Cdc42 regulate actin polymerisation *via* the WASp/WAVE proteins which act on Arp2/3 complex and also through p65PAK activating LIM kinase (adapted from Raftopoulou and Hall) [162].

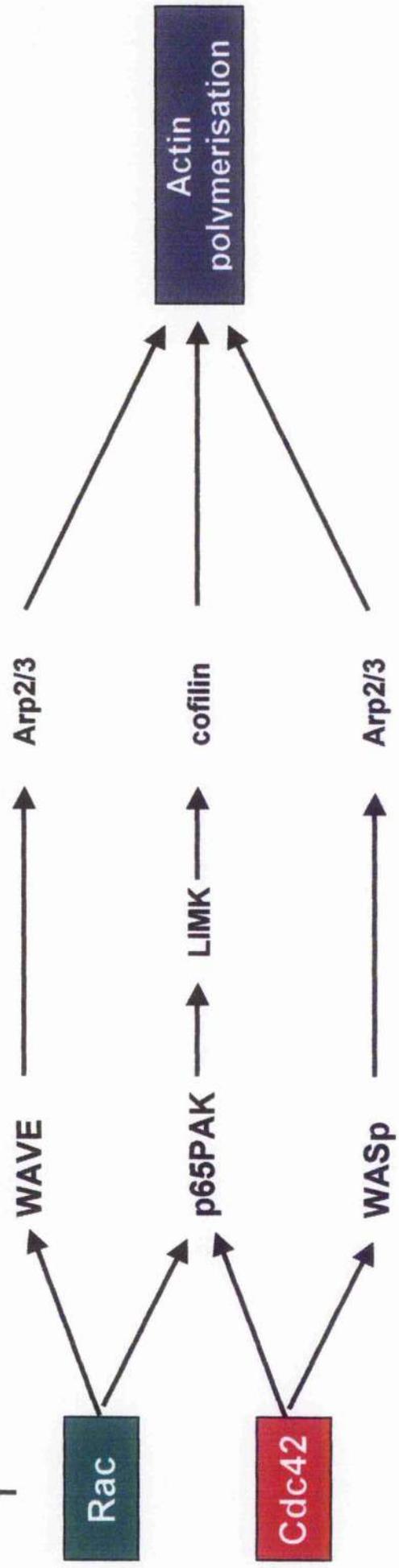
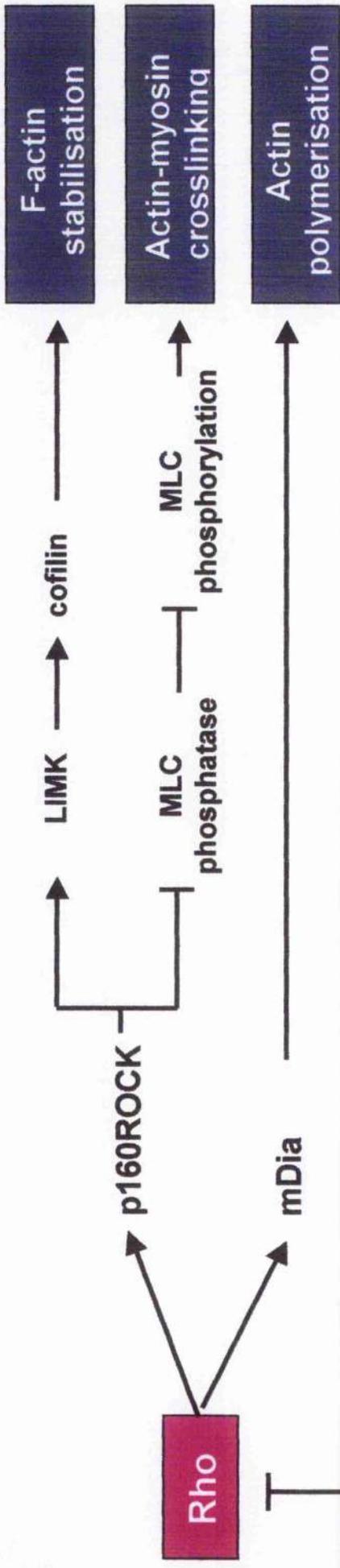


Figure 1.15 S1P₁ Activates Rac in a PKB-Dependent Manner

The model proposed by Lee *et al* [126] suggests that transactivation of S1P₁ by PKB is essential for S1P₁-mediated Rac activation. S1P binds to S1P₁ results in PI 3-kinase mediated translocation of PKB to the plasma membrane. PKB is activated by the phosphorylation at Ser473 and Thr308. This results in an increased association of PKB with the third intracellular loop of S1P₁ which leads to the PKB-mediated phosphorylation of Thr236. This action is required for the Rac activation and subsequent actin assembly.

PDK= 3-phosphoinositide-dependent kinase

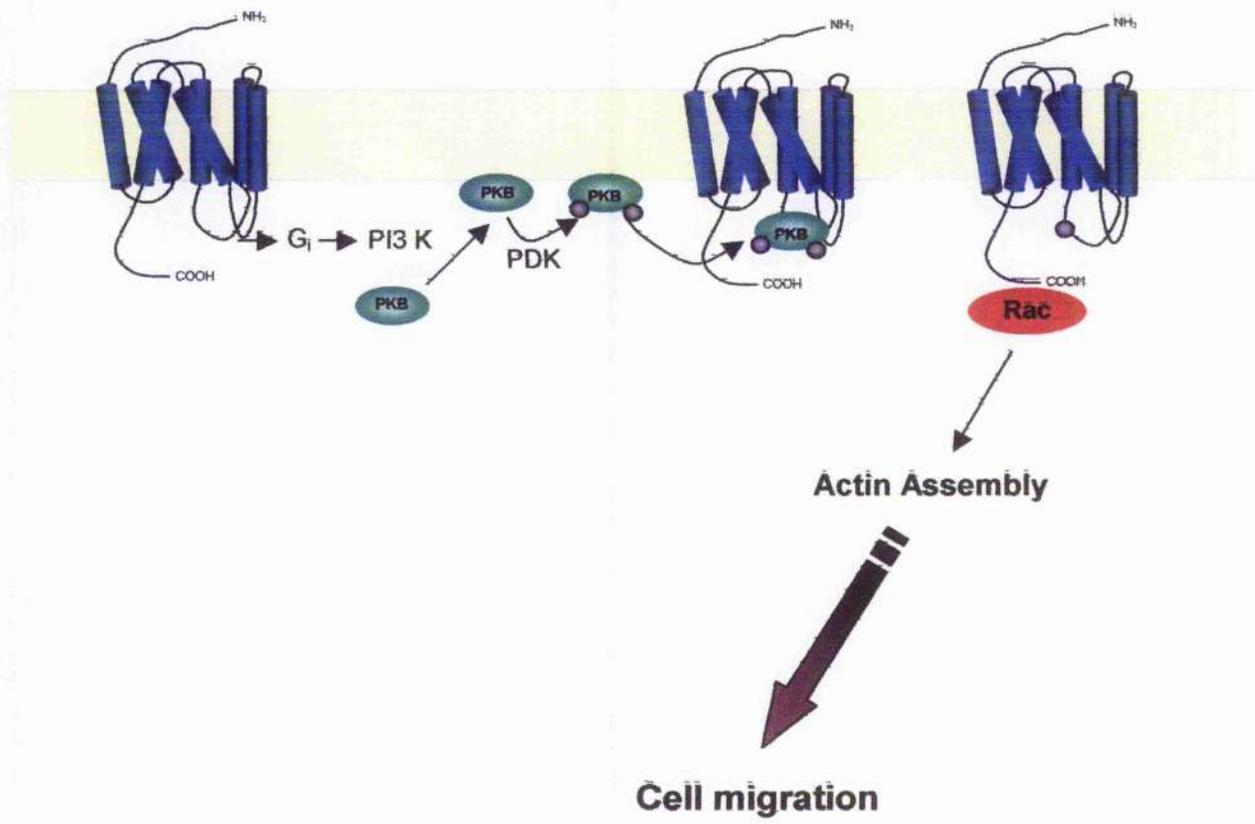
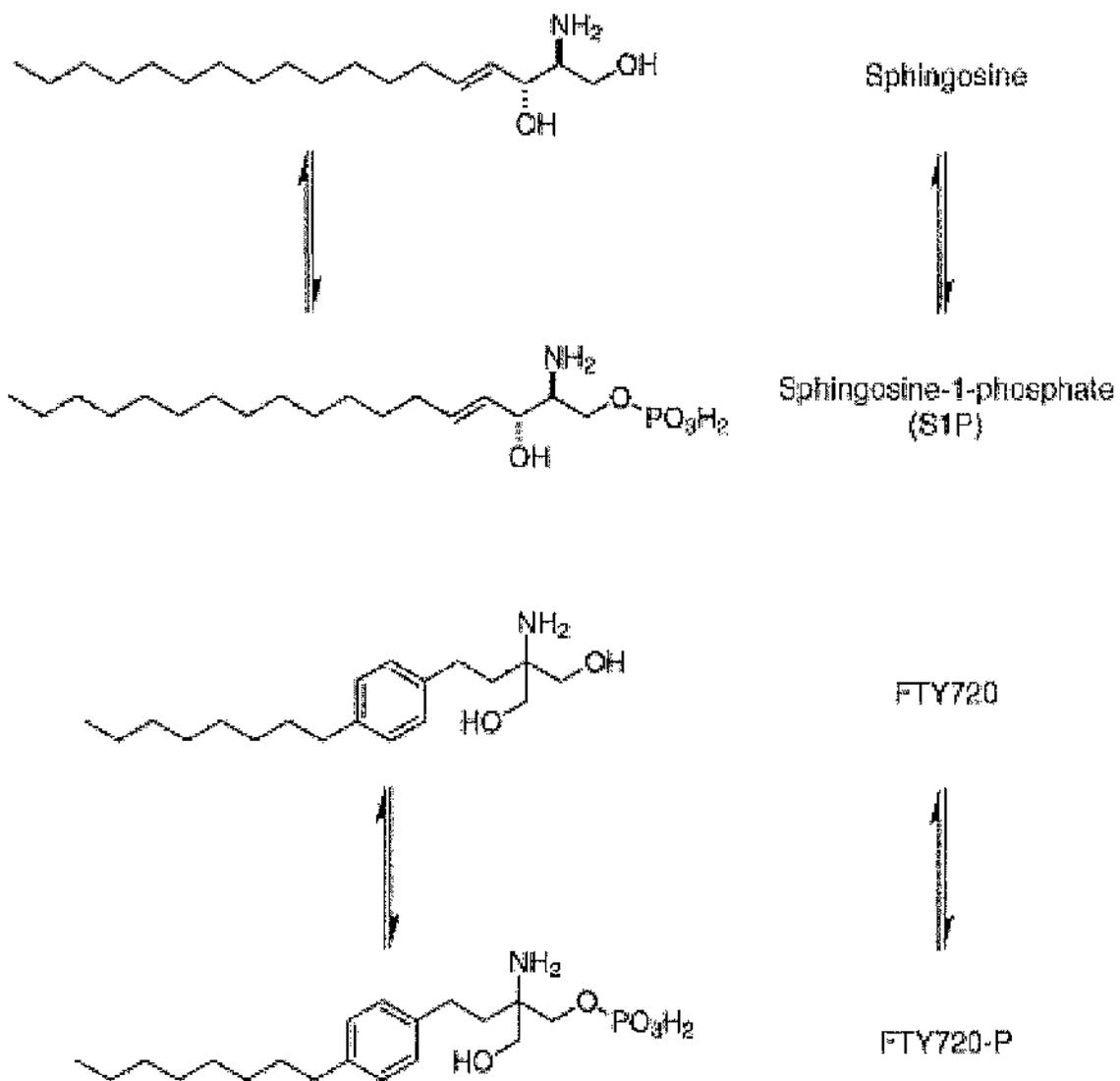


Figure 1.16 The Compound FTY720P is Structurally Similar to S1P

FTY720 is a weak agonist for the S1P receptors but addition of the phosphate ester group to yield FTY720P increases the potency by a 1000 fold. FTY720P acts on S1P₁, S1P₃, S1P₄ and S1P₅ receptors but not S1P₂ (image taken from Rosen and Liao) [250].



1.6 Morphological features of Apoptosis

Apoptosis (or active cell death) can be defined as a form of cell death in which a well ordered sequence of events leads to the elimination of the cell without releasing harmful substances into the surrounding area [251]. In contrast to apoptosis, the other form of cell death, necrosis (or passive cell death) is characterised by progressive loss plasma membrane integrity and influx of Na^+ , Ca^{2+} and H_2O [252, 253]. This causes cytoplasmic swelling and eventually the plasma membrane will rupture releasing the lysosomal and granular contents of the cell into the extracellular space causing an inflammatory response [254]. The word apoptosis is derived from Greek and alludes to the dropping of leaves from a tree [255]. The morphological features of apoptosis include:

- blebbing of plasma membrane,
- chromatin condensation,
- nuclear fragmentation,
- loss of adhesion and cell shrinkage [255, 256]

The biochemical features of apoptosis include:

- internucleosomal cleavage of DNA resulting in an oligonucleosomal ladder [257],
- phosphatidylserine (PS) externalisation [258],
- proteolytic cleavage of intracellular substrates [259].

The dying cell fragments into apoptotic bodies which are recognised by neighbouring cells and consequently phagocytosed. Programmed cell death has a distinct morphology and the mechanism of cell dismantlement is the same regardless of the stimulus that initiated the process [260].

1.6.1 The Bcl-2 Family

The Bcl-2 family of proteins constitutes a critical intracellular checkpoint in the intrinsic cell death pathway. The prototypic member, Bcl-2 was first identified as a proto-oncogene in B cell lymphoma [261]. It was subsequently identified as the mammalian homologue of *ced-9* which has an pro-survival role in *C.elegans* [262]. There are 19 members identified to date, each of which contains at least one of the four conserved domains Bcl-

2 homology domains (BH1-4). The Bcl-2 family can be divided into three categories:

1. Anti-apoptotic proteins; Bcl-2, Bcl-X_L, Bcl-w (BH1-4), Mcl-1, A1, NR-13 and Boo (contain at least BH1 & 2, those most similar to Bcl-2 have BH1-4),
2. Pro-apoptotic proteins; Bax, Bak and Bok (BH1-3, also known as multi-domain proteins),
3. Pro-apoptotic 'BH3-only' proteins; Bid, Bad, Bim, Bik, Blk, Hrk, Bnip3, Puma and Noxa (Figure 1.17) [248, 263, 264].

The caspase cascade is regulated by the Bcl-2 family of proteins. Anti-apoptotic Bcl-2 proteins reside in the mitochondrial outer membrane to protect mitochondrial integrity and prevent cytochrome c release by binding and sequestering pro-apoptotic BH3-only proteins, thus preventing Bax and Bak activation [265].

Bim, Bid and Puma are termed as activators of Bax and Bad and pro-survival Bcl-2 proteins act to divert these molecules' activity, whereas other BH3 proteins like Bad and Noxa do not have the ability to activate Bax directly and are described as sensitizers or depressors [266]. Apoptosis is initiated by BH3-only proteins, which are stringently regulated by transcriptional and/or post-transcriptional controls. In particular relevance to this thesis, the BH3-only protein Bim is believed to have a prominent role in apoptosis caused by removal of trophic factors [267, 268].

1.6.2 The BH3-only protein Bim

The Bim (Bcl-2 interacting mediator of cell death) locus encodes three isoforms: Bim_S (Bim short), Bim_L (Bim long) and Bim_{EL} (Bim extra long) which is the most abundant isoform. Recently, it has been predicted that there may be up to eighteen splice variants [269]. However, these variant gene products have not been detected endogenously [267]. All three major isoforms are potent inducers of apoptosis although Bim_S is the most cytotoxic [270]. Bim is expressed *de novo* following the withdrawal of survival factors in various cell types including haematopoietic cells [271], neurons [272], osteoclasts [273] and fibroblasts [274].

Bim activity can be regulated by phosphorylation of Bim_{EL} by ERK1/2. This antagonises the ability of Bim to induce apoptosis in response to loss of trophic factors by promoting its proteosomal-mediated degradation following poly-ubiquitylation. [274, 275]. The ERK-mediated phosphorylation of Bim_{EL} also protect cells from apoptosis by preventing its interaction with Bax [276].

Another way in which Bim_L and Bim_{EL} activity is regulated is interaction with microtubule complexes by binding to dynein light chain (DLC1) which is a component of the dynein motor complex. Growth factor deprivation and other apoptotic stimuli result in the release of Bim-DLC1 complex and its translocation to mitochondria where it is activated neutralises the anti-apoptotic effects of Bcl-2 proteins [277]. The stress-activated kinase JNK has been shown to phosphorylate Bim_L on Thr56, which is located within the DLC1 binding-motif and can also phosphorylate either Ser44 or Ser58 residues. This disrupts the Bim_L-DLC1 complex which facilitates its release thereby promoting cytochrome c release and activation of downstream cell death machinery [278].

1.6.3 The Caspase Family

The cellular machinery for apoptosis is well conserved in all multicellular organisms [264]. Caspases (cysteiny aspartate-specific proteinases) mediate very specific proteolytic events in apoptotic cells [279]. The role of caspases in cell death was discovered when genetic analysis of *C.elegans* revealed the genes *ced-3*, *-4* and *-9* are crucial in apoptotic processes during development [280]. The discovery that *ced-3* has sequence homology to a mammalian cysteine protease called interleukin1 β -converting enzyme (ICE/caspase-1) [281] led to the search for other mammalian homologues. Caspase-3 was found to have the most similar substrate preferences [282]. To date there are fourteen mammalian caspases known, eleven of which are human. The caspase family at present is divided into ICE-like or CED-like categories (Figure 1.18).

Caspases exist as zymogens and a have similar structural organisation and substrate specificity [279]. They each contain three domains:

- N-terminal pro-domain (N-peptide)
- large subunit containing the active site cysteine within a conserved, QACXG motif (17-20 kDa),
- C-terminal small subunit (9-12 kDa).

Caspases are activated by two cleavage events; the first divides the large and small subunits leaving a second that separates the N-peptide from the large subunit. The active enzyme is a tetramer containing two large and two small subunits [260].

Caspases are grouped according to pro-domain length into initiator caspases or effector caspases. Long pro-domain caspases (such as caspases-2,-8,-9 and -10) are recruited by adaptor proteins into membrane receptor complexes so therefore function to initiate the effector caspases i.e. those with short pro-domains (such as caspases-3,-6 and -7) [283].

1.6.4 Inhibitors of Caspases

Protease activity is crucial throughout the life of the cell but in order to control their destructive abilities this group of enzymes requires careful regulation. As well as being expressed as zymogens caspases have another level of negative regulation placed upon them – namely inhibitors of apoptosis (IAPs) which function to inhibit inappropriate cell death. There are five IAPs identified in mammals: X-linked inhibitor of apoptosis (X-IAP), c-IAP1, c-IAP2, neuronal IAP and survivin [263]. X-IAP displays the most extensive and robust activity, and alongside c-IAP1 and c-IAP2 can directly inhibit caspase activity by binding and inactivating caspases-3,-7 and-9 but not caspases-1, -6, -8 or -10 [284, 285].

The binding of IAPs to caspases is mediated *via* the baculovirus IAP repeats (BIR) which each consist of approximately seventy amino acids [286]. Within each IAP there are three tandem copies of the BIR domain (BIR1-3), one of which (BIR2) is required for inhibition of caspase activity [287]. Furthermore, there is a RING domain within each IAP at the C-terminal which displays E3 ubiquitin ligase activity by binding E2 ubiquitin-conjugating enzyme and so catalysing the recruitment of ubiquitin. Thereby the IAP is functioning to promote both its own proteasomal-mediated degradation and any caspase to which it is bound [288, 289]. c-IAP1 and c-

IAP2 each contain caspase recruitment domains (CARD) [290] which implies they may also function in the regulation of caspase processing and activation.

The activity of caspases may also be attenuated by additional class of inhibitor termed pseudocaspases which evolved by gene duplication. Pseudocaspases lack protease activity and inhibit caspase activation by interaction with caspase activation complexes [283]. For example, FLICE (Fas-associated death domain protein-like interleukin-1 β -converting enzyme) binds to the death effector domain (DED) of FADD thereby inducing apoptosis [291]. c-FLIP (FLICE-like inhibitory protein) is a catalytically inactive caspase-8/-10 homologue which competes with caspase-8 for binding to FADD at the cytoplasmic face of the Fas receptor, thereby attenuating activation of the caspase cascade [292]. The various stimuli that can trigger a cell's apoptotic machinery can be divided into extrinsic (receptor-mediated) or intrinsic (non-receptor-mediated) pathways. Despite the variety of factors able to induce apoptosis, these all converge at the mitochondria and the release of cytochrome *c* [283].

1.6.5 The Extrinsic Pathway of Apoptosis

Extrinsic apoptosis is driven by the activation of death receptors belonging to the TNF receptor family (TNFR) including Fas/CD95, DR3, and DR4/TRAIL. These receptors each contain an intracellular amino acid stretch in the C-terminus called the death domain (DD) [293], which is responsible for activation of the caspase cascade which ultimately ends in the cell undergoing apoptosis. For instance, Fas associates with the adaptor protein Fas-associated death domain protein (FADD) *via* a DD-DD interaction. Fas-bound FADD can then associate with pro-caspase-8 through another functional domain called the death effector domain (DED). The aggregation of two or more pro-caspase-8 molecules enables them to process one another into their active form, thereby triggering the caspase cascade [263, 294].

Activated caspase-8 not only cleaves caspase-3 but can further amplify the apoptotic signal by causing mitochondrial injury *via* the cleavage and release of the pro-apoptotic C-terminal fragment of the BH3-only protein

Bid (tBid). tBid then serves as a membrane-targeted ligand that triggers the oligomerisation of Bax or Bak resulting in cytochrome *c* release [295].

Another set of death receptors (DR4/5) share a different death ligand known as TRAIL (TNF-related Apoptosis Inducing Ligand) [296, 297]. In healthy cells Bim is sequestered by the Bcl-2 protein Mcl-1 but TRAIL-mediated activation of granzyme B (a serine protease) or caspase-3 causes the degradation of Mcl-1. This allows the liberated Bim to activate Bax which leads to cytochrome *c* release thereby further propagating the apoptotic signal [298].

1.6.6 The Intrinsic Pathway of Apoptosis

Intrinsic cell death is mediated by the release of cytochrome *c* from the mitochondria. Stimuli that can provoke apoptosis by this route include cytotoxic drugs, radiation, heat shock and, of relevance to this thesis, the removal of trophic factors [299]. This serves to initiate the caspase cascade in a similar mechanism to death receptors.

DNA damage can initiate apoptosis if not repaired by checkpoint genes [300]. Up-regulation of p53 caused by DNA damage results in the transcription of Bax and its subsequent translocation to the mitochondria [301]. Bax and Bak exist as monomers in viable cells and it is believed upon receipt of a pro-apoptotic signal they form homo-oligomers that insert into the mitochondrial membrane. This causes the formation of pores resulting in the release of cytochrome *c* [295]. DNA damage also results in the up-regulation of the BH3-only proteins Bax, Noxa and Puma driven by the checkpoint kinases CHK1 and 2 activation and p73 up-regulation [302].

Pro-apoptotic BH3-only proteins are localised in the cytosol and can induce apoptosis in two ways: inactivation of anti-apoptotic Bcl-2-like proteins by direct binding or by modification of Bax-like proteins. For example, Bad function is believed to be modulated by phosphorylation. Phosphorylation of Bad in response to survival factor signalling promotes its sequestration by 14-3-3, however de-phosphorylation of Bad leaves it free to act as an antagonist to anti-apoptotic Bcl-2 proteins [209, 303]. Both Bad and Bid are substrates of caspases, and truncation of these proteins results in their

translocation to the mitochondria and the promotion of cytochrome *c* release [295, 304].

A second protein with the ability to activate caspases [Smac (second mitochondria-derived activator of caspases)/DIABLO (direct IAP binding protein with low pI)] was discovered to be released at the same time as cytochrome *c*. Smac/DIABLO eliminates the inhibitory effect of many IAPs including X-IAP, c-IAP-1, c-IAP-2 and survivin [305, 306]. Studies of its crystal structure show Smac/DIABLO to be complexed with the BIR3 domain which primarily targets pro-caspase-9 [287, 307, 308], thus allowing the activation of pro-caspase-9 and the caspase cascade.

1.6.7 Apaf-1 and the Apoptosome

Cytochrome *c* release is an early event in apoptosis and regardless of the initial stimulus all mitochondria release cytochrome *c* within approximately 5 minutes [263]. Cytochrome *c* then binds to Apaf-1 (apoptotic protease activating factor) *via* its WD40 domain. Apaf-1 is the mammalian homologue of the aforementioned *ced-4* [309]. Apaf-1 then promotes the clustering of pro-caspase-9 and in association with ATP causes a conformational change facilitating heptamer assembly in the shape of a wheel called the 'apoptosome' [310, 311]. This is comprised of several Apaf-1 molecules each bound to a molecule of caspase-9 *via* their caspase-recruitment domains (CARD) which causes the proximity-induced activation of caspase-9 [312]. Caspase-9 cleaves and activates both caspase-3 and -7. Caspase-3 then drives the activation of caspase-2 and -6 leading to the activation of caspases-8 and -10 (Figure 1.19) [313].

The executioner caspases (-3, -6, and -7) have a varied range of substrates including structural proteins, signalling proteins and regulators of DNA replication and transcription (Table 1.3). These include Bcl-2 and Bcl-X_L which are cleaved by caspase-3 thereby destroying their protective function by revealing the C-terminal fragments which are pro-apoptotic [260, 314].

As the signal propagates other components of the apoptotic machinery are activated. CAD (caspase-activated deoxyribonuclease) exists in an inactive state bound to its inhibitor iCAD which acts as a specific

chaperone during its synthesis. Caspase-3 action cleaves iCAD causing the dissociation allowing CAD to enter the nucleus and display DNase activity thereby leading to DNA fragmentation [315]. The DNA repair enzyme poly (ADP-ribose) polymerase (PARP), which catalyses the addition of ADP-ribose to nuclear proteins such as histones, is also cleaved by caspase-3 [316, 317].

Caspase activity also functions in the destruction of the nuclear envelope by cleavage of lamins which leads to nuclear shrinkage and contributes to chromatin condensation. Lamins are arranged in head-to tail polymers of intermediate filaments which is cleaved at a single site by caspases [318-320]. Loss of adherence and overall cell shape (blebbing) is caused by the cleavage of proteins that regulate the cytoskeleton such as fodrin [321], gelsolin [322] and FAK [323]. In addition, caspase-mediated cleavage of p21-activated kinase (PAK2) contributes to the formation of apoptotic bodies [324, 325].

1.6.8 The Role of SphK in Cell Survival

Ceramide and sphingosine are known inducers of apoptosis whereas SphK activation and concomitant S1P production is associated with growth and survival [77]. It has been proposed that it is not absolute amounts of ceramide/sphingosine to S1P but the proportion of each that determines cell fate. This is known as the “sphingolipid rheostat” model [78].

Nevertheless, there is increasing body of evidence that suggests that SphK1 and 2 may have opposing roles in determining cell fate [326, 327]. SphK1 stimulates growth and proliferation, promotes G₁-S transition and protects cells from serum deprivation- and ceramide-induced apoptosis [328]. Activation of SphK1 in HUVECs by TNF α and subsequent production of S1P has also been shown to protect cells from TNF α -induced apoptosis [92]. The mechanism for this protection is thought to be *via* the activation of SphK1 by TNF α receptor-associated factors (TRAFs). Within SphK1 there is a TRAF-2-binding motif that mediates this interaction. This is required for the TRAF-2-induced activation of the anti-apoptotic transcription factor NF- κ B but not for JNK [329]. In addition to this, S1P has been implicated in the up-

regulation of another anti-apoptotic transcription factor, activator protein-1 (AP-1) [330].

There are several mechanisms by which intracellular S1P is thought to act to prevent programmed cell death. In a A-375 melanoma cell line the over-expression of the pro-survival protein Bcl-2 markedly increased SphK1 expression and activity and this conferred protection from Fas- and ceramide-mediated apoptosis [94]. In Jurkat cells, S1P has been shown to inactivate the BH3-only molecule Bad *via* the ERK pathway and to also obstruct the translocation of Bax to the mitochondria, thereby preventing the release of cytochrome c. This action could not be attributed to S1P binding its receptors as concentrations of <50nM did not confer cytoprotection [331]. Moreover, the S1P analogue sphingosine-1-phosphonate, which does not bind to S1P receptors, inhibited apoptosis in these cells [332]. In addition, over-expression of SphK1 has been associated with the down-regulation of the pro-apoptotic protein Bim in endothelial cells [333].

In contrast to SphK1, SphK2 has been associated with suppression of proliferation and induction of apoptosis [334]. SphK2 contains a nine amino acid motif that is similar to the BH3 domain found in pro-apoptotic BH3-only proteins [335, 336]. However, mutation of conserved leucine residues in the BH3 domain did not abolish SphK2-induced apoptosis, indicating that there may be other factors involved [335]. In contrast to initial studies, it has been demonstrated in various cells types that SphK2 activity is localised to the nucleus where its activity has been shown to inhibit DNA synthesis. SphK2 localisation to the nucleus appears to be essential for this action although no substrate has been identified [95].

The pro-apoptotic actions of SphK2 appear to be independent of cell surface receptors S1P₁₋₃. Attenuation of S1P₁ signalling by treatment with pertussis toxin was shown to have no effect SphK2-induced apoptosis. Also, the utilisation mouse embryonic fibroblasts (MEFs) lacking S1P₂, S1P₃ or both had no effect on the ability of SphK2 to induce apoptosis [335]. Another study using the neuronal cell line PC12 showed that SphK1 over-expression prevents serum withdrawal-induced apoptosis in a G_i-independent manner. Cell death is averted by the inhibition of the JNK pathway and the executioner caspases 2, 3 and 7 [337]. Since SphK1 activation and

translocation is associated with secretion of S1P it is very difficult to differentiate any intracellular actions from receptor-mediated responses [96].

1.6.9 The Role of S1P Receptors in Cell Survival

Although there are many studies examining a role for S1P signalling intracellularly, no intracellular target has been identified in higher eukaryotes. The intracellular level of S1P in HUVECs is relatively low whereas activated platelets are an abundant source of S1P. It has been proposed that S1P acts as a survival factor for endothelial cells and that platelets assist in the maintenance of endothelial cell integrity by making and releasing S1P which binds and activates cell surface S1P receptors [338]. Extracellular S1P is found mainly in circulating lipoproteins in the order of high density lipoproteins (HDL)>low density lipoproteins (LDL)>very low density lipoproteins (VLDL) [339]. The mild oxidation of LDLs is characteristic of the onset of atherosclerosis [340]. The level of S1P present in LDLs is approximately four times lower than in HDLs [341]. Furthermore, the oxidative modification of LDL (ox-LDL) further reduces the content of S1P and reduces their affinity for each other [342]. Both S1P and HDL protect endothelial cells from serum deprivation and ox-LDL [343, 344]. The binding of S1P to plasma components is believed to regulate its ability to interact with cell surface receptors [339]. Lipoprotein delivery of S1P to its receptors may offer an explanation why higher doses of S1P are required to elicit receptor-mediated responses *in vitro* than occur naturally in the circulation [345].

Indeed the extensively documented protective effect of HDLs in vasculature is now thought to be due to their bioactive lipid element. HDL binds to the scavenger receptor type 1 (SR-B1) on endothelial cells and stimulates NO release *via* activation of endothelial NO synthase (eNOS) [346, 347]. However the component known to bind SR-B1 apolipoprotein A1 (apoA1) does not activate eNOS [347]. A further study revealed that S1P₃ receptor activation was required for NO production and release. This indicates that HDL is acting as a carrier for S1P, bringing it in close enough proximity to bind S1P receptor(s) [348].

NO can promote either survival or apoptosis depending on concentration and cell type [349]. NO is produced by enzymatic oxidation of

L-arginine by nitric oxide synthase (NOS) and subsequently binds to guanylyl cyclase (GC)-coupled receptors which trigger the production of guanosine 3', 5' monophosphate (cGMP) from GTP [350]. cGMP interacts with various downstream targets such as cGMP dependent kinases, phosphodiesterases (PDEs) and ion channels [350]. NO also confers protection against the pro-apoptotic effects of $TNF\alpha$ via cGMP-mediated activation of protein kinase G and the inhibition sphingomyelinase. However, this mechanism of protection operates during the early stages of apoptosis [351]. Another cGMP dependent effect is believed to be inhibition of the release of cytochrome c from the mitochondria via the up-regulation of Bcl-2 proteins and the down-regulation of the adaptor protein p66^{shc} [352].

NO also inhibits apoptosis independently of cGMP by the post-translational modification of caspases. One such modification is S-nitrosylation (addition of NO) to the thiol group of the cysteine residue in the active site which is conserved in all caspases [353-355]. In addition to this, NO is believed to interfere with the assembly of the apoptosome complex by inhibiting interaction between Apaf-1 and pro-caspase-9 via their CARD domains. Consequently this attenuates the sequential activation of caspases-9, -3 and -8 which are required for programmed cell death [356]. Furthermore, endothelial cells in particular, undergo a specific type of apoptosis in response to reactive oxygen species (ROS)-mediated endothelial injury called anoikis (a type of cell death induced by the loss of cell-matrix interactions) [357]. NO acts as an anti-oxidant mopping up ROS generated by ceramide thereby preventing programmed cell death by DNA damage [358].

In endothelial cells NO production has cytoprotective effects [358]. eNOS is constitutively expressed in endothelial cells and is localised in caveolae through association with caveolin-1. There is evidence that S1P₁ is targeted to caveolae upon agonist stimulation [359]. It has also been suggested that S1P₁ may facilitate eNOS activation by allowing its release from caveolin-1 [360]. Stimulation of S1P₁ has also been shown to activate eNOS via the serine/threonine kinase PKB which phosphorylates eNOS on serine 1177 [207, 361]. VEGF has also been identified as an activator of

eNOS through the same pathway *via* the Flk-1 receptor. S1P is believed to augment this action by increasing the tyrosine phosphorylation of Flk-1 [362].

In addition to this, S1P₁ signalling is also thought to enhance Ca²⁺ sensitive NOS activity in endothelial cells. This was demonstrated using anti-sense oligonucleotides against S1P₁ which significantly reduced S1P-induced protection from serum withdrawal. Anti-sense S1P₃ oligonucleotides partially reduced this effect. NO production was suppressed by treatment with pertussis toxin, the PLC inhibitor U73122 and the Ca²⁺ chelator BAPTA-AM. The inhibition of caspase-3 activity was also observed [344].

S1P₂ and S1P₃ have been shown not only to confer protection from serum withdrawal but stimulate cell proliferation by activating ERK in HTC4 hepatoma cells. The transient induction of the AP-1 transcription factors c-Jun and c-Fos leading to that activation of *c-fos* and serum response element was observed [363]. Another study in HEK cells suggests a role for S1P₂ and S1P₃ but not S1P₁ in the activation of NFκB. This action is thought to be mediated by G_q or G₁₃ and requires PKC and Ca²⁺ to activate NFκB [364]. NFκB up-regulates the transcription of many survival factors including, TRAF-1, -2, Bcl-2 and cellular inhibitors of apoptosis proteins (cIAPs) [365]. S1P production also protects against TNFα-induced cell death in hepatocytes by activation of the PI3K/PKB pathway [366]. The S1P₁₋₃ receptors are known to activate this pathway. One of the downstream targets of PKB is the pro-apoptotic protein Bad and the subsequent phosphorylation attenuates Bad activity [209].

Figure 1.17 The Bcl-2 Family

There are three subfamilies within the Bcl-2 family: anti-apoptotic Bcl-2 family member promote cell survival (the members with the most homology to Bcl-2 contain all four BH domains), pro-apoptotic and BH3-only proteins promote cell death. Bid and Bad do not contain a membrane-anchoring domain [263].

Anti-apoptotic
Bcl-2, Bcl-X_L, Bcl-w, Mcl-1



Pro-apoptotic
Bax, Bak, Bok



'BH3-only' pro-apoptotic
Bim, Puma, Bik, Blk, Bnip3



Bid, Bad

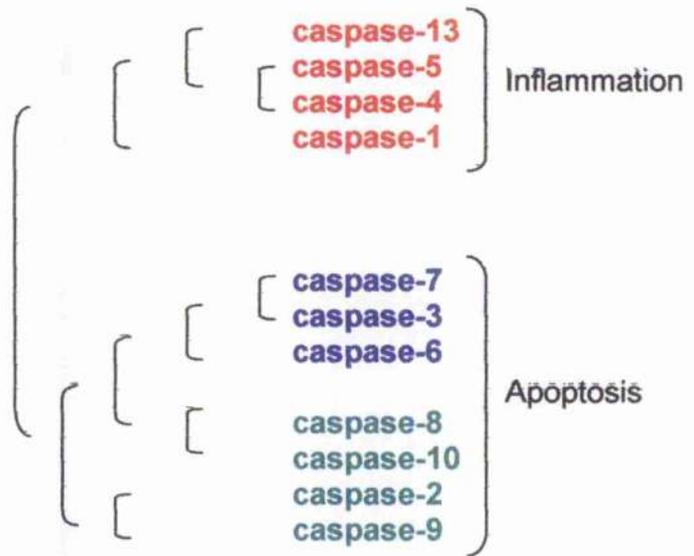


Figure 1.18 The Caspase Family

The caspase family divides into two major phylogenic sub-families (ICE and CED-3). They can be further segregated based on their substrate specificity: inflammatory caspases which regulate cytokine maturation (red), effectors of apoptosis (blue) or initiators of apoptosis (green) (adapted from Zimmermann *et al*) [263].

**ICE
Subfamily**

**CED-3
Subfamily**



■ Mediators of cytokine maturation

■ Effectors of cell death

■ Upstream activators of cell death

Figure 1.19 Apaf and the Apoptosome

Despite the variety of insult on the cell the various pathways of apoptosis converge at the mitochondria where cytochrome *c* release causes the clustering of pro-caspase-9 and Apaf-1. The subsequent activation of caspase-9 and caspase-3 initiates the caspase cascade. Caspase-3 can also activate upstream caspases such as caspase-8 which further amplifies the pro-apoptotic signal. The proteolysis of cell and nuclear components ultimately results in apoptosis.

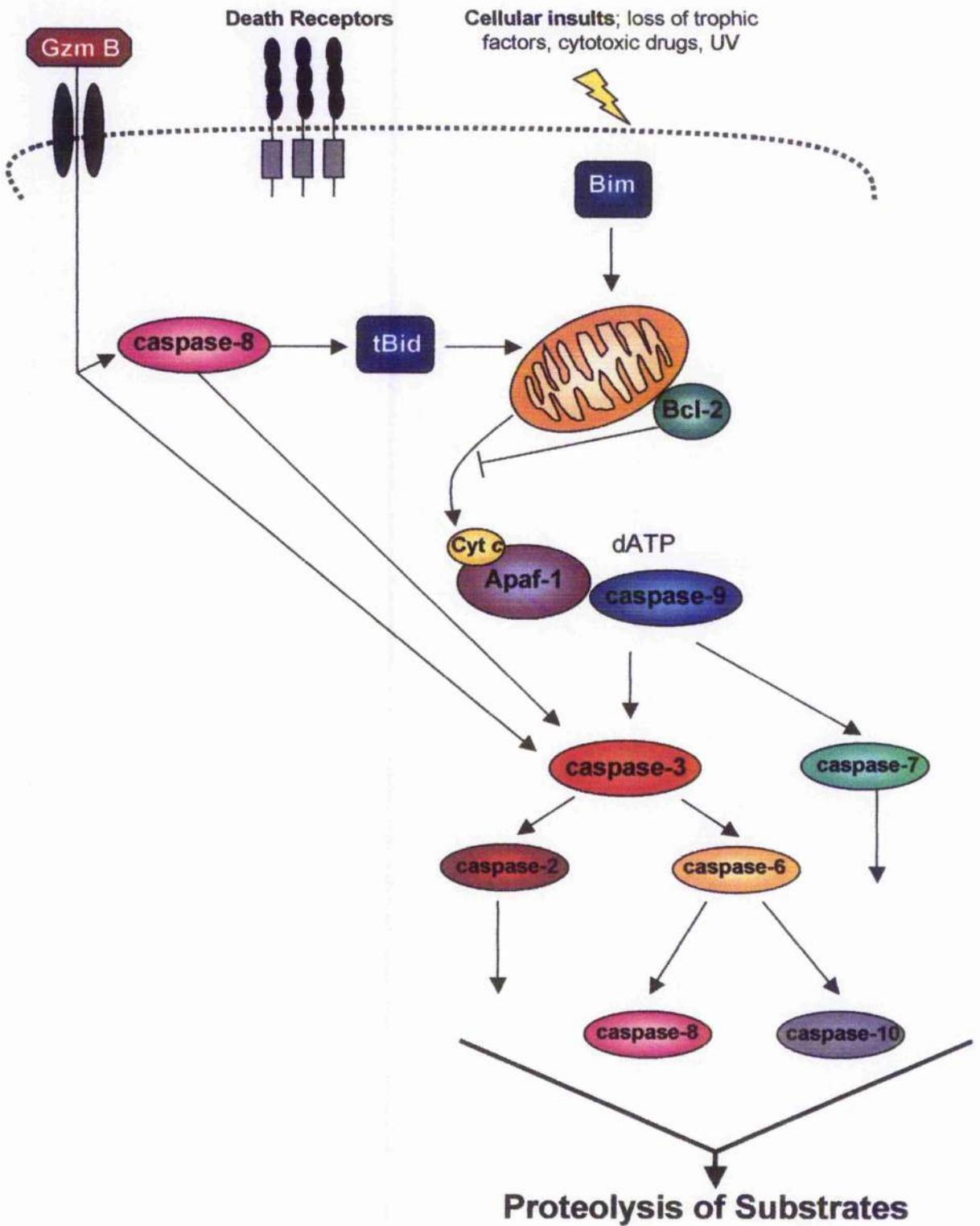


Table 1.3 Caspase Substrates

Substrate Class	Function	Examples
Pro- and anti-apoptotic proteins	Signal amplification Inactivation of apoptotic inhibitors	Pro-caspases Bcl-2 Bcl-X _L Bid
Components of apoptotic machinery	Inducing the apoptotic phenotype	ICAD Gelsolin PAK2 MEKK1 PKC δ
Structural proteins	Dissolution of cell integrity Cellular packaging	Lamins Fodrin Keratins Actin Catenins FAK
Homeostatic proteins	Disruption of macromolecular synthesis and cellular repair mechanisms	DNA-PK _{cs} PARP HnRNPs Transcription factors

Aims

The bioactive lipid sphingosine-1-phosphate is recognised as a mediator of cell processes such as small G protein activation and cell survival through the receptor S1P₁, but the mechanisms involved are not yet fully understood. Therefore the aim of this PhD is to further characterise aspects of S1P₁ receptor regulation and signal transduction with respect to control of cytoskeletal dynamics and cell survival.

Chapter 2
Materials and Methods

2.1 Materials

Abcam Ltd., Cambridge, UK

Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (cat # ab8243, used at 1:20,000)

Affinity Bio Reagents, Colorado, USA

S1P₁ antibody (cat # PA1-1040 used at a 5µg/ml)

Amersham Biosciences, Golden, CO, USA

[³²P] orthophosphate (10mCi/ml), glutathione-Sepharose beads

Biorad, Laboratories Inc., Herts UK

Protein assay dye reagent concentrate

BDH-MERCK, Chemicals Ltd., Poole, UK

Ammonium persulphate, sodium dodecyl sulphate, sodium chloride, vitronectin

Calbiochem-Novabiochem, Nottingham, UK

D-ethyrosphingosine-1-phosphate (S1P), LY294002, U0126, GF109203X, *L-threo-dihydrosphingosine* (DHS), dihydrochloride (emetine)

Cell Signalling Technology, Beverly, MA, USA

Anti-ERK antibody (cat # 9102, used at 1:1000), anti-phospho (Tyr202/Tyr204) ERK antibody (cat # 9106, used at 1:1000), anti-cleaved caspase-3 (Asp175) antibody (cat # 9661, used at 1:500), anti-phospho (Ser473) PKB antibody (cat # 9271, used at 1:1000)

Chemicon International, USA

Caspase-3 Colorimetric Activity Assay Kit, anti-Bim antibody (cat # AB17003, used at 1:1000)

Duchefa Biochemie, Haarlem, Netherlands

Yeast extract, tryptone, microagar

Eurogentec, Belgium

9E10 ascites fluid (used at 1:1000)

Fisher Scientific, Loughborough, Leicestershire, UK

Glycine, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), ethylenediaminetetra-acetic acid (EDTA), dimethyl sulfoxide (DMSO), glacial acetic acid, methanol, ethanol, concentrated hydrochloric acid, sodium hydroxide, hydroxymethyl-aminomethane (TRIS) base, sodium carbonate, sodium hydrogen carbonate, sodium dihydrogen ortho-phosphate, disodium hydrogen ortho-phosphate

GIBCO BRL Life Technologies, Paisley, UK

Phosphate-free Dulbecco's Modified Eagle Medium (PFDMEM), cell culture grade phosphate-buffered saline (PBS), Optimem, Lipofectamine

Inverclyde Biologicals, Strathclyde Business Park, Bellshill, Lanarkshire, UK

Protran nitrocellulose Schleicher and Schuell membrane (0.2 μ m pore size)

Invitrogen Molecular Probes, Inchinnan Business Park, Paisley, UK

Alexa fluor 594-conjugated phalloidin, Alexa Fluor 594-conjugated goat anti-rabbit (cat # A5163, used at 1:200), Alexa Fluor 488-conjugated goat anti-rabbit (cat # A31628, used at 1:200)

Melford Laboratories, Chelworth, Ipswich, Suffolk, UK

Dithiothreitol (DTT), isopropyl- β -D-thiogalactopyranoside (IPTG)

New England Biolabs Inc., Beverly, MA, USA

Rainbow markers (ranging from 6.5-175kD)

Perkin Elmer Life Sciences, Monza, Italy

Enhanced chemiluminescence (ECL) reagents

Pierce, Rockford, IL, USA

EZ-link™ Biotin-LC-hydrazide, western blot stripping solution

Promega, Southampton, UK

G-418 sulphate, Promega™ Wizard Plus SV miniprep kit

Riedel-de Haen, Seezle, Germany

Glycerol, calcium chloride

Santa Cruz, California, USA

Anti-Cdc42 antibody (cat # sc6083, used at 1:500)

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

Horseradish peroxidase (HRP)-conjugated anti-mouse (cat # A4416, used at 1:1000) and rabbit IgG (cat # A8275, used at 1:1000) HRP-conjugated streptavidin, HRP-conjugated anti-sheep antibody (cat # A3415, used at 1:1000), rabbit anti-mouse bridging antibody (cat # S1265, used at 1:1000), anti-Rac antibody (cat # R2650, used at 1:1000), 30% (w/v) acrylamide/0.8% (w/v) bis-acrylamide, Triton X-100, soybean trypsin inhibitor, benzamidine, bovine serum albumin (BSA), protein A-Sepharose, sodium fluoride, sodium periodate, thimerosal, bromophenol blue, Coomassie Brilliant Blue R-250, bichinchonic acid (BCA), ampicillin, N,N,N',N'-tetramethylethylenediamine (TEMED), phenyl methyl sulphonyl fluoride (PMSF), Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum (FBS), cell culture grade trypsin (0.5g/L), penicillin/streptomycin, L-glutamine, fibronectin, colchicine, cytochalasin B, pertussis toxin, sodium potassium tartrate, deoxycholic acid sodium salt, Tween-20, copper (II) sulphate, hydroxypropyl-β-cyclodextrin, paraformaldehyde

Universal Biologicals Ltd., Cambridge, UK

Anti-Cdc42 antibody (cat # ACD02, used at 1:500)

FTY720P (compound 433) was a gift from Dr. Richard Munford, Merck Sharp & Dohme, Rahway, New Jersey, USA

PGEX/PAKCRIB construct was a gift from Dr. Steve Yarwood, IBLS, Glasgow, UK

PRK5mycL61Rac1 construct was donated from Prof. Alan Hall, UCL, London, UK

2.2 Cell Culture

Parental CCL39 (Chinese hamster lung fibroblasts) cells were maintained in DMEM supplemented with 10% (v/v) FBS, streptomycin (100 μ g/ml), penicillin (100units/ml) and 2mM L-glutamine, which will hereafter be referred to as "complete DMEM". CCL39 cells stably expressing the human myc epitope-tagged S1P₁ receptor were also maintained in complete DMEM further supplemented with G-418 sulphate (1.6mg/ml) [367]. Control CCL39 cells which also have antibiotic resistance were maintained in complete DMEM supplemented the same concentration of G418 sulphate. All cells were kept at 37°C, in a humidified atmosphere containing 5% (v/v) CO₂. To split the cells, confluent monolayers were washed with tissue culture grade PBS, 2ml of trypsin was added and the cells were allowed to detach from the flask. 6ml of the appropriate complete DMEM was added to neutralise the trypsin and wash any remaining cells off the flask. This mix was gently pipetted to resuspend cells, which were either seeded into dishes for experiments or passaged into flasks to maintain the cell line.

2.2.1 Transfection of cells with Lipofectamine

The following transfection method is based on cells cultured in a 6-well dish. In a sterile microfuge tube 120 μ l of Optimem and 2 μ g of DNA were gently mixed/well to be transfected. In another microfuge tube 120 μ l of Optimem and 4 μ l of Lipofectamine transfection reagent was added and mixed gently. To this, the DNA/Optimem mix was added and gently mixed. This was left to incubate at room temperature in the dark for 45 minutes. The cell monolayers were washed briefly with 2ml of Optimem which was replaced by 760 μ l of Optimem. The DNA-lipofectamine mix was then added dropwise to each well and placed into the incubator for 3 hours. The transfection mix was removed and the medium replaced with complete DMEM.

2.3 Molecular Biology Techniques

2.3.1 Preparation of Antibiotic Agar Plates

LB agar (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride and 1.5% (w/v) agar) was prepared, autoclaved and left to cool to approximately 37°C before addition of ampicillin at a final concentration of 50µg/ml. The liquid LB agar was poured into 10cm Petri dishes, allowed to solidify and then left overnight at room temperature to allow excess moisture to evaporate. The plates were stored at 4°C for a maximum of 4 weeks.

2.3.2 Preparation of Plasmid DNA

10ml of LB broth (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride) supplemented with ampicillin at a concentration of 50µg/ml was inoculated with a stab from a glycerol stock. This was placed in the shaking incubator overnight at 37°C. Plasmid DNA was then isolated by using the Promega™ Wizard Plus SV miniprep purification kit as per the manufacturer's instructions.

2.3.3 Preparation of Competent BL21 *E.coli* cells

An overnight culture of BL21 *E.coli* (protease-deficient strain) was grown in 3ml of LB broth containing 50µg/ml ampicillin. This culture was used to inoculate 250ml of LB broth which was grown at 37°C in a shaking incubator at 200 r.p.m until the OD₆₀₀ was 0.35-0.375. The bacteria were transferred to two pre-chilled centrifuge tubes and left for 1 hour on ice. The cells were sedimented by centrifugation for 20 minutes at 3500 g, and resuspended in 62.5ml of ice-cold 15% (v/v) glycerol with 0.1M calcium chloride. 250µl of bacteria were aliquoted into sterile microfuge tubes in a dry/methanol bath to induce rapid freezing, and stored at -80°C.

2.3.4 Transformation of Competent BL21 *E.coli* cells

Approximately 30-50ng of plasmid DNA was added to a pre-chilled microfuge tube on ice. An aliquot of competent bacteria was defrosted on

ice, and 80µl added to each tube of plasmid DNA. This was incubated for 30 minutes. The tubes were placed at 42°C for 2 minutes then placed back on ice and 0.5ml of LB broth was added. The tubes were then incubated at 37°C for 30 minutes to allow recovery. 0.1ml of the transformation mix was plated out onto agar plates containing 50µg/ml ampicillin and incubated overnight at 37°C.

2.3.5 GST Fusion Protein Preparation

10ml of LBamp broth (LB broth supplemented with ampicillin at a concentration of 50µg/ml) was inoculated from a glycerol stock of BL21 *E.coli* transformed with pGEX/PAKCRIB, which was then grown overnight, shaking at 37°C. This starter culture was then used to inoculate 400ml LBamp, which was grown shaking, with aeration at 200 r.p.m for 3 hours (or until the OD₆₀₀≥0.3) at 37°C. The cells were induced by addition of IPTG to at a final concentration of 10mM, then grown shaking at 200 rpm for 3 hours at 37°C.

The bacteria were harvested by centrifugation at 6700 g for 15 minutes, the supernatant was discarded, the cells resuspended in 19.5ml PBS and transferred to a fresh tube. 0.5ml of 20% (v/v) Triton X-100 was added to the resuspended cells to give a final concentration of 0.5% (v/v) and the tube was inverted 6 times to mix. The sample was probe sonicated for 1 minute on ice to ensure cell lysis and then centrifuged at 27000 g for 30 minutes to pellet any insoluble material. The resulting cleared lysate was then added to 0.6ml of 50% (v/v) glutathione-Sepharose bead suspension and placed on a rotating wheel at 4°C overnight to allow the fusion protein to bind. The following day the beads were washed three times with 10ml of PBS. After the final wash the beads were resuspended in 50% (v/v) glycerol in PBS supplemented with protease inhibitors (0.1mM PMSF, 10µg/µl soybean trypsin inhibitor and 10µg/µl benzamidine) and stored at -20°C.

GST fusion protein concentration was determined by SDS PAGE using a 10% (w/v) polyacrylamide resolving gel (as described in section 2.4.3). The GST fusion protein was eluted from the glutathione-Sepharose beads with electrophoresis sample buffer (50mM TRIS (pH 6.8), 10% (v/v) glycerol, 12% (w/v) SDS, 0.0001% (w/v) bromophenol blue, 6µM DTT) and

run on the gel in parallel with known BSA standards ranging from 0.2-4 $\mu\text{g}/\mu\text{l}$ in amount. The gel was stained with Coomassie Brilliant Blue R-250 (0.25% (w/v) Coomassie Brilliant Blue R-250, 45% (v/v) methanol, 10% (v/v) acetic acid) for 1 hour shaking. The gel was rinsed in de-stain (50% (v/v) methanol, 5% (v/v) acetic acid) and left overnight in de-stain. The gel was dried down under vacuum and the concentration of fusion protein was determined using the software TotalLab 2003.02 to detect the optical density of the bands. These values were then used to construct a straight line to ascertain the amount of fusion protein purified.

2.4 Laboratory Techniques

2.4.1 BCA Assay to Determine Protein Concentration

10 μl aliquots of BSA standards of known concentration ranging from 0-2mg/ml and 10 μl of unknown cell lysates were added in duplicate to a 96-well plate. 0.2ml of BCA solution (1% (w/v) 4,4 dicarboxy-2,2 biquinolin 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 pH 11.25, 0.08% (w/v) copper (II) sulphate) was added to each well and left to incubate for 10 minutes at 37°C. Reduction of Cu^{2+} to Cu^{+} in a protein-specific manner allows the BCA to bind the Cu^{+} ion forming an intense purple colour, which is then measured at A_{492} using a plate reader. The absorbance values of the standards were used to construct a straight line from which the unknown protein concentrations were determined.

2.4.2 Bradford's Assay to Determine Protein Concentration

10 μl aliquots of BSA standards of known concentration, ranging from 0-2mg/ml were added in duplicate to a 96-well plate. 2 μl of unknown cell lysates and 8 μl of cell lysis buffer were also added in duplicate to the same 96-well plate. The Bradford's reagent was diluted 1:5 and 50 μl added to each well. The plate was left to incubate for 5 minutes at room temperature and then read on a plate reader at A_{630} . The absorbance values of the standards were used to construct a straight line from which the unknown protein concentrations were determined.

2.4.3 SDS-PAGE and Immunoblotting

Samples to be prepared for SDS-PAGE were normalised for protein concentration using the BCA assay described in section 2.4.1. Each sample, equalised for protein concentration and volume was prepared in electrophoresis sample buffer (50mM TRIS (pH 6.8), 10% (v/v) glycerol, 12% (w/v) SDS, 0.0001% (w/v) bromophenol blue, 6 μ M DTT) and subjected to discontinuous SDS-PAGE alongside Rainbow Markers also prepared in electrophoresis buffer using a 10% or 12% polyacrylamide resolving gel (10%/12% (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 a 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 performed using Biorad Mini-Protean II/III gel electrophoresis systems with running buffer (24.7mM TRIS, 0.19M glycine and 0.1% (w/v) SDS) at 150 V (constant voltage) until the bromophenol blue dye front was at the bottom of the gel. The proteins were transferred electrophoretically to a nitrocellulose membrane at 400mA for 45 minutes in transfer buffer (24.7mM TRIS, 0.19M glycine and 20% (v/v) methanol). The membranes were rinsed briefly with PBS and non-specific protein binding sites were blocked for 1 hour at room temperature with Blotto [5% (w/v) skimmed milk containing either 0.1% (v/v) Triton X-100 or 0.1% (v/v) Tween-20 in PBS or TRIS-buffered saline (TBS)]. After blocking, the membranes were washed briefly in PBS or TBS. The primary antibody was diluted in Blotto or BSA (50mg/ml BSA in TBS) for phospho-specific antibodies. The membranes were incubated on a rotating platform overnight at 4°C, or at room temperature for 1 hour.

Following primary antibody incubation, the membranes were washed three times with Blotto or TBS supplemented with 0.1% (v/v) Tween-20 for phospho-specific antibodies. The membranes were incubated with a 1:1000 dilution of the appropriate secondary antibody conjugated to HRP on a rotating platform for 1 hour at room temperature. Following secondary antibody incubation, the membranes were washed three times with Blotto and a further two times with PBS. Blots probed with a phospho-specific

antibody were washed five times with TBS supplemented with 0.1% (v/v) Tween-20.

Membranes were exposed to enhanced chemiluminescence (ECL) reagents where HRP-specific oxidative degeneration of luminol causes the emission of light, which is detected by X-ray film to visualise immunoreactive proteins. The optical density of the bands were measured using the software TotalLab 2003.02 (Phoretix, UK).

2.4.4 Preparation of Stock S1P

0.66ml of methanol was used to resuspend 1mg of S1P giving a final concentration of 4mM. The vial was closed tightly, sealed with Nescofilm and transferred to an 80°C oven. The vial was vortexed every 10 minutes until the S1P had dissolved, it was then pipetted in 25 µl aliquots into glass vials. The methanol was evaporated off using a stream of nitrogen gas. The tubes were then capped and stored at -80°C.

S1P was reconstituted for use in serum-free DMEM supplemented with 0.5mg/ml fatty acid-free BSA. 0.25ml of vehicle was added to each vial and incubated at 37°C with repeated vortexing for 30 minutes. This gave a working S1P stock solution of 400µM.

2.4.5 Preparation of Stock FTY720P

1mg of FTY720P (compound 433) was dissolved in 50µl of 40% (w/v) hydroxypropyl-β-cyclodextrin, 5.16µl of 0.5M Na₂CO₃ and 944.84µl of distilled water in an ultrasonic water bath at 60°C for 30 minutes, giving a final concentration of 2.6mM. The solution was pipetted in 10µl aliquots into glass vials, capped and stored at -80°C. To reconstitute the FTY720P for use, 2.6ml of serum-free DMEM supplemented with 0.5mg/ml fatty acid free BSA was added to each vial. This gave a working stock solution of 10µM.

2.4.6 Cell Surface Labelling of S1P₁ with Biotin-LC-hydrazide

Confluent 75cm² flasks of CCL39mycS1P₁ cells were passaged at 1:8 and seeded into 6-well dishes and cultured overnight in DMEM. The next day the confluent cells were serum-starved for 16-20 hours in 3 ml of serum-

free DMEM. The cells were treated with drugs as described in the Figure legend and receptor internalisation was initiated by addition of 4 μ M S1P for 2 hours. The experiment was stopped by placing the cells on ice and all the following steps were carried out on ice unless otherwise stated.

Cell monolayers were washed three times with 2ml of ice cold PBS-CM (PBS supplemented with 1mM MgCl₂ and 0.1mM CaCl₂). Residual PBS-CM was removed and 1ml of 10mM sodium periodate in PBS-CM was added to each well to facilitate the oxidation of hydroxyl groups to aldehydes. The wells were covered and left to incubate for 30 minutes. After removal of sodium periodate, the monolayers were washed once with 2ml PBS-CM and then twice with 2ml of 0.1M sodium acetate-CM (0.1M sodium acetate, pH 5.5, supplemented with 1mM MgCl₂ and 0.1mM CaCl₂). 0.1mM of biotin-LC-hydrazide in sodium acetate-CM was added to each well, which were then covered and left to incubate for 30 minutes. The biotin was removed and the monolayers were washed with 2ml of PBS three times. After removal of residual PBS the cells were harvested in 0.25ml of RIPA buffer (50mM HEPES, pH 7.5, 150mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS with 10mM NaF, 5mM EDTA, 10mM Na₂PO₄, 0.1mM PMSF, 10 μ g/ml soybean trypsin inhibitor and 10 μ g/ml benzamidine) and each well was rinsed with a further 0.25ml of RIPA buffer, which was transferred to the appropriate tube. The samples were then solubilised on a rotating wheel at 4°C for 1 hour. Any insoluble material was pelleted by centrifugation at 20000 g for 15 minutes and 0.4ml of the supernatants were transferred to fresh microfuge tubes.

The concentrations of protein for each sample was determined by performing a BCA assay (as described in section 2.4.1). 0.4ml of sample normalised for protein concentration was added to a chilled microfuge tube containing 20 μ l of a 1:1 suspension of protein A-Sepharose beads, 0.2% (w/v) IgG-free BSA, 2 μ l of rabbit anti-mouse bridging antibody and 5 μ l of 9E10 ascites fluid. These were incubated on a rotating wheel at 4°C overnight. The immune complexes were isolated by brief centrifugation, washed with 1ml of RIPA buffer three times, then eluted from the beads by addition of 50 μ l electrophoresis sample buffer and incubation at 37°C for 1

hour with brief vortexing every 15 minutes. Following elution, the samples were pelleted and the eluates removed with a Hamilton syringe. 25µl of each sample was fractionated by SDS-PAGE, transferred to nitrocellulose and blocked with Blotto supplemented with 0.1% (v/v) Triton X-100 (as described in section 2.4.3).

Labelled cell surface receptors were identified by incubation of the membrane with 1µg/ml of HRP-conjugated streptavidin, at room temperature for one hour and visualisation on autoradiography film by ECL.

2.4.7 Whole Cell Phosphorylation Assay

Confluent 75cm² flasks of CCL39mycS1P₁ cells were passaged at 1:8 and seeded into 6-well dishes and cultured overnight in DMEM supplemented with G418 sulphate. The following day the cells were serum-starved for 16-20 hours in serum-free DMEM, then washed twice with phosphate-free DMEM and incubated with 0.75ml of the same media supplemented with 50µCi/well [³²P]-orthophosphate for 90 minutes at 37°C.

The cells were treated with drugs as indicated in Figure legends and then exposed to S1P at a concentration of 5µM for 5 minutes. The reaction was terminated on ice and the monolayers were washed twice with ice-cold PBS. All subsequent steps were carried out on ice unless indicated otherwise. The cells were harvested in 0.25ml of RIPA buffer and transferred to a microfuge tube. Each well was then washed with a further 0.25ml of RIPA buffer, which was transferred to the appropriate tube. The cells were solubilised by rotation on a wheel for 1 hour at 4°C and analysed for protein content using the BCA assay (as described in section 2.4.1). Equivalent amounts of soluble protein from each sample were made up to 0.4ml in RIPA buffer and added to microfuge tubes containing 100µl of 0.2% (w/v) IgG-free BSA, 2µl goat anti-mouse IgG bridging antibody and 5µl of ascites fluid generated against the myc-tagged receptor (9E10). The samples were incubated for 1 hour on a rotating wheel at 4°C. The immune complexes were then isolated by brief centrifugation and washed three times with 1ml of RIPA buffer. Following washing, the samples were eluted from the beads by addition of 30µl of electrophoresis buffer and incubation at 37°C for 1 hour,

with vortexing every 15 minutes. The eluates were then analysed by SDS-PAGE using a 10% (w/v) polyacrylamide gel (as described in section 2.4.3), dried down under vacuum and phospho-proteins were visualised by autoradiography for between 16 and 20 hours at -80°C.

2.4.8 GST Pull Down Assay to Detect Small G protein Activation

Confluent 75cm² flasks of CCL39 cells or CCL39mycS1P₁ cells were passaged 1:8 and seeded into either 6-well or 10cm dishes on plain tissue culture plastic, or tissue culture plastic coated with fibronectin or vitronectin (5µg/ml). The cells were cultured overnight in complete DMEM or complete DMEM supplemented with G418, respectively. The following day the monolayers were washed and incubated in serum-free media for 4 hours.

S1P was then added to monolayers at a concentration of 5µM over a time course as indicated in the Figure legend. The time course was terminated by placing the cells on ice. Each subsequent step was carried out on ice unless otherwise stated. The cells were washed twice with ice cold PBS and harvested in 0.25ml of lysis buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 1% (v/v) Triton X-100, supplemented with 10mM MgCl₂, 10mM NaF, 5mM EDTA, 10mM Na₂PO₄, 0.1mM PMSF, 10µg/ml soybean trypsin inhibitor and 10µg/ml benzamidine). Each well was washed with a further 0.25ml of lysis buffer, transferred to the appropriate tube and vortexed briefly. The insoluble material was then pelleted by centrifugation of the samples at 20000 g for 15 minutes. 0.4ml of the cleared lysate was added to microfuge tubes containing 15µl of GST-PAKCRIB glutathione sepharose beads (concentration ranging between 70-90µg/µl) and 100µl of 0.2% (w/v) IgG-free BSA. The remaining lysate was kept for a BCA assay (as described in section 2.4.1) and analysis by immunoblotting (as described in section 2.4.3).

The samples were placed on a rotating wheel at 4°C for 1 hour. The GST complexes were isolated by brief centrifugation and the supernatant discarded. The beads were then washed three times with 1ml of lysis buffer. GTP-bound small G proteins were eluted from the beads by addition of 30µl of electrophoresis buffer and incubation at 65°C for 20 minutes, with brief vortexing every 5 minutes. A BCA assay was used to calculate the volume to

be added to each lane to ensure equal concentration of input protein. The eluate and lysates were analysed by SDS PAGE using 12% (w/v) polyacrylamide resolving gels, transferred to nitrocellulose membranes and blocked with Blotto supplemented with 0.1% (v/v) Tween-20 (as described in section 2.4.1 and 2.4.3). GTP-bound small G proteins were detected by immunoblotting with the appropriate primary antibody as described in the Figure legend [368].

2.4.9 Assay of Caspase-3 Activity

Confluent 75cm² flasks of CCL39 cells and CCL39mycS1P₁ cells were subjected to serum deprivation for 16 hours and control flasks were cultured in complete media concurrently. The media from each flask was collected and transferred into appropriately labelled tube. The monolayers were washed with 5ml of PBS and this was transferred into respective tubes. 2ml of trypsin was added to each flask and left to incubate at room temperature till the cells had detached. 5ml of either complete DMEM or serum-free media was added respective flasks to neutralise the trypsin and wash any remaining cells from the flasks and this was transferred to respective tubes.

The tubes were centrifuged for 10 minutes at 4000 g to pellet the cells and the supernatants were discarded. The pellets were resuspended in 250µl of 1X Cell Lysis Buffer and transferred to fresh tubes. The tubes were placed on ice for 10 minutes and centrifuged for 5 minutes at 10000 g. The supernatants were transferred to fresh tubes, put on ice and the protein concentrations were determined by a Bradford assay (as described in section 2.4.2) to ensure equal concentrations of protein were used in the activity assay. The assay mixtures were prepared in a 96-well plate (as shown in Table 2.1).

The caspase-3 inhibitor (Ac-DEVD-CHO) was incubated with the appropriate samples for 10 minutes at room temperature at a final concentration of 0.1µM prior to addition of substrate (Ac-DEVD-pNA). After substrate addition the 96-well plate was covered in aluminium foil and incubated at 37°C for 2 hours. A standard curve was prepared ranging from 10µM to 1mM with the pNA standard provided with the kit. The plate was

read at A_{405} on a plate reader. The background readings from the buffers were subtracted from the sample readings. Fold increase in caspase-3 activity was determined by comparison of the optical density of the control cells with the cells subjected to serum deprivation.

Table 2.1 Caspase-3 Activity Reaction Mixtures

Sample	Assay Mixture				Substrate	Total Volume
	5X Assay Buffer	Caspase-3 Sample	Inhibitor	dH ₂ O		
Buffer Blank	20 μ l	-	-	80 μ l	-	100 μ l
Substrate Blank	20 μ l	-	-	70 μ l	10 μ l	100 μ l
Test sample	20 μ l	50 μ l	-	20 μ l	10 μ l	100 μ l
Test sample + Inhibitor	20 μ l	50 μ l	1 μ l	19 μ l	10 μ l	100 μ l

2.4.10 Detection of S1P₁ by Immunofluorescence

CCL39 cells stably transfected with mycS1P₁ from 1:10 split were seeded out onto sterile glass coverslips in a 6-well dish and left to grow overnight. The next day the media was discarded and each monolayer washed three times in 1ml of PBS. To each well 1.5ml 4% (w/v) paraformaldehyde in a 5% (w/v) sucrose/PBS solution was added to fix the cells. This was allowed to incubate for 10 minutes at room temperature. The paraformaldehyde was discarded and the cells washed three times in 1ml of PBS. 1.5ml of blocking solution (5% (w/v) skimmed milk containing 0.1% (v/v) Triton X-100) was added to each well and left to incubate for ten minutes at room temperature. The blocking solution was discarded and coverslips were then washed in 1ml of PBS. 0.2ml of primary antibody

(5 μ g/ml) diluted in blocking buffer was dropped onto a strip of nescofilm and each coverslip was then placed inverted onto the drop of liquid. This was allowed to incubate for one hour at room temperature. The coverslips were returned to the 6-well dish and washed three times in 1ml of PBS. 0.2ml of the secondary antibody (Alexa 594- or 488- conjugated) diluted in blocking buffer was dropped onto a strip of nescofilm and each coverslip was placed inverted onto the drop of liquid. The coverslips were covered and left to incubate for one hour at room temperature. The coverslips were returned to the 6-well dishes washed three times in 1ml of PBS then allowed to dry. The coverslips were then mounted in 15 μ l of 40% (v/v) glycerol/PBS solution and stored at 4°C.

2.4.11 Detection of Stress Fibres with Alexa Fluor 594-conjugated Phalloidin

200 μ l of either CCL39 cells or CCL39 cells stably transfected with mycS1P₁ from 1:10 split were seeded out onto sterile glass coverslips in a 6-well dish and left to grow overnight. The next day the cells were serum-starved for 4 hours before stimulation with S1P (5 μ M) over a time course as indicated in the Figure legend.

The following steps were carried out at room temperature. The media was discarded and the coverslips were washed twice with 1ml of PBS. To each coverslip 1ml of fixation buffer was added (4% (w/v) formaldehyde in a 5% (w/v) sucrose/PBS solution) and left to incubate at room temperature for 15 minutes. The monolayers were washed a further two times with 1ml of PBS before addition of 1ml of 0.1% (v/v) Triton X-100 for 2 minutes to permeabilise cells. The coverslips were washed twice with 1ml of PBS before blocking with a 5% (w/v) BSA/PBS solution at room temperature for 30 minutes.

The cells were stained with phalloidin conjugated to Alexa Fluor 594 diluted 1:20 in a 5% (w/v) BSA/PBS solution. The coverslips were left to incubate for 30 minutes, then washed twice in 1ml of PBS prior to mounting in 40% (v/v) glycerol/PBS solution. Images were captured using a Zeiss fluorescence microscope and manipulated with Axiovision software.

2.4.11 Statistical Analysis

All statistical analyses were carried out using a one way ANOVA with a Bonferroni comparisons post test as described in the GraphPad software Instat 3.

Chapter 3

S1P₁ and Small G protein Activation Status

3.1 Introduction

S1P is a bioactive lipid derived from the ubiquitous component of eukaryotic membrane sphingomyelin. It mediates its effects through family of GPCRs called S1P₁₋₅. S1P₁ was the first S1P receptor to be identified and is linked to multiple signalling pathways *via* the heterotrimeric G protein G_i. S1P₁ mediates many growth-related cell processes including cytoskeletal dynamics which are important in cell migration and angiogenesis. S1P has been demonstrated to induce angiogenesis *via* PKB-mediated phosphorylation of eNOS and subsequent NO production [226]. In addition to angiogenesis, S1P has an essential role in embryonic development. S1P₁-null mice die *in utero* at E12.5-E13.5 due to massive haemorrhaging and inability to recruit stabilising pericytes to newly formed blood vessels [230].

Like all GPCRs, S1P₁ is subject to regulation by phosphorylation and internalisation. S1P₁ is regulated in part by agonist-dependent GRK-2 mediated phosphorylation but is also phosphorylated in an agonist-independent manner by PKC. Phosphorylation by either of these kinases is associated with the loss of receptor from the cell surface [367]. The most studied form of internalisation is clathrin-mediated, where receptors are clustered in clathrin rich areas which are pinched off from the plasma membrane to form vesicles. This is mediated by small G proteins like Rab and Arf [150], however, the small G proteins generally associated with regulation of cytoskeletal processes, the Rho GTPases have been demonstrated to contribute to the efficiency of internalisation. For example, expression of the dominant negative form of Rac and RhoA were shown to attenuate clathrin-mediated internalisation of the transferrin receptor [185].

The protein kinase PKB has also been demonstrated to phosphorylate the S1P₁ receptor on the residue Thr236 which is situated in its third intracellular loop [126]. S1P₁ activates the PI 3-kinase pathway which results in the translocation and association of PKB to the plasma membrane *via* its PH domain. PKB and phosphoinositides have been shown to accumulate at the leading edge of migrating fibroblast cells stimulated with PDGF [369]. It has also been demonstrated that transactivation of the S1P₁ receptor by PKB is required for S1P-mediated migration of endothelial cells [126].

Most cells respond to surfaces coated in ECM proteins by adhering to them and spreading out. This process is mediated by integrins and involves changes in cytoskeletal dynamics which are controlled by small G proteins like Rho, Rac and Cdc42. S1P₁ expression and signalling has also been demonstrated to activate Rac in CHO cells [216] which is concentrated at the leading edge of migrating cells. By regulation of microfilament dynamics Rac mediates the formation of membrane ruffles termed lamellipodia. Cdc42 regulates the formation of filopodia which are long, thin, actin-dependent protrusions of the plasma membrane [201] and is believed to control cell direction [166].

Rho-like GTPase signal transduction is tightly regulated by several classes of protein. Briefly, GEFs which activate small G proteins by catalysing the exchange of GDP for GTP, GAPs which inactivate small G protein by stimulating intrinsic GTPase activity favouring the GDP-bound state and GDIs which sequester small G proteins which also favours inactivation. There is evidence that specific GEF activity may in part determine the downstream effects elicited. For example, S1P treatment of endothelial cells results in the translocation of Rac and its GEF Tiam-1 to the plasma membrane [227]. In addition to this, expression of dominant negative Tiam-1 was also demonstrated to attenuate S1P-mediated Rac1 activation [124]. Once activated, GTP-bound Rac and Cdc42 stimulate various downstream targets, of which the first to be identified was p65PAK [370]. p65PAK targets downstream effectors such as LIMK and cofilin which ultimately results in actin polymerisation [162].

As mentioned previously, integrin interaction with ECM proteins such as fibronectin and vitronectin is important in the regulation of small G proteins. $\alpha_v\beta_1$ or β_3 integrins that clustered to focal contacts were shown to be required for S1P-mediated migration in endothelial cells. This action was also blocked by C3 toxin but unexpectedly cell spreading and migration required the presence of both S1P₁ and S1P₃ receptors [215].

Although a mechanism for Rac activation by S1P₁ has been described by Lee *et al* it is not known if this is a universal mechanism in all cell types. Chinese hamster lung fibroblast (CCL39) cells stably transfected with S1P₁ provide a tractable model system to investigate whether disruption of the

cytoskeleton has any effect on receptor regulation and what effect S1P has on Rac and Cdc42 activation in this system.

3.2 Results

S1P₁ has been demonstrated to control the activation status of cytoskeletal regulatory proteins such as Rac and Cdc42 thereby having a role in migration and angiogenesis [126, 230]. The Chinese hamster lung fibroblast cell line (CCL39) stably expressing S1P₁ (CCL39mycS1P₁) [367] was used as a model to determine whether disruption of cytoskeletal processes would have an effect on receptor regulation by phosphorylation and internalisation. Myc-tagged receptor expression was confirmed by immunofluorescence microscopy where the myc epitope and the S1P₁ antibody were shown to localise (Figure 3.1). As there is no suitable radioligand available the number of receptors expressed on the cell surface was not assessed.

Whole cell phosphorylation assays were used to detect any change in the level of agonist-induced receptor phosphorylation in response to various inhibitors of cytoskeletal dynamics. An inhibitor of microtubule dynamics, colchicine had no effect on the level of receptor phosphorylation (Figure 3.2). Another inhibitor, cytochalasin B which prevents the polymerisation of microfilaments also had no effect on agonist-induced phosphorylation (Figure 3.3). Furthermore, pertussis toxin which inactivates G_i had no effect on agonist-induced receptor phosphorylation (Figure 3.4). To examine whether receptor internalisation was affected by these same inhibitors cell surface receptors were labelled with biotin-LC-hydrazide. Again, none of these inhibitors had any significant effect on receptor internalisation (Figure 3.5). Thus, it appears that disruption of the cytoskeleton with inhibitors has no effect on receptor regulation by phosphorylation or internalisation.

The level of Rac expressed in CCL39mycS1P₁ cells is significantly lower than in control CCL39 cells but removal of serum rescues partially this effect (Figure 3.6a and Figure 3.6b). To assess how this would affect the ability of S1P₁ to activate Rac a pull down assay was employed using the fusion protein GST-PAKCRIB which detects GTP-bound Rac (Figure 3.9b) compared to total levels of Rac by western blot. In control CCL39 cells no S1P-induced Rac activation was detected over a time period of 15 minutes (Figure 3.7). As the GST fusion protein PAKCRIB also binds to GTP-bound Cdc42 the levels of active Cdc42 were also determined by western blot and

like Rac, there was no detectable activation of Cdc42 in control CCL39 cells (Figure 3.8). To determine whether expression of S1P₁ would result S1P-induced Rac and Cdc42 activation the same experiments were repeated in CCL39mycS1P₁ cells. However in contrast to the expected result there was no significant increase in Rac activation above the basal levels of GTP-bound Rac detectable, which is not reflected in the total levels of Rac (Figure 3.9a). This effect was not restricted to Rac alone but was observed in Cdc42 activation levels also (Figure 3.10).

As integrin engagement is a known inducer of small G protein activation [200] the level of GTP-bound Rac and Cdc42 was assessed in CCL39mycS1P₁ cells that had been cultured on the ECM protein fibronectin. However, there was no significant increase in the level of GTP-bound Rac or Cdc42 in response to S1P and integrin engagement (Figures 3.11 and 3.12). The same was observed in CCL39mycS1P₁ cells cultured on vitronectin (Figures 3.13 and 3.14).

It is well documented that S1P₁ induces Rac activation [126, 216], however, the S1P₂ receptor has a negative effect on the activation status of Rac. S1P₂ is reported to be expressed in this cell type [64] so to investigate whether endogenous S1P₂ was inhibiting S1P-mediated Rac activation, the selective S1P receptor agonist FTY720P, which does not stimulate the S1P₂ receptor [244] was utilised. FTY720P is a potent agonist of S1P₁ and activates ERK in a dose dependent manner in CCL39mycS1P₁ cells (EC₅₀=12.2pM) (Figure 3.15). FTY720P also activates ERK in a time-dependent manner in a comparable manner to S1P in CCL39mycS1P₁. Stimulation by either S1P or FTY720P causes a significant increase in the level of phosphorylated ERK detected by 2 minutes and peaks at 5 minutes. This indicates that the recombinant receptor is functioning correctly. It also suggests that if there is an endogenous receptor negating the S1P₁ receptor's ability to activate ERK then the receptor responsible does not activate ERK (Figures 3.16 and 3.17). However, the selective agonist FTY720P does not stimulate Rac or Cdc42 activation in CCL39mycS1P₁ cells (Figures 3.18 and 3.19). Thus, it appears that the ability of S1P to activate Rac and Cdc42 in this system is not due to expression of endogenous S1P₂.

Thus, to confirm the lack of Rac activation in response to S1P both control cells and CCL39mycS1P₁ cells were stained with Alexa fluor 594-conjugated phalloidin which binds to actin microfilaments. In both control CCL39 and CCL39mycS1P₁ cells by 30 minutes there were detectable stress fibres present in CCL39mycS1P₁. The formation of lamellipodia was also observed (Figure 3.20 and 3.21).

3.3 Discussion

S1P₁ has been shown to regulate the activation status of small G proteins in a number of cell types and is thereby considered an important mediator of not only cell migration but angiogenesis also. Gaining insight into S1P₁ signalling has therapeutic potential in a number of disease states including atherosclerosis and cancer.

The current explanation for transactivation of Rac by PKB is unlikely to be a universal mechanism since using CCL39mycS1P₁ cells it was demonstrated that PKB does not phosphorylate S1P₁ (data not shown) in this system. However, the inability to detect Rac or Cdc42 activation in CCL39mycS1P₁ cells was an unexpected result as there are several reports implicating S1P₁ as an activator of small G proteins in various cell types [126, 216, 371]. The GST-fusion protein employed to detect small G protein activation-GST-PAKCRIB contains the recognition site for GTP-bound Rac/Cdc42 of their downstream target p65PAK. The constitutively active form of Rac, L61 was isolated using GST-PAKCRIB confirming that the fusion protein was functioning correctly (Figure 3.9b). The non-hydrolysable analogue GTP_γS was also detected in the pull down assays also indicating that the assay was working. Although it may have been beneficial to use an assay detecting GTPase activity as GTP-bound small G proteins have intrinsic GTPase activity in addition to this [371].

CCL39mycS1P₁ showed a significant decrease in the level of Rac expression compared to parental cells Figure (3.5a). Serum starvation of CCL39mycS1P₁ cells partially rescued this effect (Figure 3.5b). Constitutive activity of S1P₁ in this model system was observed with respect to protection from serum withdrawal-induced apoptosis (see Chapter 4) so it is therefore probable that receptor over-expression caused the down-regulation of Rac observed in CCL39mycS1P₁. However the western blots of cell lysates from pull down assays show consistent levels of total Rac (and Cdc42) which were easily detectable. Although not significant, the basal levels of active Rac present in untreated CCL39mycS1P₁ cells were elevated compared to S1P treated cells which is also indicative of constitutive activity (Figures 3.9a and 3.11). The recombinant receptor appears to be functioning correctly as

agonist stimulation results in ERK activation in a time dependent manner (Figure 3.16).

S1P₂ has been documented to inhibit Rac activation and migration [216] but S1P₂ RNA was not detected in CCL39 cells by northern blot analysis (data not shown). In addition to this the use of the selective inhibitor FTY720P which is a potent activator of all S1P receptors bar S1P₂ did not result in detectable Rac or Cdc42 activation in response to S1P in CCL39mycS1P₁ cells (Figure 3.18 and 3.19). This agonist was functioning correctly as it stimulated ERK in a time and dose-dependent manner (Figures 3.15 and 3.17). Thus, it can be deduced that the lack of S1P-induced Rac and Cdc42 activation is not due to endogenous S1P₂ expression.

Since S1P stimulation does not induce Rac or Cdc42 activation in this cell type it might have been beneficial to try cell line where the receptor is expressed endogenously. The rate limiting step in activation of small G protein is the exchange of GDP for GTP [150] and if the appropriate GEF was not expressed or available in the locality this may effect activation levels. For example, the Rac-GEF Tiam-1 translocates to the plasma membrane where Rac is also concentrated to induce Rac activation and subsequent migration [164]. In both control CCL39 cell and CCL39mycS1P₁ cells stress fibres were detected (Figure 3.20 and 3.21). At 30 minutes lamellipodial structures were observed in CCL39mycS1P₁ cells treated with S1P (Figure 3.21). The presence of lamellipodia suggests there may be low levels of localised Rac activation but it is undetectable biochemically. Although well documented in other cell types it appears that S1P-induced Rac and Cdc42 activation does not occur in this model system.

Figure 3.1 Confocal Image of CCL39 Cells Stably Expressing the Myc Epitope Tagged Receptor S1P₁

CCL39 cells stably expressing the recombinant mycS1P₁ were incubated with antibodies recognising the myc epitope tag (9E10 1:200) and the S1P₁ receptor (5µg/ml). The secondary antibodies used were Alexa 488- and 594-conjugated. The image on the far right shows the merge and co-localisation of the myc tag and the S1P₁ receptor. The image was captured while using a 40 X objective lens.

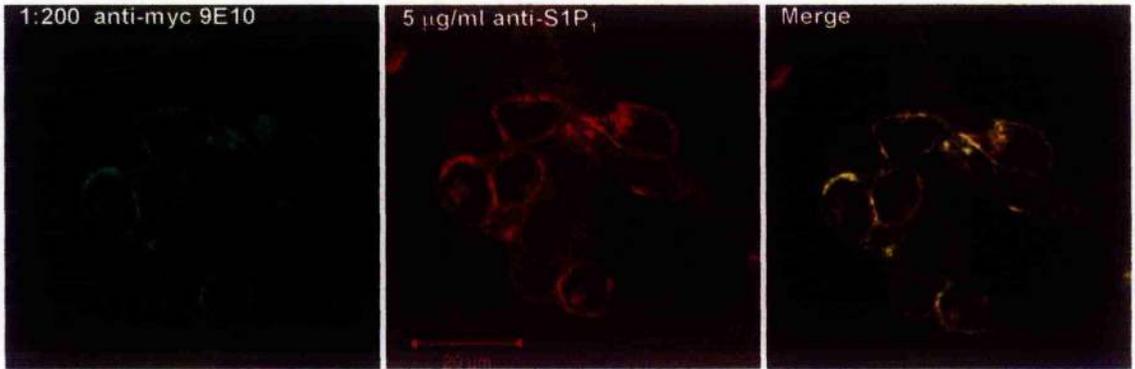


Figure 3.2 The Effect of the Cytoskeletal Inhibitor Colchicine on Levels of S1P₁ Receptor Phosphorylation.

The autoradiographs show the level of mycS1P₁ receptor phosphorylation. CCL39 cells that stably express mycS1P₁ were labelled with [³²P] orthophosphate for 90 minutes prior to a 30 minute treatment with an inhibitor of microtubule polymerisation, colchicine in concentrations ranging from 0.01μM to 10μM. The cells were then stimulated with S1P at a concentration of 5μM for 5 minutes. Labelled mycS1P₁ was detected by immunoprecipitation and visualised by autoradiography. The level of total receptor was detected by western blotting. This Figure is representative of 3 experiments and the graph shows the percentage maximal stimulation.

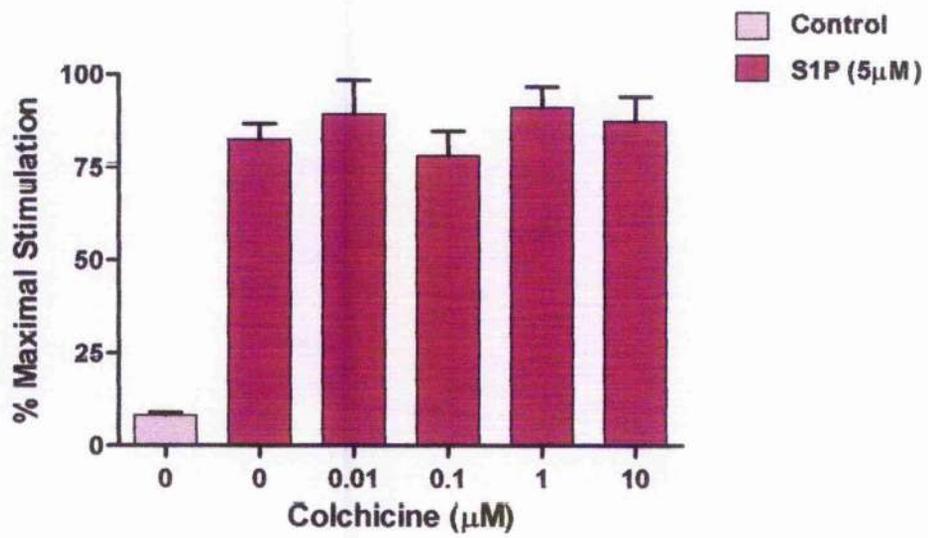
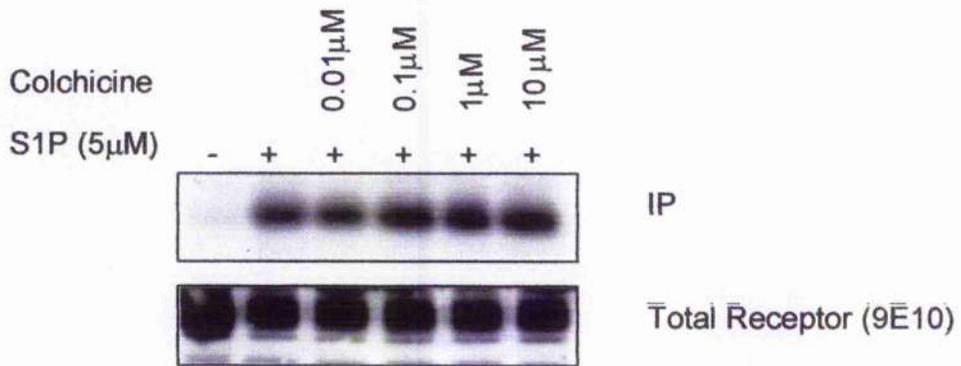


Figure 3.3 The Effect of the Cytoskeletal Inhibitor cytochalasin B on Levels S1P₁ Receptor Phosphorylation.

The autoradiographs show the levels of mycS1P₁ receptor phosphorylation. CCL39 cells stably expressing mycS1P₁ were labelled with [³²P] orthophosphate for 90 minutes prior to a 30 minute treatment with cytochalasin B, an inhibitor of actin polymerisation in concentrations ranging from 0.01µM to 10µM. The cells were then stimulated with S1P at a concentration of 5µM for 5 minutes mycS1P₁ was detected by immunoprecipitation. Labelled mycS1P₁ was detected by immunoprecipitation and visualised by autoradiography. The level of total receptor was detected by western blotting. This Figure is representative of 3 experiments and the graph shows the percentage maximal stimulation.

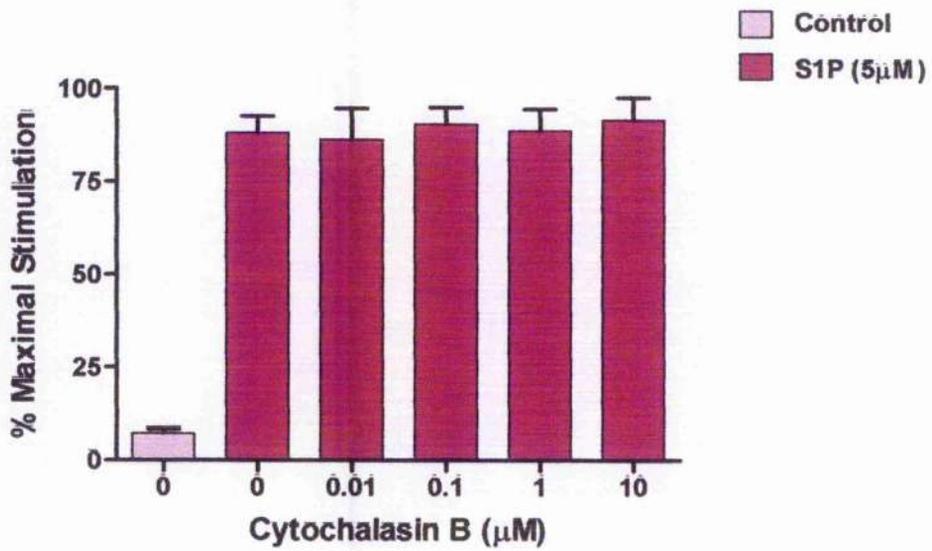
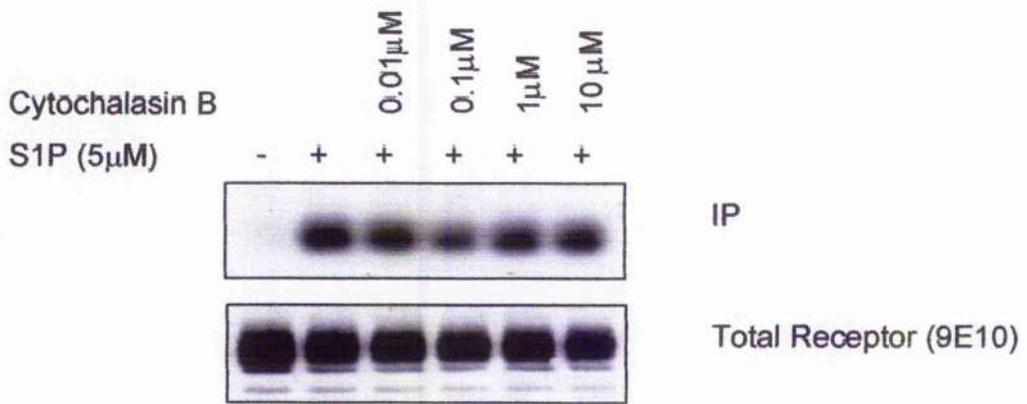


Figure 3.4 The Effect of Pertussis Toxin on Levels S1P₁ Receptor Phosphorylation.

The autoradiographs show levels of mycS1P₁ receptor phosphorylation. CCL39 cells stably expressing mycS1P₁ were treated with pertussis toxin (PTX) for 18 hours in concentrations ranging from 1ng/ml to 100ng/ml. The cells were labelled with [³²P] orthophosphate for 90 minutes prior to stimulation with S1P at a concentration of 5μM for 5 minutes. MycS1P₁ was detected by immunoprecipitation. Labelled mycS1P₁ was detected by immunoprecipitation and visualised by autoradiography. The level of total receptor was detected by western blotting. This Figure is representative of 3 experiments and the graph shows the percentage maximal stimulation.

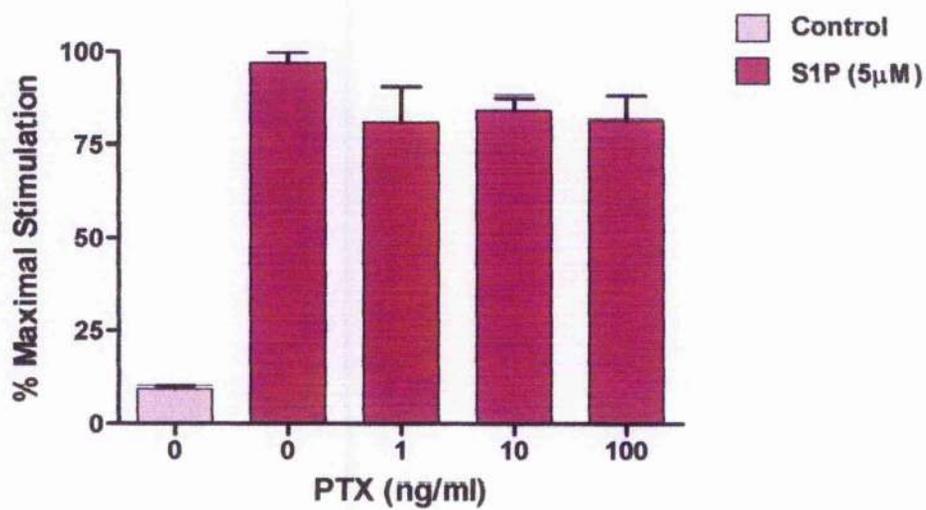
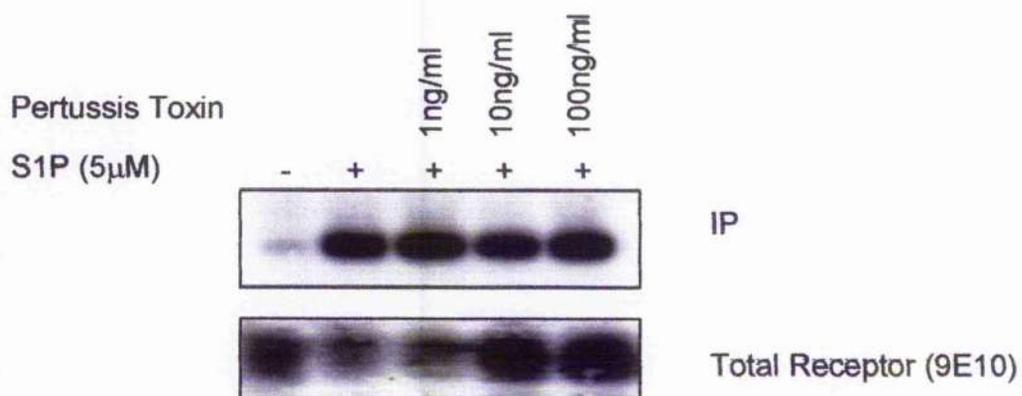


Figure 3.5 The Effect of Cytoskeletal Inhibitors on Agonist-induced S1P₁ Receptor Internalisation.

This Figure shows the detection of cell surface labelling of mycS1P₁ with biotin-LC-hydrazide. CCL39 cells stably expressing mycS1P₁ were treated with the cytoskeletal inhibitors colchicine and cytochalasin B at a concentration of 10 μ M and pertussis toxin (PTX) at a concentration of 100ng/ml. The cells were stimulated with S1P at a concentration of 5 μ M for 90 minutes. The receptors were immunoprecipitated and cell surface receptors detected by HRP-streptavidin. The level of total receptor was detected by western blotting. This Figure is representative of 3 experiments and the graph shows cell surface receptor detected.

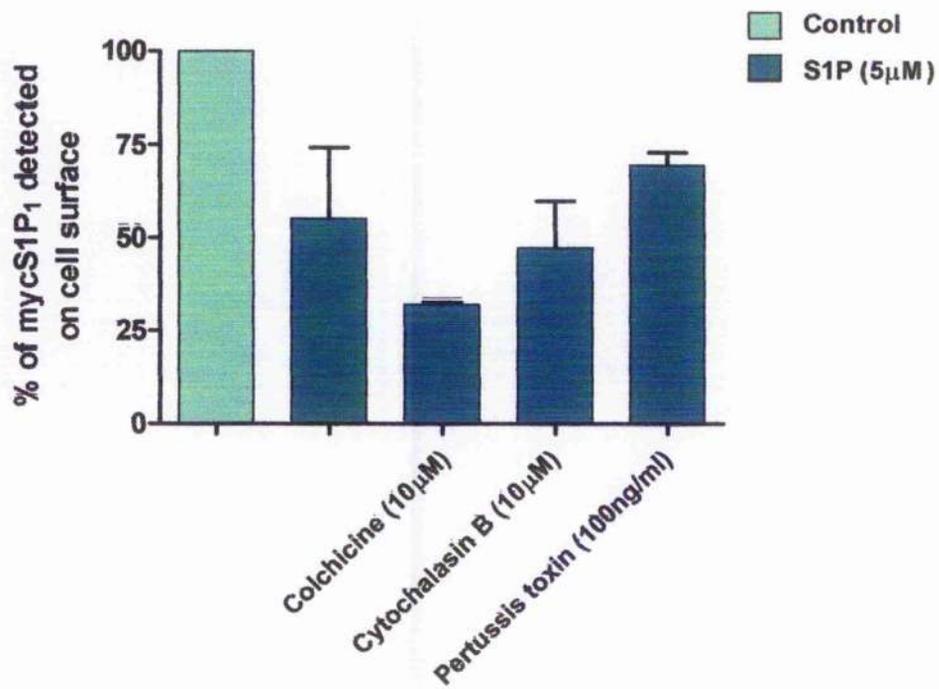
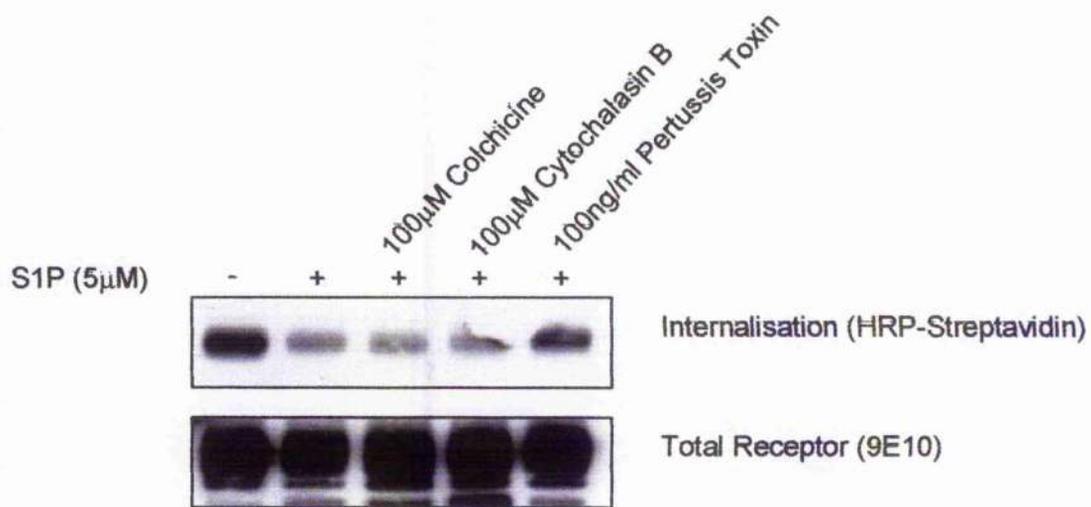


Figure 3.6 Detection of Rac in CCL39 Cells Over-expressing mycS1P₁

- (a) Detection of Rac in CCL39 over-expressing mycS1P₁ compared to parental CCL39 cells, which have been cultured in complete media by western blot,
- (b) Detection of Rac in CCL39 cells over-expressing mycS1P₁ compared to parental CCL39 cells which have been serum-starved for 4 hours by western blot.

The cells were cultured, lysates prepared and the concentration of protein equalised. *p* values are from a one way ANOVA with a Bonferroni comparisons test, bars marked with *** have a $p < 0.001$ and those marked with * have a $p < 0.05$. Levels of mycS1P₁ and GAPDH were also determined by western blot.

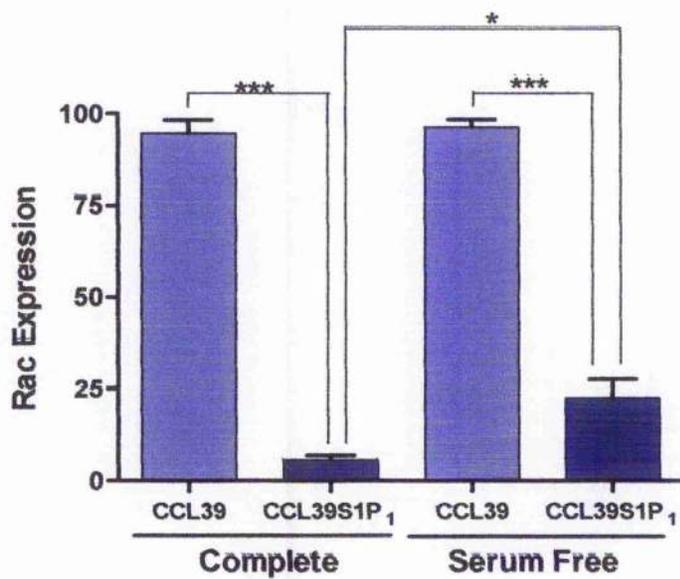
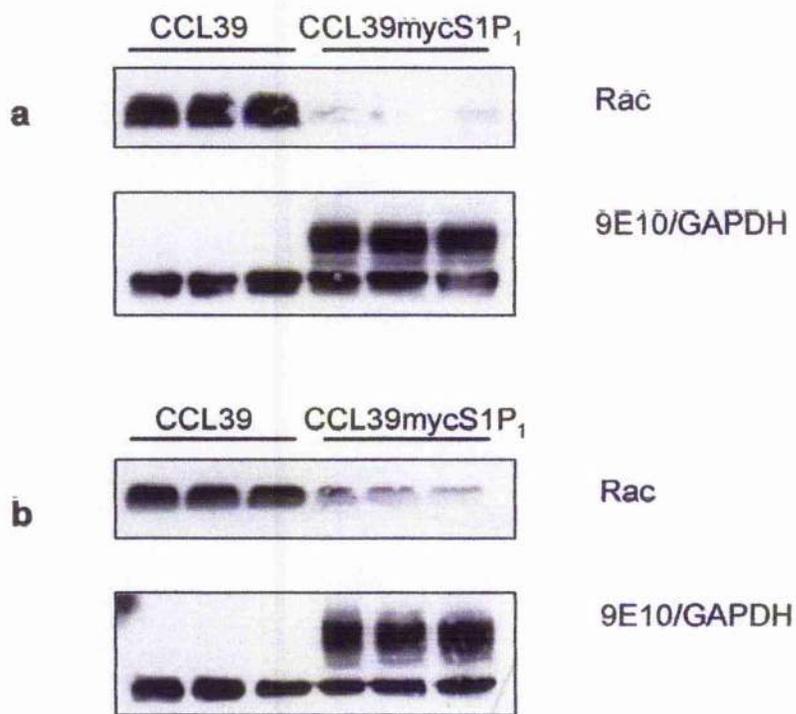


Figure 3.7 The Effect of S1P Stimulation on Rac Activation Status in Parental CCL39 Cells.

Detection of GTP-bound Rac in parental CCL39 cells that have been stimulated with S1P (5 μ M) by western blot. Parental CCL39 cells were serum-starved for 4 hours prior to stimulation with S1P for 0.5, 1, 5 and 15 minutes. GTP-bound Rac was isolated using the GST fusion protein PAKCRIB. Total Rac was also determined by western blot and this Figure is representative of three experiments.

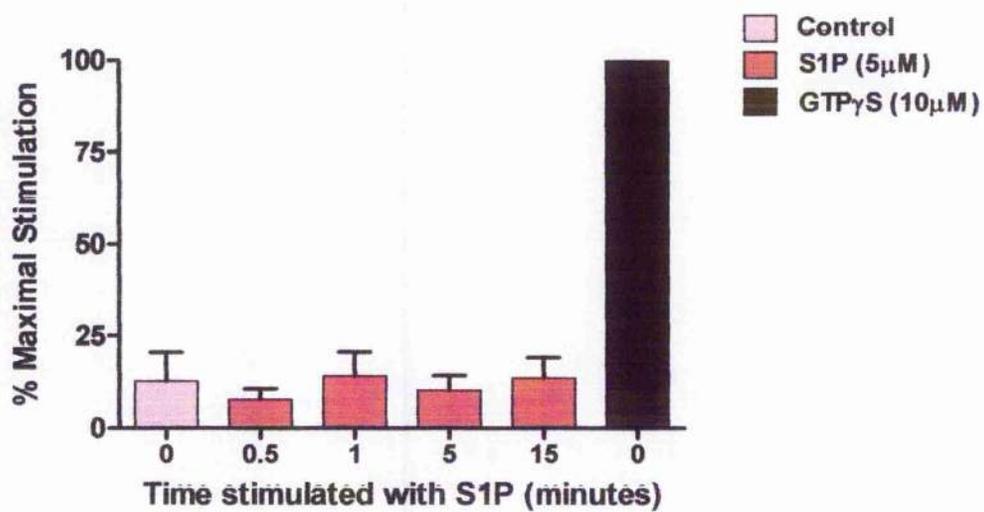


Figure 3.8 The Effect of S1P Stimulation on Cdc42 Activation Status in Parental CCL39 Cells.

Detection of GTP-bound Cdc42 in parental CCL39 cells that have been stimulated with S1P by western blot. CCL39 cells were serum-starved for 4 hours prior to stimulation with S1P at a concentration of 5 μ M for 0.5, 1, 5 and 15 minutes. GTP-bound Cdc42 was isolated using the GST fusion protein PAKCRIB. The total level of Cdc42 was also determined by western blot. This Figure is representative of two experiments.

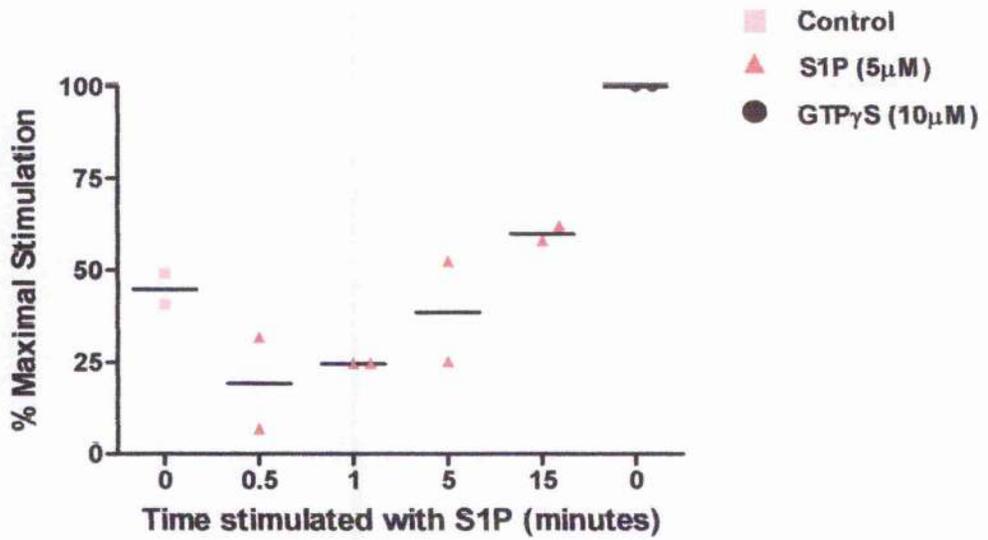
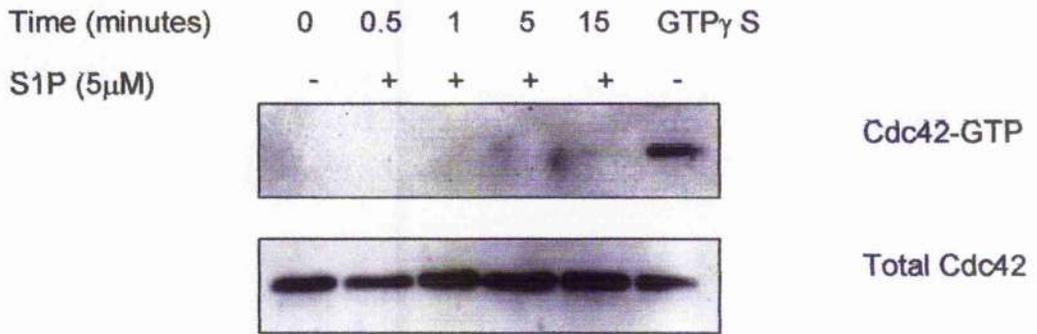


Figure 3.9 Rac Activation Status in S1P-stimulated CCL39mycS1P₁ Cells

- (a) CCL39mycS1P₁ cells were serum-starved for 4 hours prior to stimulation with S1P at a concentration of 5 μ M for 0.5, 1, 5 and 15 minutes. GTP-bound Rac was isolated using the GST fusion protein PAKCRIB and detected by western blot. The level of total Rac was also detected by western blot. The Figure is a representative of three experiments,
- (b) The above experiment was repeated but with the constitutively active L61Rac control included. L61Rac was detected by western blot as above.

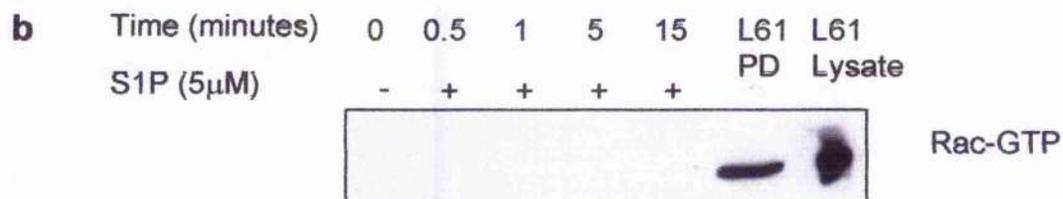
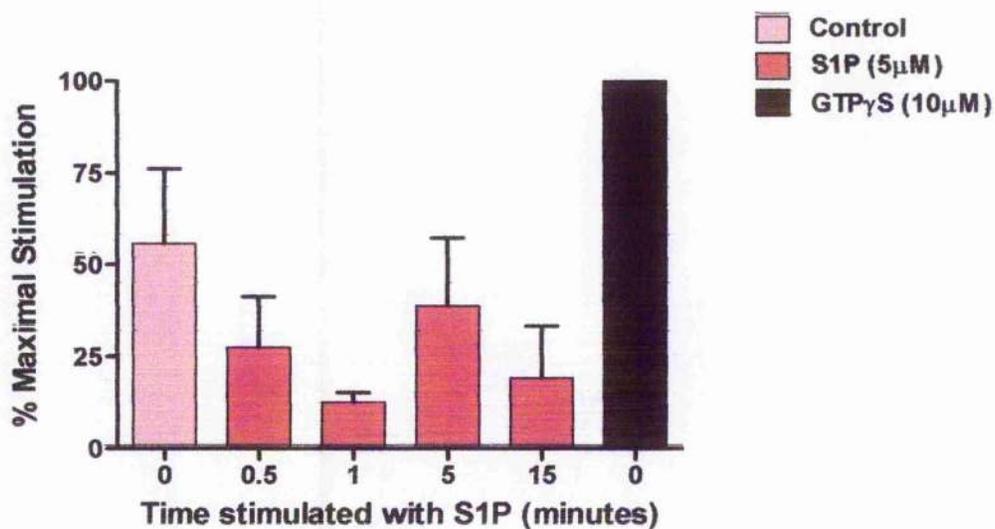


Figure 3.10 Cdc42 Activation Status in S1P-stimulated CCL39mycS1P₁ Cells

CCL39mycS1P₁ cells were serum-starved for 4 hours prior to stimulation with S1P at a concentration of 5 μ M for 0.5, 1, 5 and 15 minutes. GTP-bound Cdc42 was isolated using the GST fusion protein PAKCRIB and detected by western blot. The level of total Cdc42 was also determined by western blot. This Figure is a representative of three experiments.

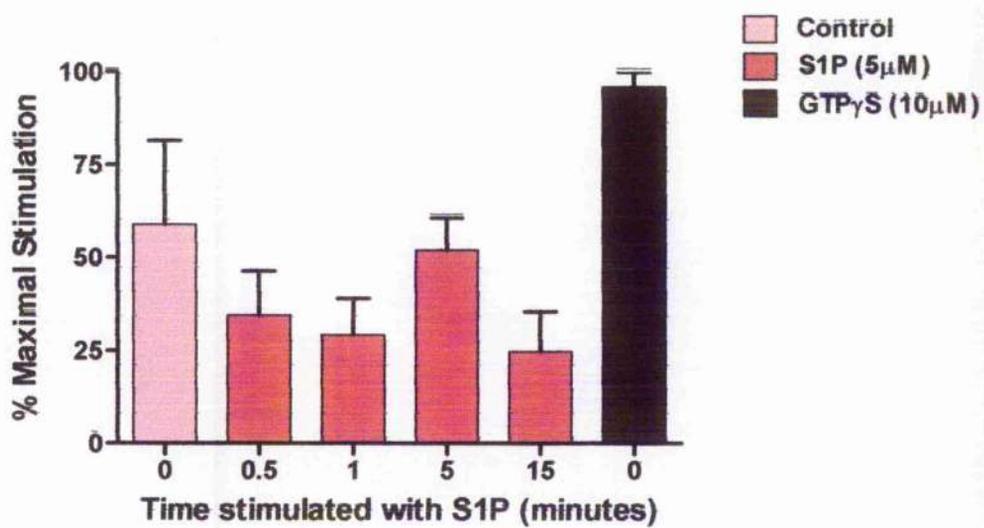
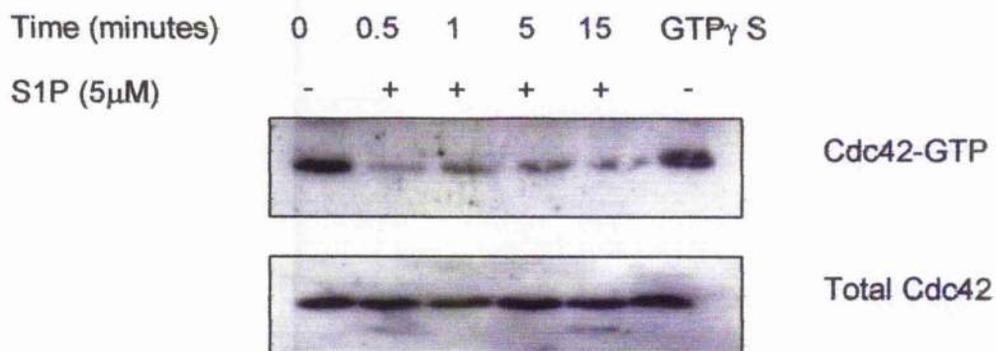


Figure 3.11 The Effect of S1P Stimulation on Rac Activation Status in CCL39mycS1P₁ Cells Cultured on Fibronectin

Levels of GTP-bound Rac in S1P stimulated CCL39mycS1P₁ cells cultured on fibronectin. CCL39mycS1P₁ cells were plated out on tissue culture dishes coated with fibronectin at a concentration of 5µg/ml. The next day, the cells were serum-starved for 4 hours prior to stimulation with S1P at a concentration of 5µM for 0.5, 1, 5 and 15 minutes. GTP-bound Rac was isolated using the GST fusion protein PAKCRIB and detected by western blot. The level of total Rac present was also detected by western blot and this Figure is a representative of three experiments. p values are from a one way ANOVA with a Bonferroni comparisons test, bars marked with * have a $p < 0.05$.

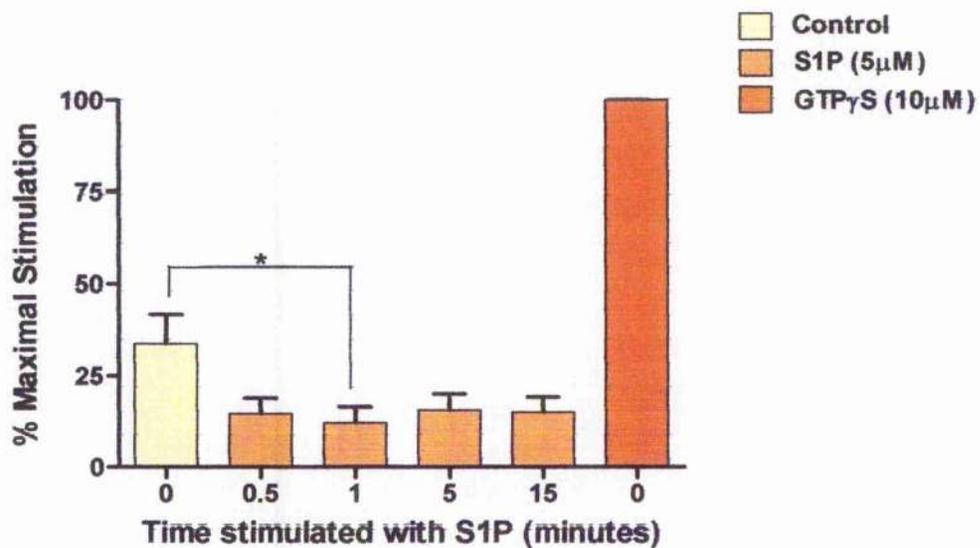
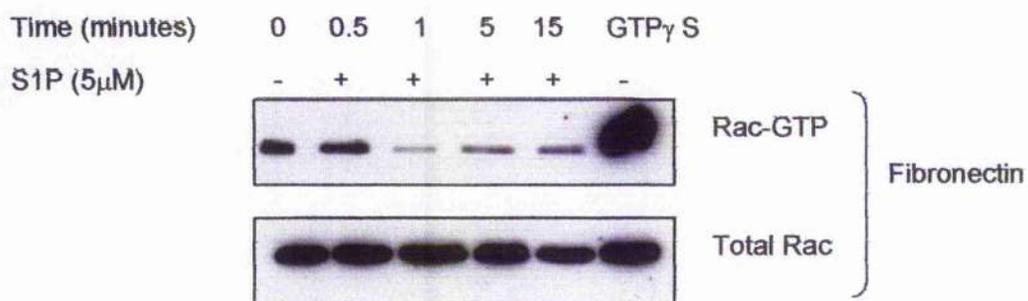


Figure 3.12 The Effect of S1P Stimulation on Cdc42 Activation Status in CCL39mycS1P₁ Cells Cultured on Fibronectin

The levels of GTP-bound Cdc42 in S1P stimulated CCL39mycS1P₁ cells cultured on fibronectin. CCL39mycS1P₁ cells were plated out on tissue culture dishes coated with fibronectin at a concentration of 5µg/ml. The next day, the cells were serum-starved for 4 hours prior to stimulation with S1P at a concentration of 5µM for 0.5, 1, 5 and 15 minutes. GTP-bound Cdc42 was isolated using the GST fusion protein PAKCRIB and detected by western blot. The levels of total Cdc42 present were also determined by western blot and this Figure is a representative of two experiments.

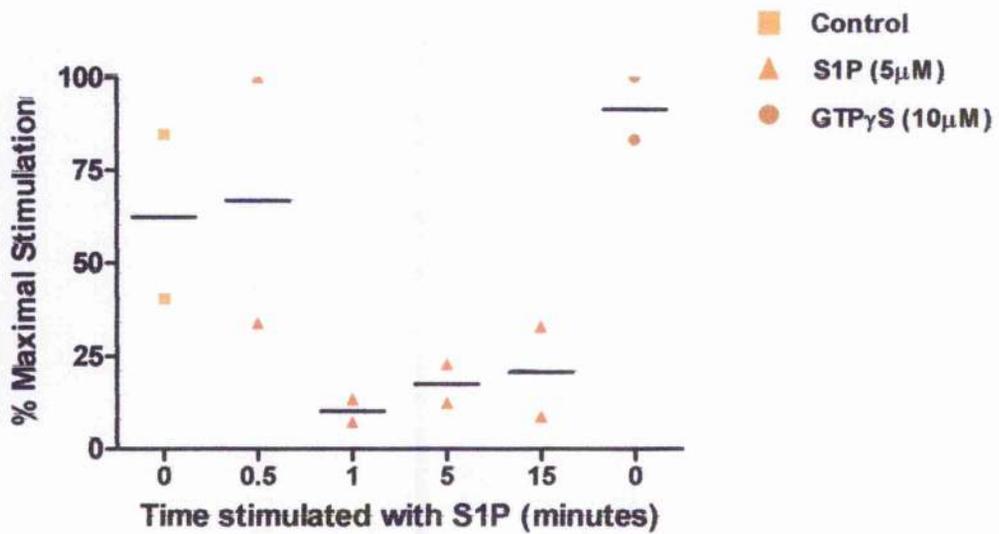
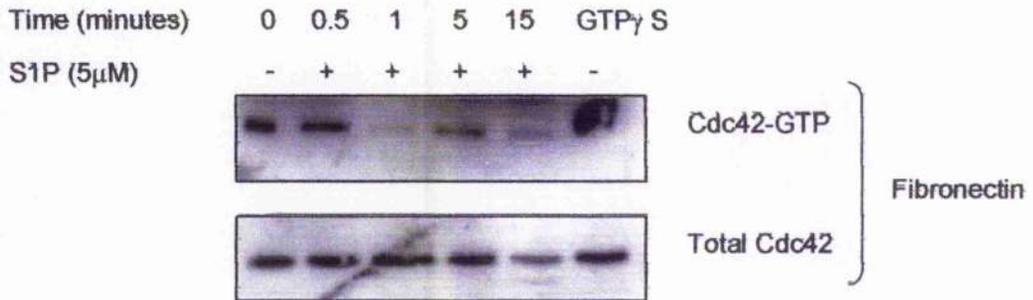


Figure 3.13 The Effect of S1P Stimulation on Rac Activation Status in CCL39mycS1P₁ Cells Cultured on Vitronectin

The levels of GTP-bound Rac in S1P stimulated CCL39mycS1P₁ cells cultured on vitronectin. CCL39mycS1P₁ cells were plated out on tissue culture dishes coated with vitronectin at a concentration of 5µg/ml. The next day, the cells were serum-starved for 4 hours prior to stimulation with S1P at a concentration of 5µM for 0.5, 1, 5 and 15 minutes. GTP-bound Rac was isolated using the GST fusion protein PAKCRIB and detected by western blot. The level of total Rac was also determined by western blot and this Figure is a representative of three experiments.

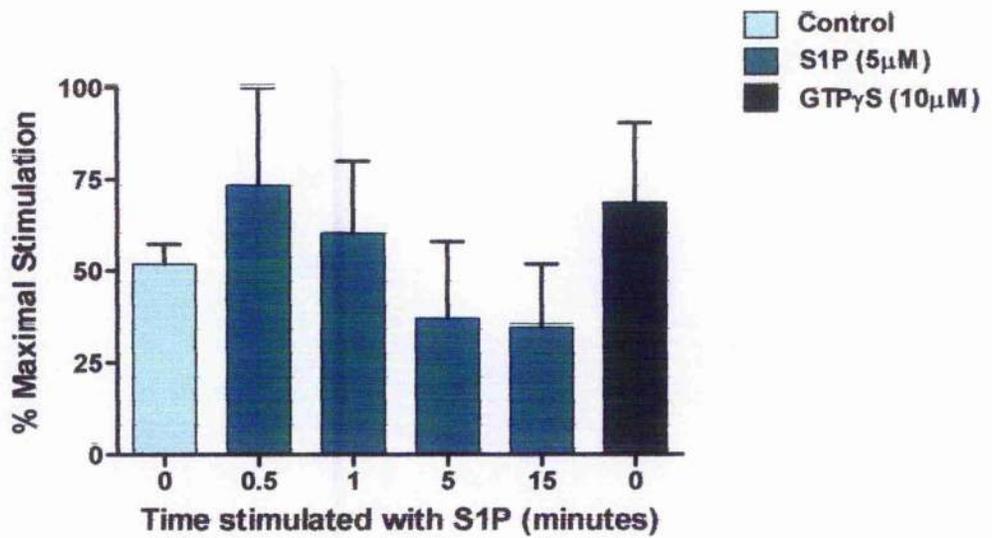


Figure 3.14 The Effect of S1P Stimulation on Cdc42 Activation Status in CCL39mycS1P₁ Cells Cultured on Vitronectin

The levels of GTP-bound Cdc42 in S1P stimulated CCL39mycS1P₁ cells cultured on vitronectin. CCL39mycS1P₁ cells were plated out on tissue culture dishes coated with vitronectin at a concentration of 5µg/ml. The next day, the cells were serum-starved for 4 hours prior to stimulation with S1P at a concentration of 5µM for 0.5, 1, 5 and 15 minutes. GTP-bound Cdc42 was isolated using the GST fusion protein PAKCRIB and detected by western blot. The level of total Cdc42 present was also determined by western blot and this Figure is a representative of two experiments.

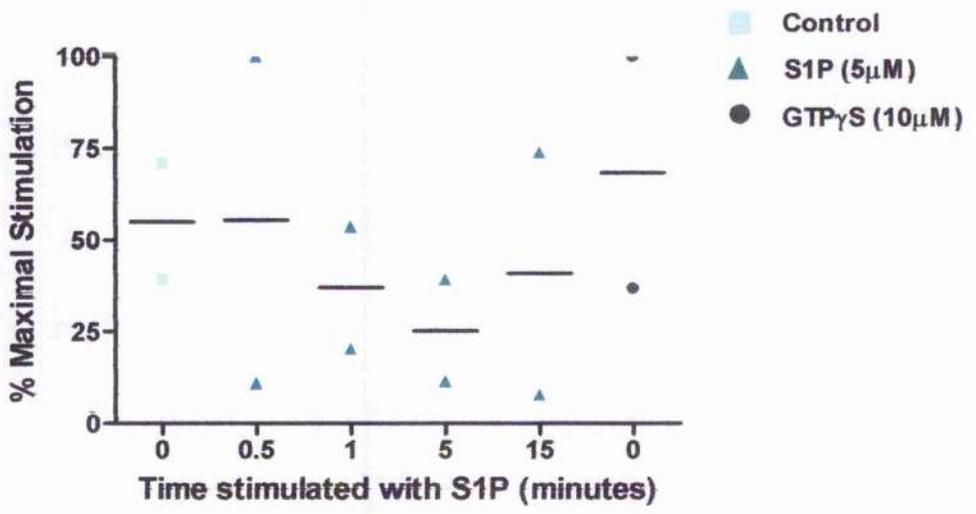
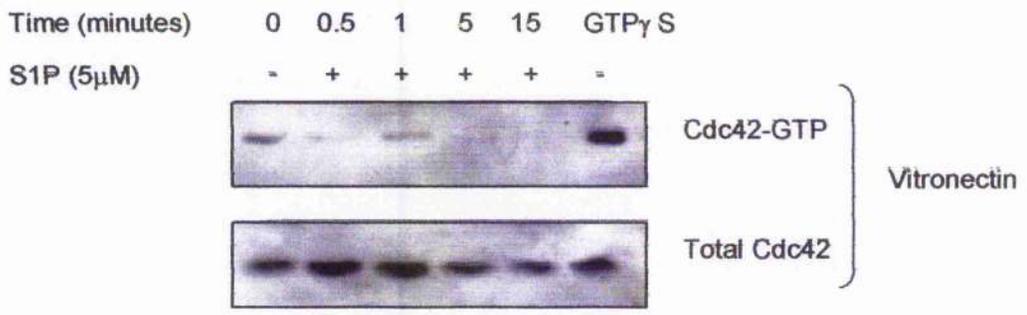


Figure 3.15 Detection of Dose-Dependent ERK Activation in CCL39mycS1P₁ Cells Stimulated with FTY720P

Both parental CCL39 cells and CCL39mycS1P₁ cells were deprived of serum for 4 hours prior to stimulation with FTY720P at concentrations ranging from 10⁻¹¹ to 10⁻⁵, for 15 minutes. The level of phospho-ERK and total ERK was determined by western blot. This Figure is a representative of three experiments.

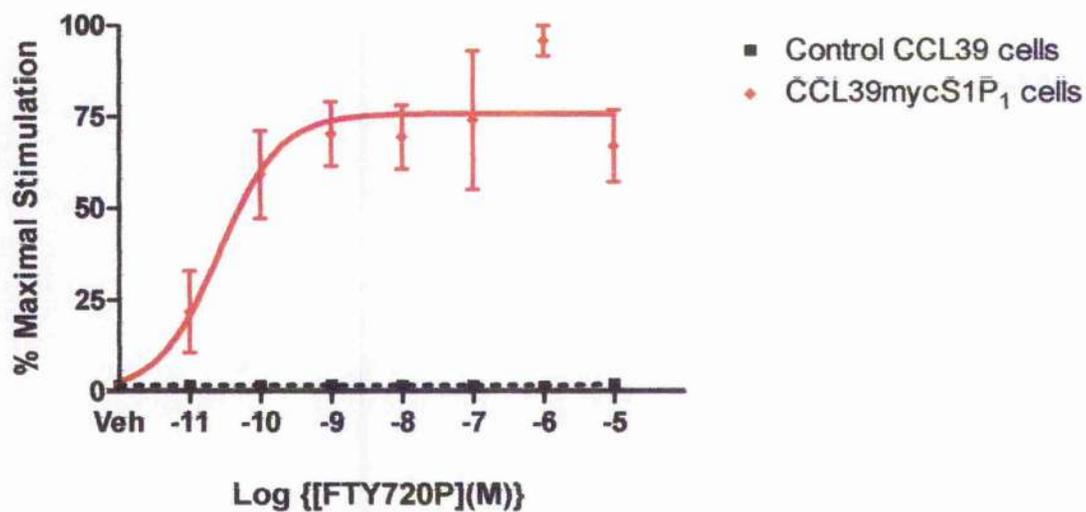
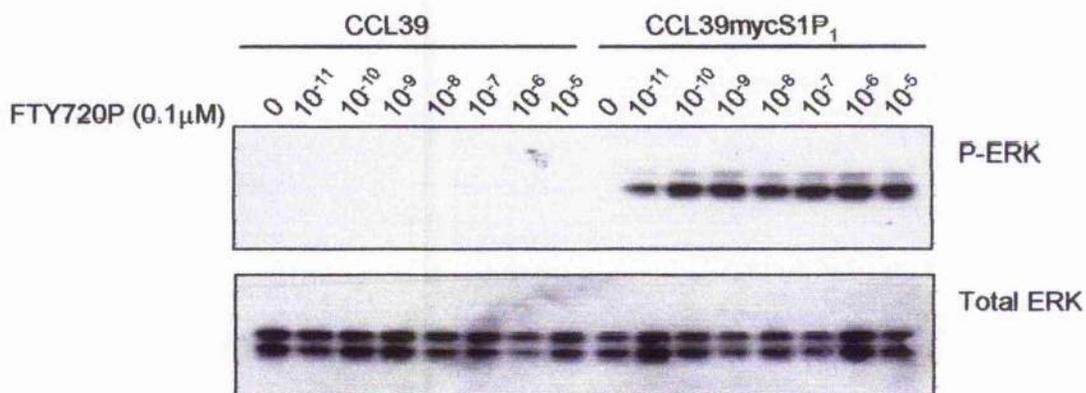


Figure 3.16 Detection of ERK Activation in CCL39mycS1P₁ Cells Stimulated with S1P

The time dependent effect of S1P stimulation on ERK activation in both parental CCL39 cells and CCL39mycS1P₁ cells. These cells were serum-starved for 4 hours prior to stimulation with S1P at a concentration of 5 μ M for 2, 5, 15 and 30 minutes and FTY720P for 15 minutes at a concentration of 0.1 μ M. Phospho-ERK (P-ERK) and total ERK were detected by western blot. This Figure is a representative of three experiments and bars marked with *** have a *p* value of <0.001.

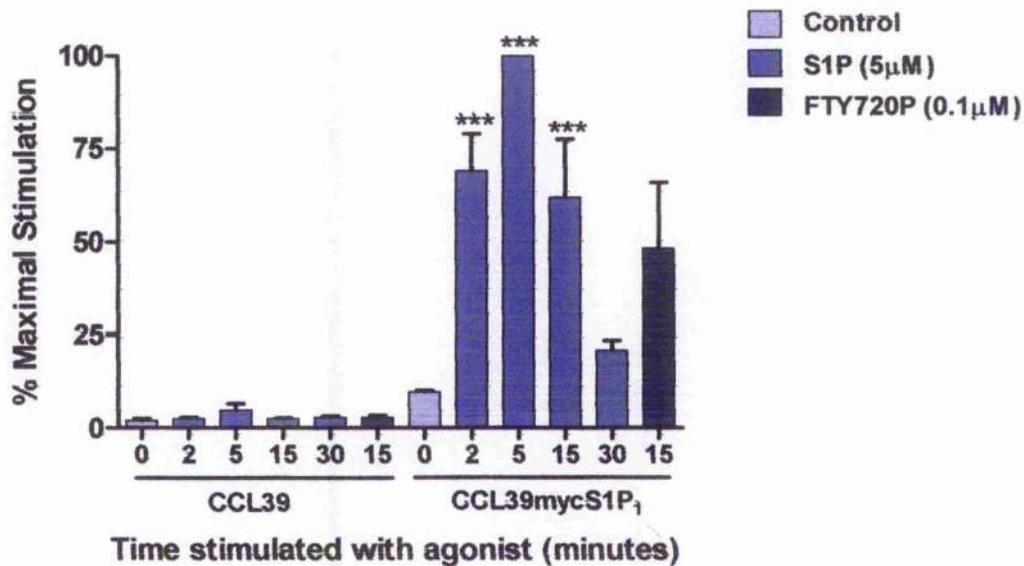
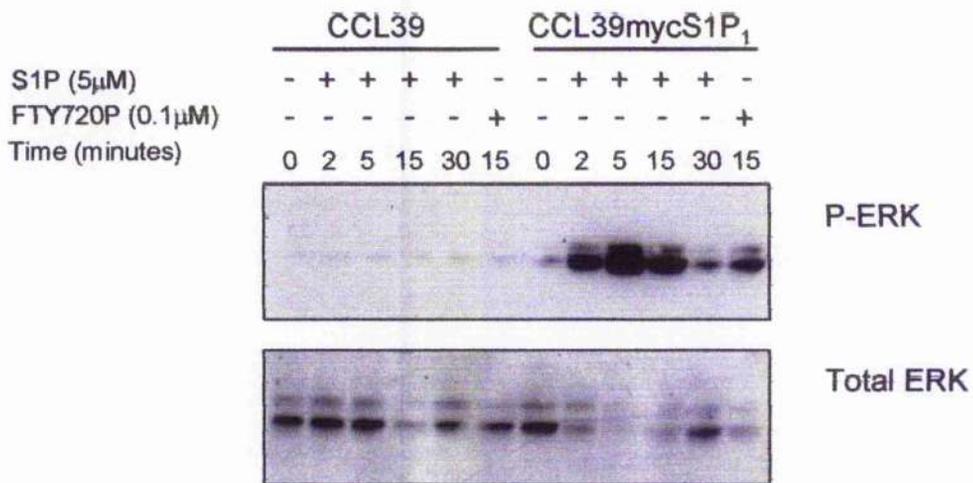


Figure 3.17 Detection of ERK Activation in CCL39mycS1P₁ Cells Stimulated with FTY720P

The time-dependent effect of FTY720P on ERK activation in both control CCL39 cells and CCL39mycS1P₁ cells. The cells were serum-starved for 4 hours prior to stimulation with FTY720P at a concentration of 0.1 μ M for 2, 5, 15 and 30 minutes and S1P for 15 minutes at a concentration of 5 μ M. Phospho-ERK (P-ERK) and total ERK were detected by western blot. This Figure is a representative of three experiments and bars marked with *** have a *p* value of <0.001.

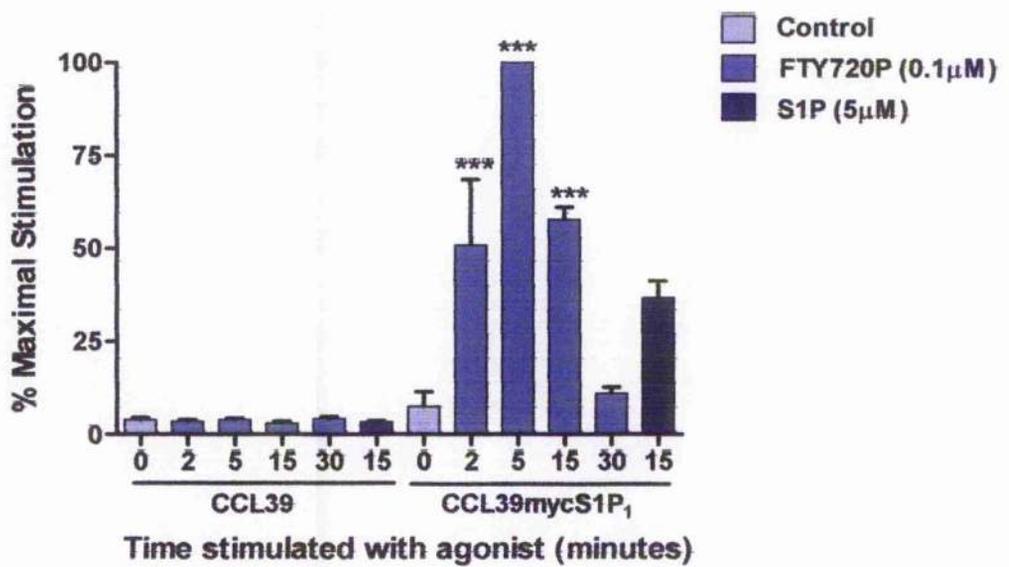
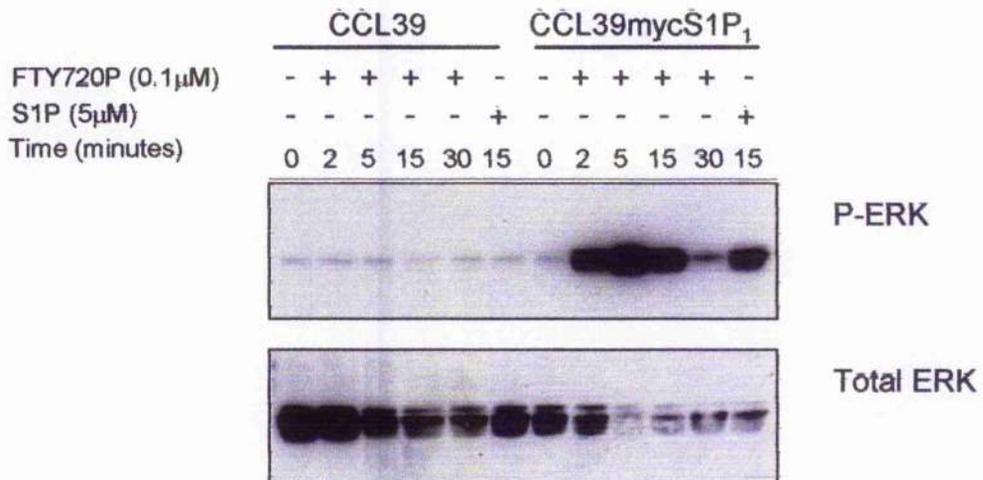
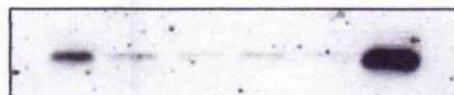


Figure 3.18 The Effect of FTY720P Stimulation on the Rac Activation Status in CCL39mycS1P₁ Cells

The level of GTP-bound Rac in FTY720P stimulated CCL39mycS1P₁ cells. CCL39mycS1P₁ cells were serum-starved for 4 hours prior to stimulation with FTY720P at a concentration of 0.1 μ M for 0.5, 1, 5 and 15 minutes. GTP-bound Rac was isolated with the GST fusion protein PAKCRIB and detected by western blot. The level of total Rac was also detected by western blot and this Figure is a representative of three experiments.

Time (minutes)	0	0.5	1	5	15	GTP γ S
FTY720P (0.1 μ M)	-	+	+	+	+	-



Rac-GTP



Total Rac

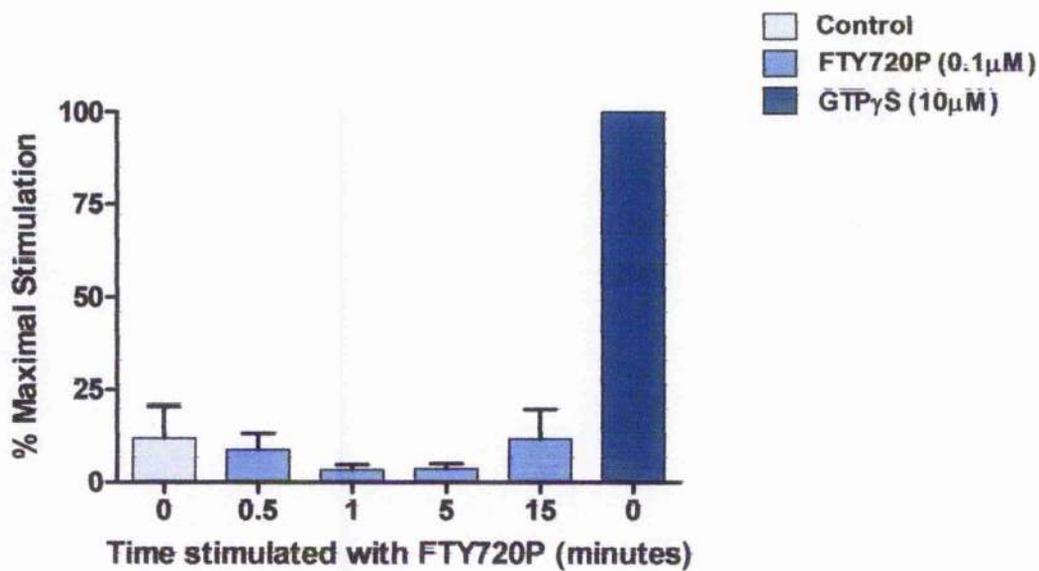


Figure 3.19 The Effect of FTY720P Stimulation on the Cdc42 Activation Status in CCL39mycS1P₁ Cells

The level of GTP-bound Cdc42 in FTY720P stimulated CCL39mycS1P₁ cells. CCL39mycS1P₁ cells were serum-starved for 4 hours prior to stimulation with FTY720P at a concentration of 0.1 μ M for 0.5, 1, 5 and 15 minutes. GTP-bound Cdc42 was isolated with the GST fusion protein PAKCRIB and detected by western blot. The level of total Cdc42 was also detected by western blot and this Figure is a representative of three experiments.

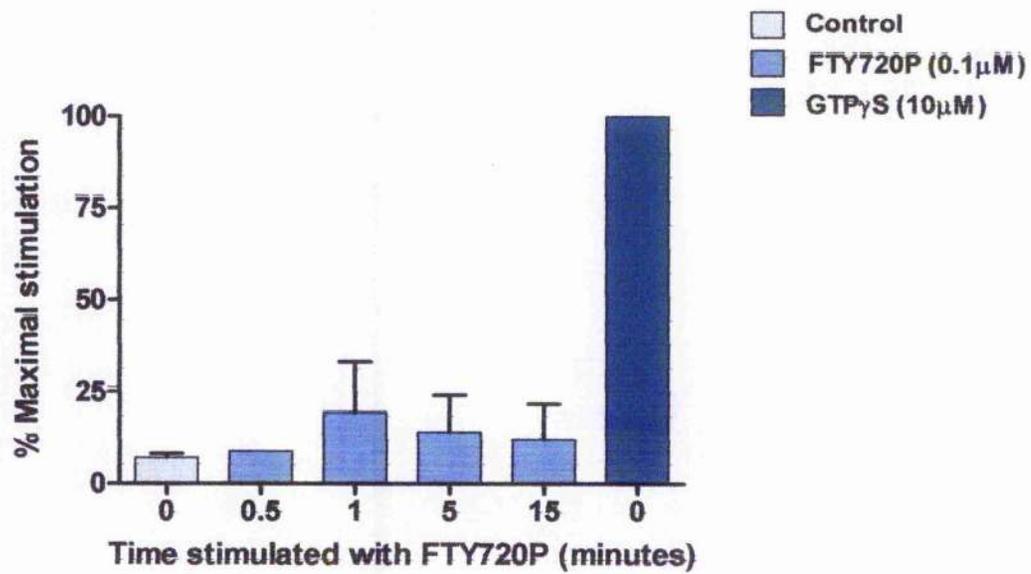
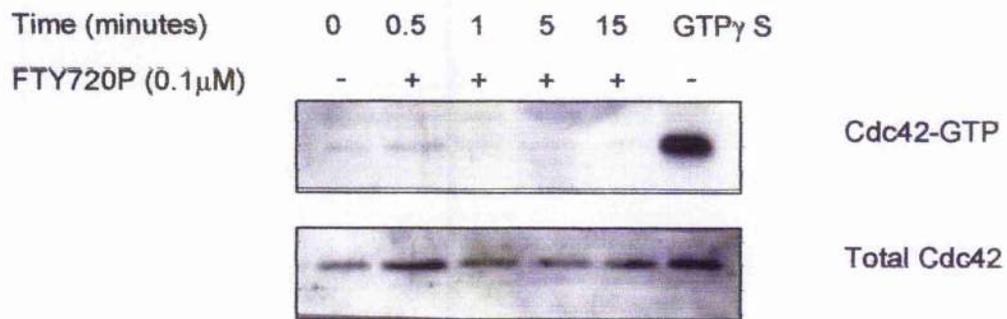
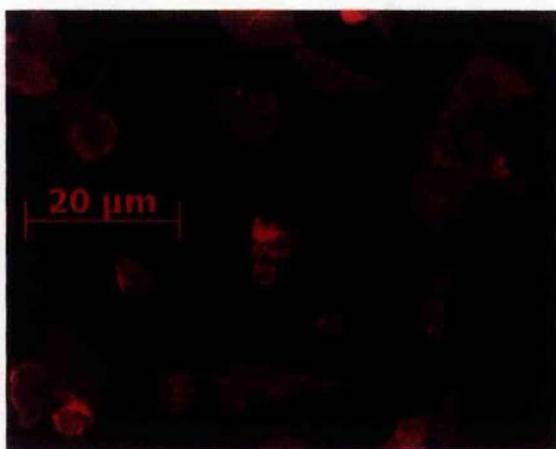
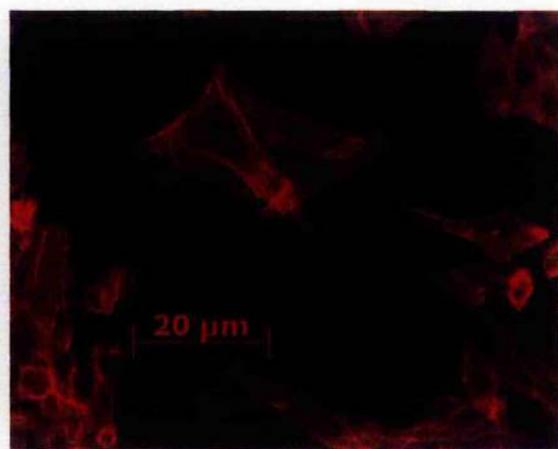


Figure 3.20 Detection of Stress Fibres in Control CCL39 Cells Treated with S1P

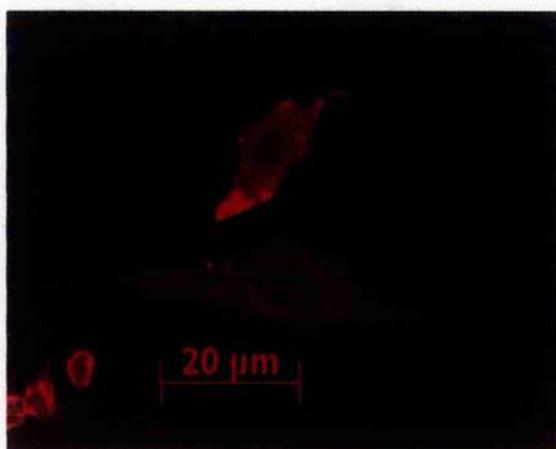
Control CCL39 cells treated with S1P (5 μ M) over a time course of 2, 5, 15 and 30 minutes. Stress fibres were detected by staining with Alexa fluor 594-conjugated phalloidin. Images were captured using a Zeiss microscope (40 x objective lense) and manipulated with Axiovision software.



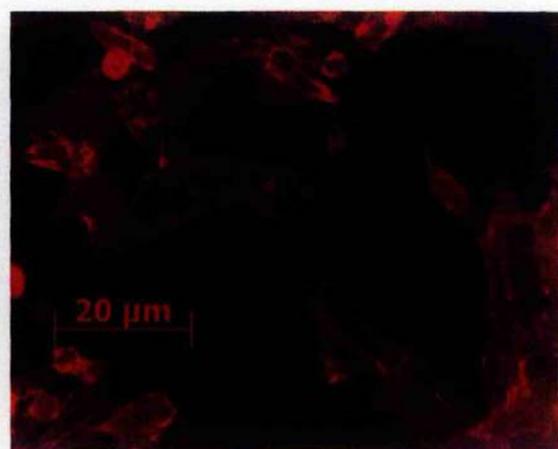
0 min



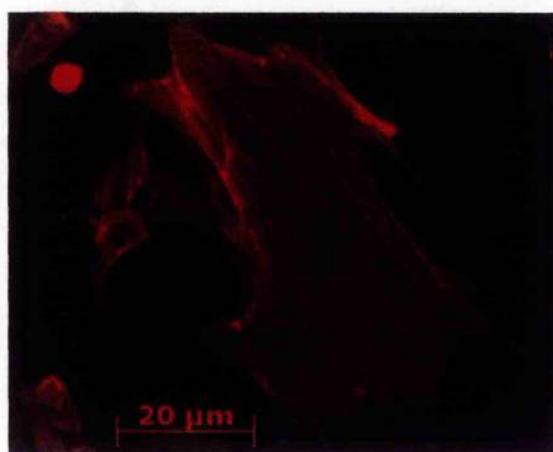
2 min



5 min



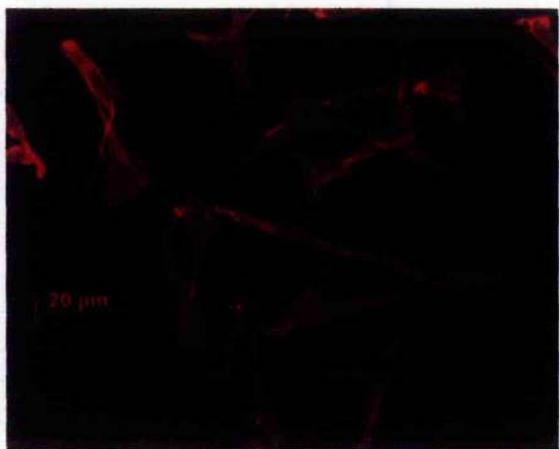
15 min



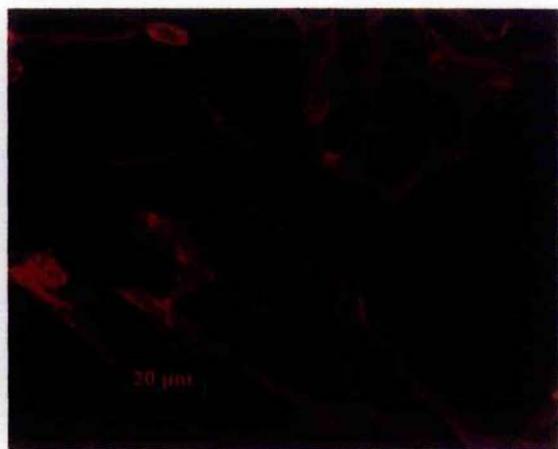
30 min

Figure 3.21 Detection of Stress fibres in CCL39mycS1P₁ Cells Treated with S1P

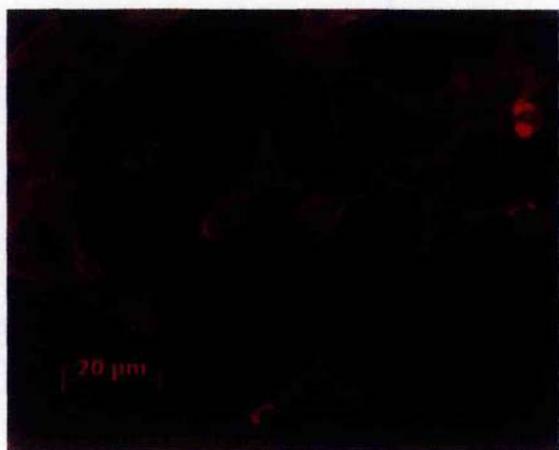
CCL39mycS1P₁ cells treated with S1P (5μM) over a time course of 2, 5, 15 and 30 minutes. Stress fibres were detected by staining with Alexa fluor 594-conjugated phalloidin. Images were captured using a Zeiss microscope (40 x objective lense) and manipulated with Axiovision software.



0 min



2 min



5 min



15 min



30 min

Chapter 4
Regulation of Apoptosis by S1P₁

Regulation of Apoptosis by S1P₁

4.1 Introduction

Apoptosis is a strictly regulated process of cell destruction that plays an essential role in both embryonic development and tissue homeostasis in the adult organism. It is characterised by nuclear and cytoplasmic condensation which results in the formation of apoptotic bodies that are recognised, ingested and degraded by neighbouring cells [256].

Programmed cell death can be instigated by the activation of death receptors such as the Fas receptor, but can also be initiated by extrinsic means like the removal of trophic factors [293, 299]. The removal of survival factors has been demonstrated to cause the increase in activity of pro-apoptotic BH3-only proteins, such as Bim in various cells types including fibroblasts [274] and haematopoietic cells [271]. In viable cells, Bim remains sequestered by the anti-apoptotic protein Mcl-1. However, another BH3-only protein [298], Noxa, regulates the proteosomal degradation of Mcl-1, which allows the liberated Bim to promote cytochrome *c* release by activating pro-apoptotic Bcl-2 proteins [372]. Bim is subject to negative regulation *via* ERK-mediated phosphorylation which promotes its degradation [274, 275], whereas phosphorylation by JNK promotes the release of Bim from microtubules allowing the liberated Bim to promote the release of cytochrome *c* from the mitochondria [278]. Cytochrome *c* release triggers the initiation of the caspase cascade and the subsequent activation of caspase-3 [311]. Caspase-3 is termed an executioner caspase as its activation is deemed to be the point of no return for the cell [283]. Substrates for proteolysis by caspases include structural proteins homeostatic proteins and DNases which induce the apoptotic phenotype and also pro-caspases which serve to amplify the apoptotic signal [260].

S1P and its derivative sphingosine have long been recognised as important mediators of programmed cell death. For example, activation of SphK and the concomitant production of S1P protects cells from apoptosis by serum deprivation, in fact, SphK1 activation has been associated with the down-regulation of Bim in HUVECs but it is unknown if this action is sufficient to promote survival [333]. S1P is secreted from various cell types, including fibroblasts and activated platelets and exerts its effects *via* the cell surface

receptors S1P₁₋₅. Extracellular S1P is mainly bound to HDLs which act as carriers that promote the binding of S1P to S1P₃ resulting in the activation of eNOS and production of cryoprotective NO [345, 348]. The S1P₁ receptor is believed to localise to caveolae upon agonist binding and is also believed to promote the activation of eNOS [359]. Stimulation of S1P₁ also results in the PKB-mediated activation of eNOS [207, 361].

Bim is also believed to be involved with a specific form of cell death termed anoikis which is caused by the detachment of cells from their substratum [373]. An investigation of anoikis in an immortalised breast epithelial cells showed an elevation in expression of all three endogenous Bim isoforms which is reversed by the hyper-activation of EGF receptor mediated activation of the MAPK/ERK pathway [374]. In particular relevance to this thesis, the down-regulation of PKB by proteolytic degradation in CCL39 cells undergoing anoikis and that activation of PKB required adhesion to the substratum [197]. The transcription factor FoxO3a has been implicated in the up-regulation of Bim expression induced by the cancer drug paclitaxel. The FoxO transcription factors are subject to regulation by PKB phosphorylation which not only impairs their ability to bind DNA but increases their affinity for 14-3-3 proteins [375-378].

S1P₁ receptor signalling is associated with many growth-related cell processes and is acknowledged to enhance survival. However, the mechanism S1P₁ utilises to promote survival remains unclarified. The response of CCL39 cells to the removal of trophic factors is well documented [379, 380] so CCL39 stably expressing mycS1P₁ cells were utilised as a tractable model system to investigate the mechanisms by which the S1P₁ receptor may employ.

4.2 Results

The cryoprotective role of S1P₁ is documented in endothelial cells but it is unknown if (a) this is a general feature of S1P₁ signalling irrespective of cell type, or (b) the mechanism is conserved between different types of cells. The regulation of Bim by phosphorylation by JNK and ERK has been characterised in CCL39 cells [274, 380]. To determine how CCL39 cells and CCL39 cells stably expressing the myc epitope-tagged S1P₁ receptor (CCL39mycS1P₁) would respond to removal of trophic factors they were cultured in serum-free conditions over a period of 24 hours. In response to serum deprivation the majority of control CCL39 cells had rounded up and detached from the substratum by 24 hours (Figure 4.1). However, CCL39mycS1P₁ cells were resistant to serum deprivation-induced anoikis and continued to proliferate even after subjection to serum-free conditions for 24 hours (Figure 4.2). To assess whether the CCL39 cells were apoptotic, the level of cleaved, active caspase-3 was determined by western blotting. In response to serum deprivation, control CCL39 cells showed a significant increase in the levels of cleaved caspase-3 at 6 hours, which peaked at 12 hours and was still detected at 24 hours. However, the level of cleaved caspase-3 in CCL39mycS1P₁ cells did not rise significantly above the basal level at any point over the 24 hour time course (Figure 4.3). To ensure that detection of cleaved caspase-3 by western blot was an accurate marker for cells undergoing apoptosis a colorimetric assay of caspase-3 activity was employed. In serum-deprived control CCL39 cells, caspase-3 activity was elevated (3pmol/min) compared to those cultured in serum, in which no significant levels of caspase-3 activity could be detected. This increase in activity was blocked by the presence of the specific caspase-3 inhibitor AcDEVD-CHO. However, caspase-3 activity was decreased (0.23pmol/min) in CCL39mycS1P₁ cells, compared to control CCL39 cells. In addition to this, caspase-3 activity was undetectable in the presence of serum or the inhibitor AcDEVD-CHO. This correlates with the serum withdrawal-induced caspase-3 cleavage detected by western blotting (Figure 4.4).

The apoptosis initiator protein, Bim, has been shown to be up-regulated in CCL39 cells in response to serum deprivation [274]. So to determine if the expression of S1P₁ has any effect on Bim induction, the level

of Bim present in serum-deprived control CCL39 cells and CCL39mycS1P₁ was also detected. The expression of Bim was promoted in response to serum deprivation and had increased significantly above the basal level by 3 hours in control CCL39 cells, thereby preceding caspase-3 activation as expected. Bim levels did not rise significantly above the basal in CCL39mycS1P₁ cells at any point over the 24 hour time course (Figure 4.5). Thus, serum deprivation of CCL39 cells induces an apoptotic phenotype which is confirmed by the concurrent increase in Bim expression and caspase-3 activation. However, the over-expression of S1P₁ attenuates the rise in Bim expression and caspase-3 activation thereby promoting survival.

To determine whether agonist stimulation of S1P₁ would enhance this effect, S1P and the selective agonist FTY720P were introduced to both control CCL39 cells and CCL39 mycS1P₁ cells after 16 hours of serum starvation. FTY720P, which is a potent agonist of all S1P receptors except S1P₂, significantly decreased the level of cleaved caspase-3 in CCL39mycS1P₁ cells (Figure 4.6). However, FTY720P stimulation of S1P₁ had no significant effect on the expression levels of Bim (Figure 4.7). To assess whether intracellular levels of S1P may influence the observed effect of S1P₁ expression, the competitive SphK inhibitor dihydrosphingosine (DHS) was added to prevent endogenous S1P production. This had no effect on either caspase-3 cleavage or Bim expression in control CCL39 cells or CCL39mycS1P₁ cells (Figure 4.8 and 4.9). The constitutive protective effect of S1P₁ over-expression is not due to the autocrine production of S1P. Furthermore the S1P₁-mediated down-regulation of caspase-3 cleavage is not dependent on the down-regulation of Bim.

To determine which signalling pathways may be involved in the S1P₁-mediated inhibition of apoptosis a number of selective inhibitors of S1P-activated pathways were tested for their effects on Bim induction and caspase-3 cleavage. The MEK inhibitor U0126 had no effect on caspase-3 cleavage suggesting that serum-withdrawal induced apoptosis in CCL39 cells is not mediated by ERK. Pertussis toxin caused a significant increase in caspase-3 cleavage in CCL39mycS1P₁ cells confirming that the observed protection is G_i-linked. Although not significant, an increase in Bim expression was observed in U0126 treated cells which did not result in

increased caspase-3 cleavage, consistent with the possibility that an increase in the level of Bim expression alone may not be sufficient to induce caspase-3 cleavage and apoptosis (Figure 4.10).

To determine the specific signalling pathways involved, selective inhibitors of other S1P₁-mediated pathways were used. Treatment with PI3 kinase inhibitor LY294002 showed a significant increase in caspase-3 cleavage and although the PKC inhibitor GF109203X also showed an increase in caspase-3 cleavage it was not significant. However, the addition of both inhibitors together shows a dramatic increase in the amount of cleaved caspase-3 detected, completely blocking its down-regulation by S1P₁ (Figure 4.11). Furthermore, the level of Bim in the cell appears to be unaffected by the attenuation of S1P₁-mediated inhibition of caspase-3 cleavage which suggests another level of regulation (Figure 4.12). To assess whether the S1P₁ receptor was driving the synthesis of a pro-survival mediator, the chemical emetine was used to block protein synthesis. This treatment resulted in a 6-fold increase in caspase-3 cleavage despite the presence of S1P₁. The expression of Bim appears to decrease over the time course, however, the rate of change between control CCL39 cells and CCL39mycS1P₁ cells is not significantly different (Figure 4.13). This suggests that the S1P₁ receptor is driving the expression of a pro-survival mediator that protects the cell from serum withdrawal-induced apoptosis.

4.3 Discussion

S1P is an important regulator in apoptosis, this chapter shows that the receptor S1P₁ has a protective effect against serum-withdrawal induced apoptosis in CCL39 fibroblasts. Although the level of the S1P₁ receptor expression in the stably transfected CCL39 cell is unknown, its presence seems to be sufficient to confer protection against apoptosis by serum deprivation. This effect is observed in the absence of agonist and whilst inhibiting endogenous S1P production by SphK (Figures 4.3 and 4.8). This implies that there may be sufficient numbers of receptors spontaneously adopting an active conformation, thereby driving receptor signalling. This could be determined by using the radioligand [³H]-dihydrosphingosine-1-phosphate [381]. This phenomenon has been observed with β -adrenergic receptor where over-expression the G_s linked receptor in NG108-15 cells (mouse neuroblastoma and glioma hybrid) results in the constitutive activity of AC [382]. However, it is unlikely that this is the case for S1P₁ there is no constitutive agonist-independent phosphorylation of ERK (Figure 3.16). Although not significantly high there is detectable basal levels of PKB phosphorylation thus the constitutive action of the S1P₁ receptor may be restricted to specific pathways that are responsible for promotion of survival (Figure 4.11).

A role for S1P₁ in the receptor tyrosine kinase (RTK) PDGF β -mediated activation of ERK has been established, where S1P₁ and the PDGF receptor form a signalling complex [383, 384]. In a recent study in HEK and aortic smooth muscle cells this was confirmed using an inverse agonist of S1P₁ called SB649146 which disrupted PDGF β activation of ERK [385]. This would be expected to attenuate S1P₁-mediated survival. PDGF β receptors are expressed in CCL39 cells so it is possible that the S1P₁ receptor and PDGF β receptor are required to associate to initiate downstream survival signals. This could be determined by using a PDGF receptor kinase inhibitor of which many are used in anti-cancer therapies, which would block S1P₁ receptor-induced survival [386]. Another RTK involved in PI 3-kinase/PKB mediated cell survival is the insulin-like growth factor (IGF) receptor which has recently been linked to transactivation of S1P receptors. The neutral

antagonist of the S1P₁ and S1P₃ receptors, VPC23019, which has been demonstrated to block IGF-stimulated ERK activation would be expected to have no effect on the effect on S1P₁-mediated survival [387].

The inhibition of protein synthesis by emetine treatment attenuates S1P₁-mediated survival (Figure 4.13). This implicates S1P₁ in the control of the expression of one or more anti-apoptotic proteins which may bind and sequester pro-apoptotic proteins such as Bim. An anti-apoptotic protein such as Mcl-1 which has a short half-life of approximately three hours [388, 389] and is known to bind and negate the pro-apoptotic effects of Bim is a good candidate. Furthermore, PKB has been implicated in Mcl-1 stability as inhibition of its downstream target GSK-3 by inhibitors, resulted increased proteosomal degradation of Mcl-1 [390]. In CCL39 cells it has been demonstrated that treatment with the PI 3-kinase inhibitor LY294002 causes the down-regulation of Mcl-1 in response to serum starvation which results in Bax activation and apoptosis [391]. However, the up-regulation of Bim due to treatment with the MEK inhibitor U0126 (as shown in Figure 4.10), does not correspond with an increase in caspase-3 activity. This suggests that actual Bim levels do not induce apoptosis in this system, and the increase in expression levels of Bim observed, is due to the lack of ERK activity mediating its degradation.

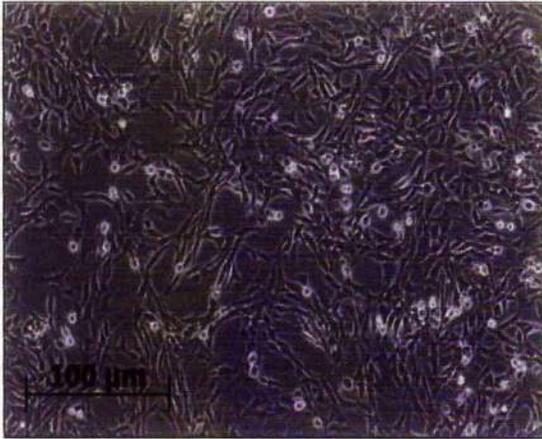
The protective effect of S1P₁ in the absence of serum was reversed using LY294002 and the broad spectrum-specific PKC inhibitor GF109203FX. The use of each of these inhibitors singly did increase apoptosis but only when used together did they completely reverse S1P₁'s protective effect. S1P₁ can be phosphorylated independently of agonist by PKC which is associated with loss of the receptor from the cell surface [367]. Therefore, the presence of PKC may be responsible for attenuating S1P₁ signalling by internalisation. PKC has also been demonstrated to stabilise newly formed Mcl-1 by phosphorylation [392]. S1P₁ activates eNOS via PKB; the subsequent production of NO is presumably responsible for the cryoprotective effect of S1P₁ signalling. PKB also promotes survival through the phosphorylation and inactivation of the pro-apoptotic protein Bad [209].

As these observations were made in an artificial model system where S1P₁ was over-expressed it is difficult to put these data in context. However,

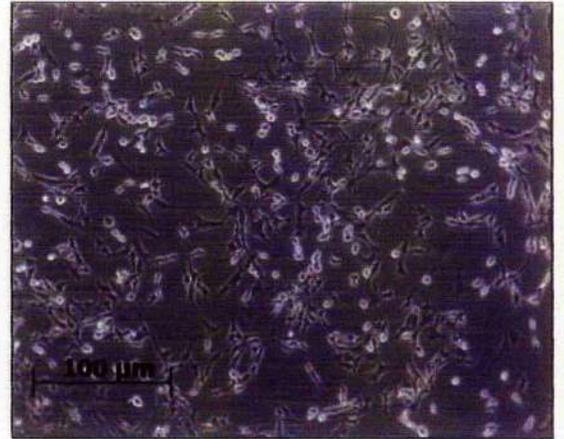
it does provide an excellent basis to explore the mechanisms used in other cells types where S1P is an important mediator of survival such as endothelial cells, VSMCs and also immune cells such as B and T lymphocytes where Mcl-1 expression is required for both development and maintenance in mature cells [213, 333, 393]. Experiments in cells with endogenous receptor would give more insight into the physiological role of S1P₁ signal transduction in survival. It would also be interesting to look at the effects of cytotoxic drugs such as paclitaxel to determine whether this response is confined to apoptosis induced by removal of trophic factors. Furthermore, confirmation that CCL39mycS1P₁ cells do not undergo the phenotypic changes expected from apoptotic cells by annexin V and propidium iodide staining and flow cytometry, would also be beneficial.

Figure 4.1 Control CCL39 Cell Morphology in Response to Serum Deprivation

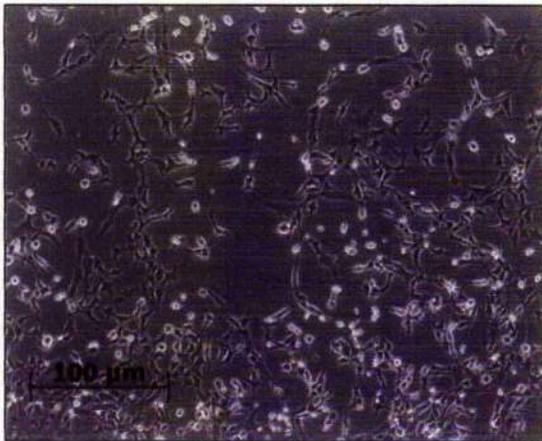
Light micrograph images of control CCL39 cells deprived of serum for 3, 6, 12 and 24 hours. Images were captured using a Zeiss microscope (10 x objective lense) and manipulated with Axiovision software.



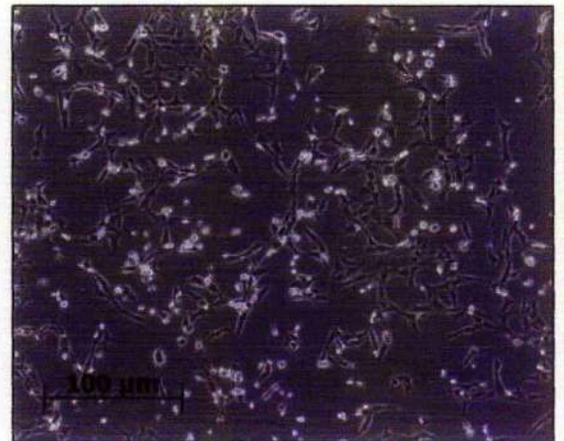
0 hours



3 hours



6 hours



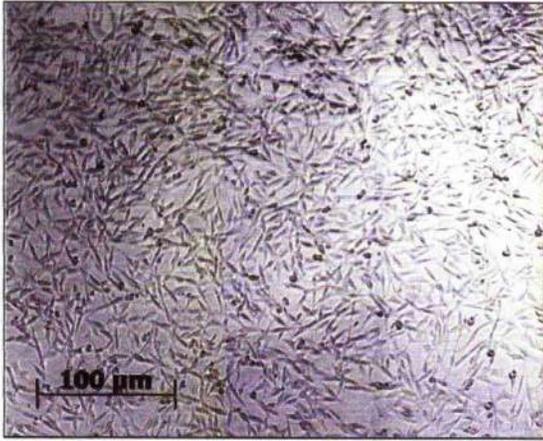
12 hours



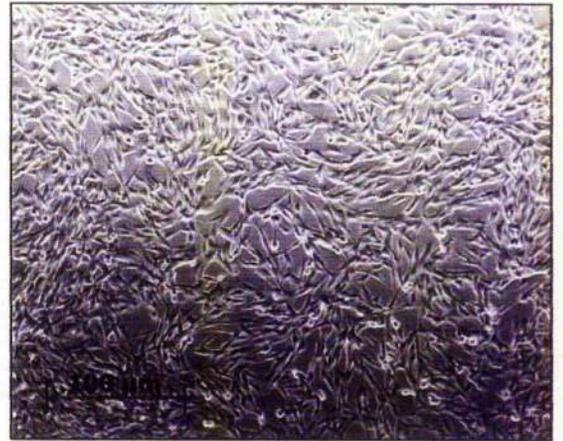
24 hours

Figure 4.2 CCL39mycS1P₁ Cell Morphology in Response to Serum Deprivation

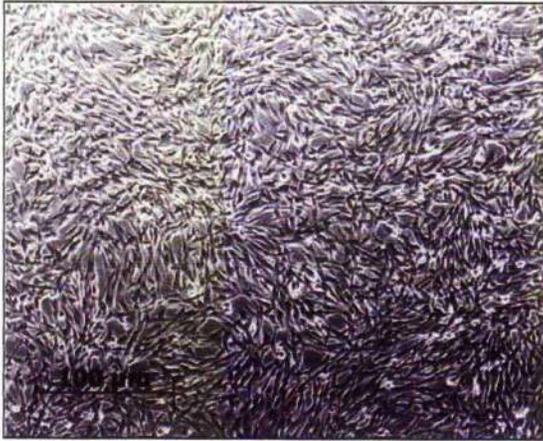
Light micrographs of CCL39 cells stably transfected with mycS1P₁ deprived of serum for 3, 6, 12 and 24 hours. Images were captured using a Zeiss microscope (10 x objective lense) and manipulated with Axiovision software.



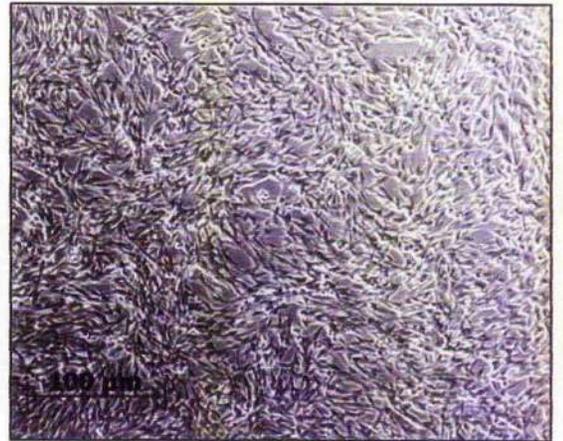
0 hours



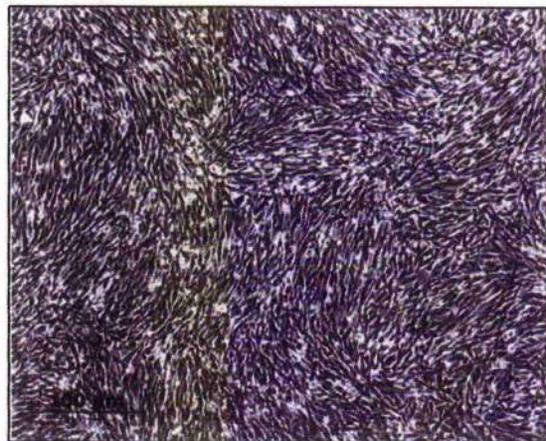
3 hours



6 hours



12 hours



24 hours

Figure 4.3 Serum Deprivation of Control CCL39 cells and CCL39mycS1P₁ Cells and its Effect on Caspase-3 Cleavage

Detection of cleaved caspase-3 in CCL39 cells and CCL39mycS1P₁ cells which were deprived of serum for 3, 6, 12, and 24 hours by western blot. Receptor and GAPDH levels were also detected by western blot. *p* values are from a one way ANOVA with a Bonferroni comparisons test, bars marked with *** have a *p*<0.001. This Figure is a representative from three experiments.

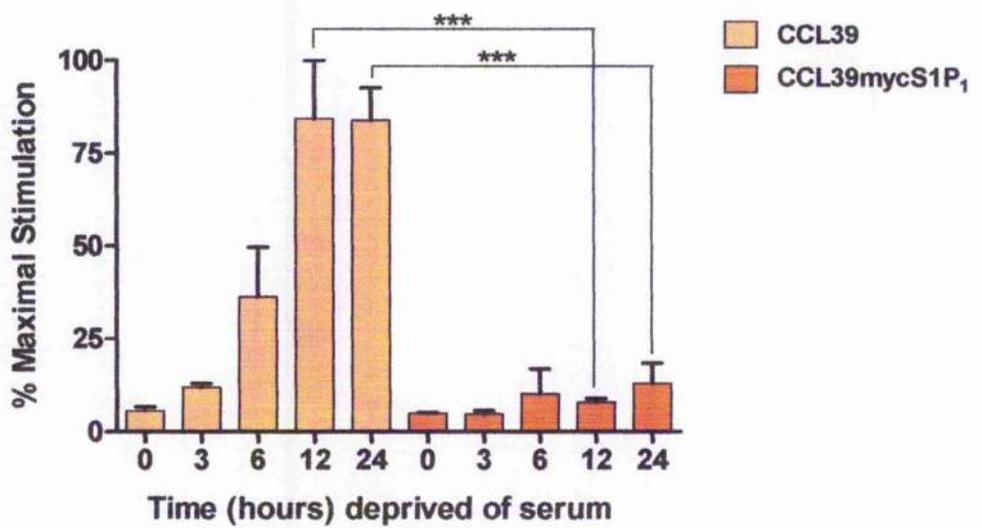
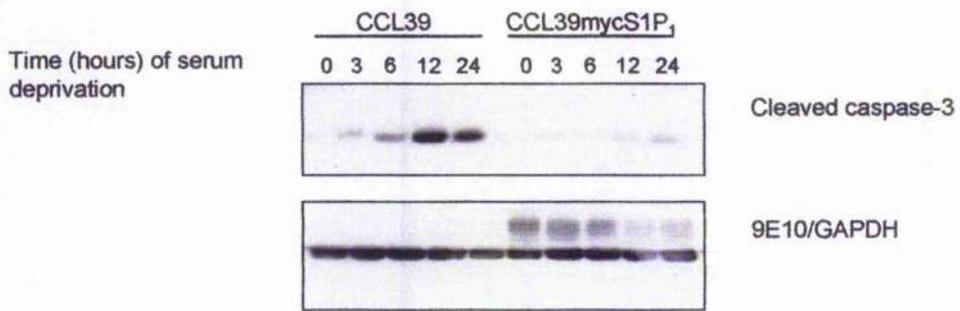


Figure 4.4 Colorimetric Determination of Caspase-3 Activity in Control CCL39 Cells and CCL39mycS1P₁ Cells

Apoptosis was induced by serum deprivation for 16 hours, extracts were prepared and analysed as per manufacturer's instructions. The colour change detected is caused by cleavage of chromophore *p*-nitroanaline (*p*NA) from the labelled substrate DEVD-*p*NA. A standard curve was constructed and from which the activity of each sample determined. The colorimetric change was determined by reading the samples at OD₄₀₅ and the activity calculated from the standard curve (see section 4.2).

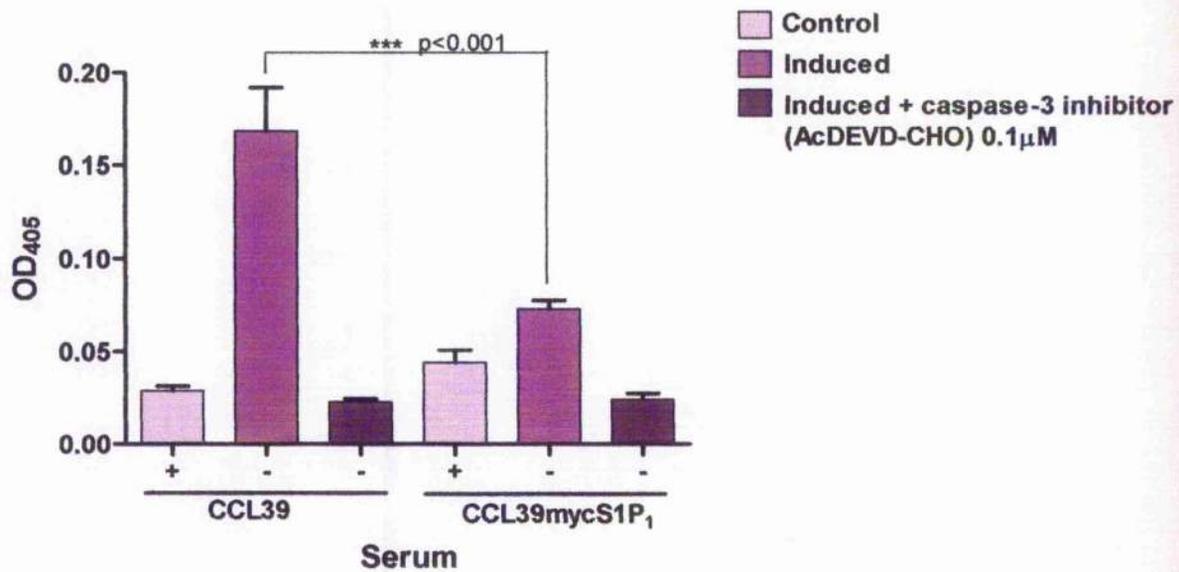
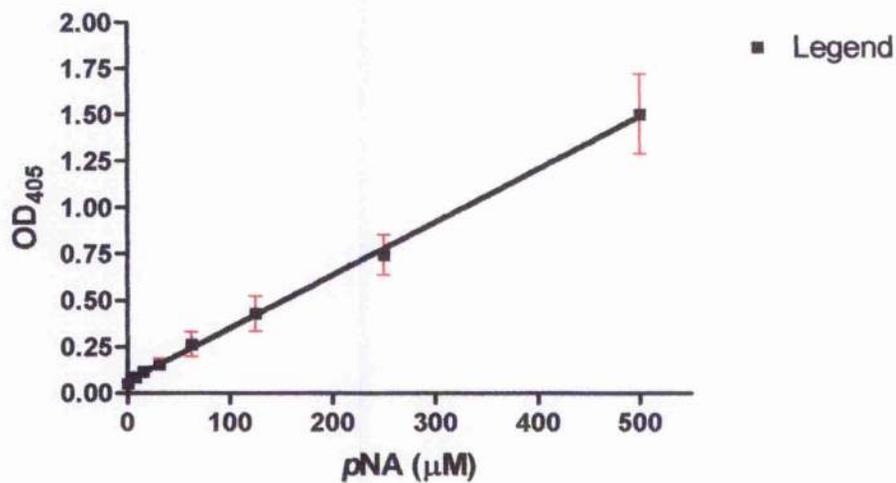


Figure 4.5 the Effect of Serum Deprivation of Control CCL39 Cells and CCL39mycS1P₁ Cells on the Level of Bim Expression

Detection of Bim in CCL39 cells and CCL39mycS1P₁ cells which have been serum-starved for 3, 6, 12, and 24 hours by western blot. Receptor and GAPDH levels were also detected by western blot. p values are from a one way ANOVA with a Bonferroni comparisons test, bars marked with *** have a $p < 0.001$. This Figure is a representative from three experiments.

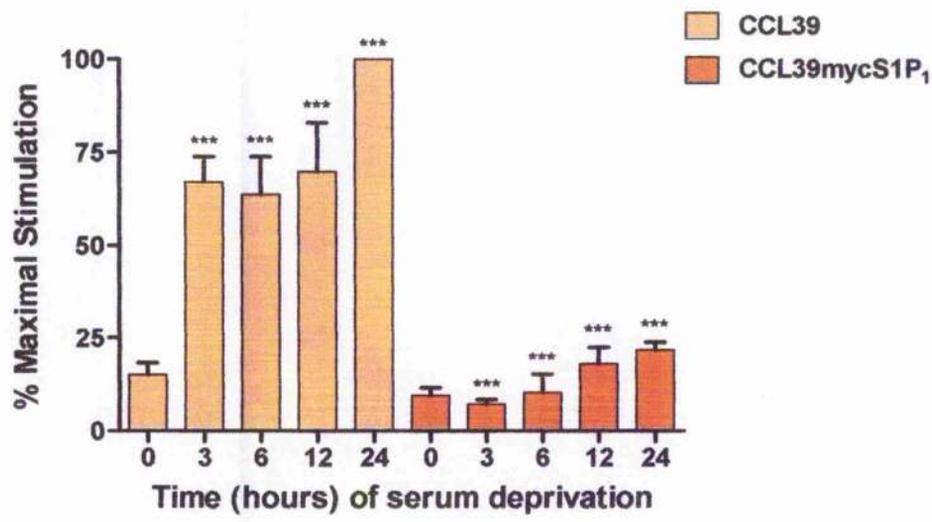
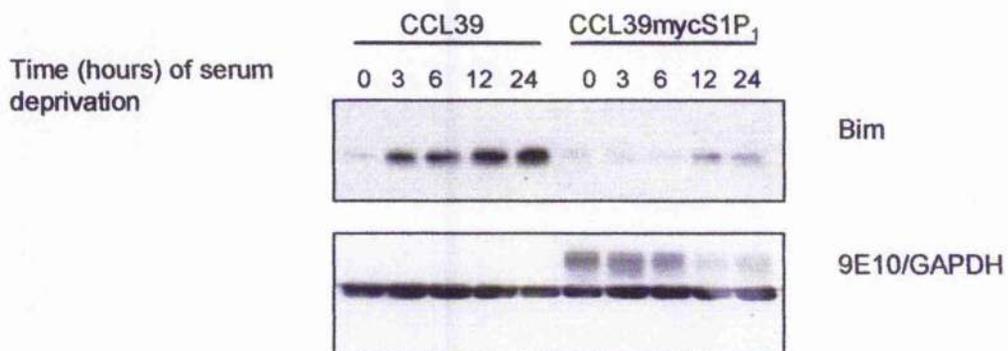


Figure 4.6 The Effect of Agonist Stimulation on Caspase-3 Cleavage in Control CCL39 Cells and CCL39mycS1P₁ Cells

Detection of caspase-3 cleavage by western blot in CCL39 cells and CCL39mycS1P₁ cells serum-starved for 16 hours and treated concurrently with S1P (5 μ M) or FTY720P (0.1 μ M). Receptor level and GAPDH levels were also detected by western blot. *p* values are from a one way ANOVA with a Bonferroni comparisons test, bars marked with *** have a *p*<0.001. This Figure is a representative from three experiments.

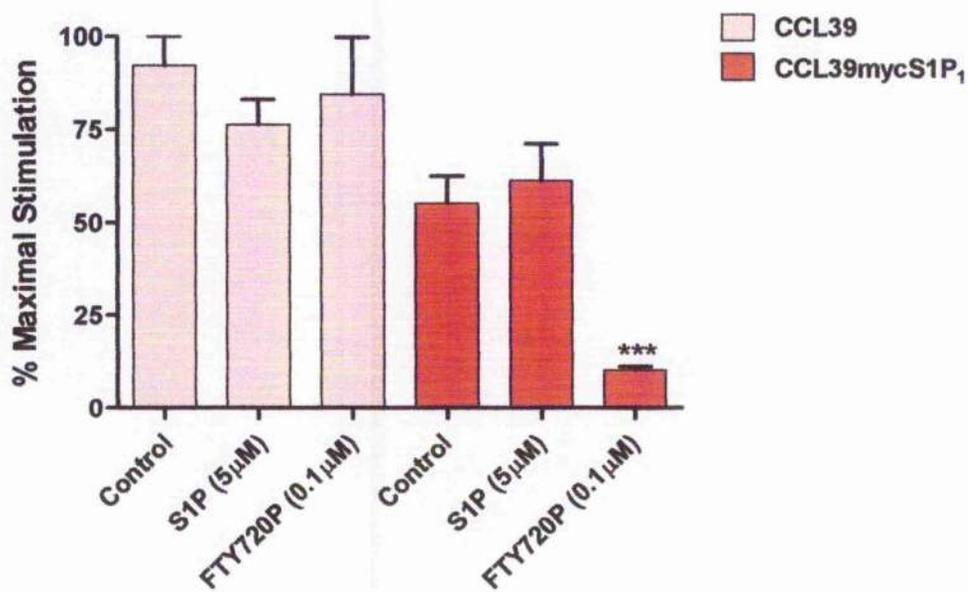
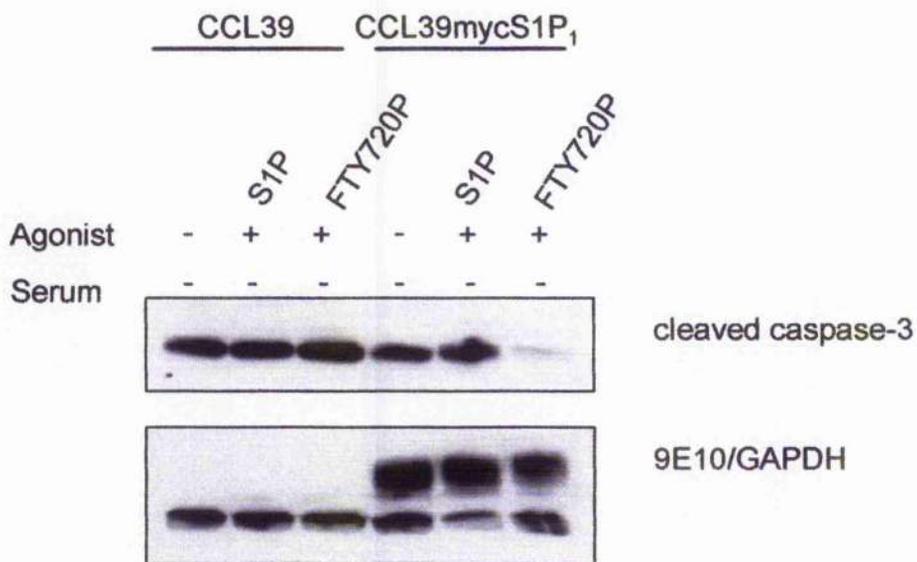


Figure 4.7 The Effect of Agonist Stimulation on Bim Expression in Control CCL39 Cells and CCL39mycS1P₁ Cells

Detection of Bim of CCL39 cells and CCL39mycS1P₁ cells by western blot which have been serum-starved for 16 hours treated concurrently with S1P (5 μ M) or FTY720P (0.1 μ M). Receptor levels and GAPDH levels were also detected by western blot and this Figure is a representative of three experiments.

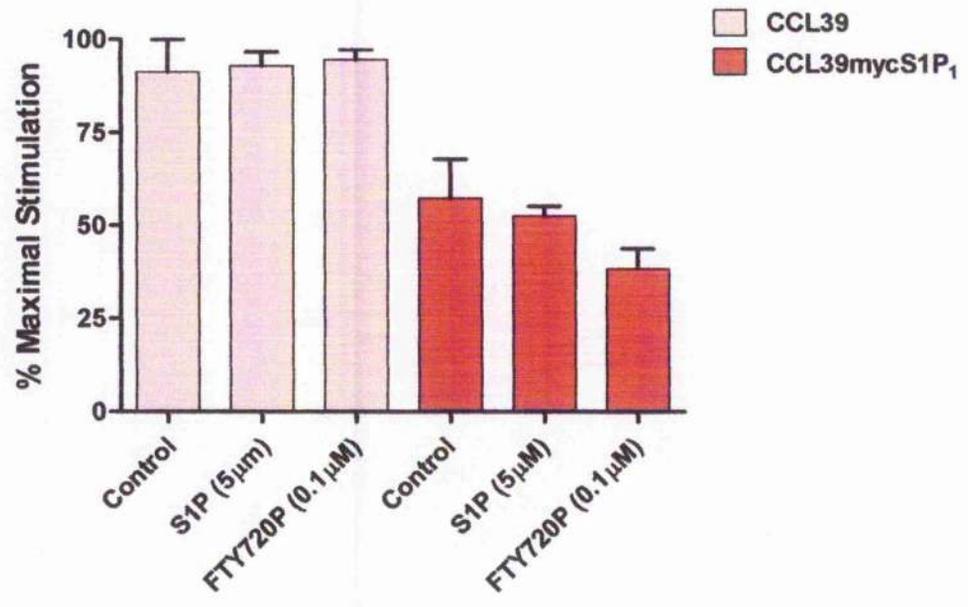
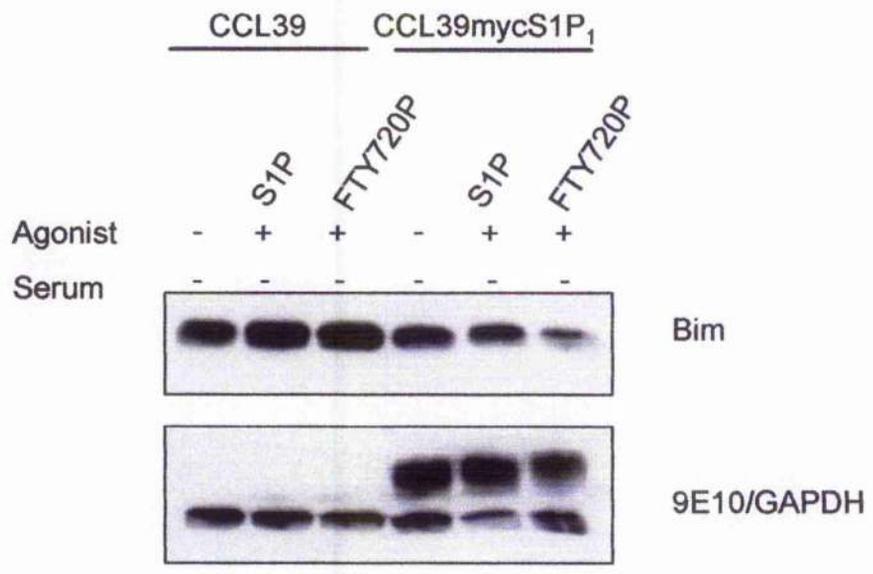


Figure 4.8 The Effect of the Sphingosine kinase Inhibitor DHS on Caspase-3 Cleavage

Detection of cleaved caspase-3 western blot in both CCL39 cells and CCL39mycS1P₁ cells which have been serum-starved and treated concurrently with the sphingosine kinase inhibitor L-*threo*-dihydrosphingosine (DHS) for 16 hours. Receptor levels and GAPDH levels were also detected by western blot and this Figure is representative of three experiments.

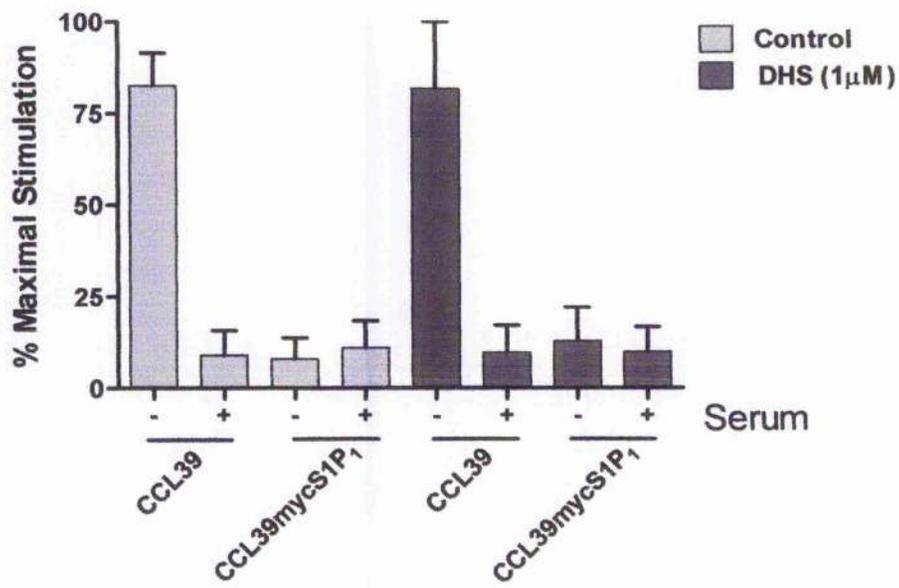
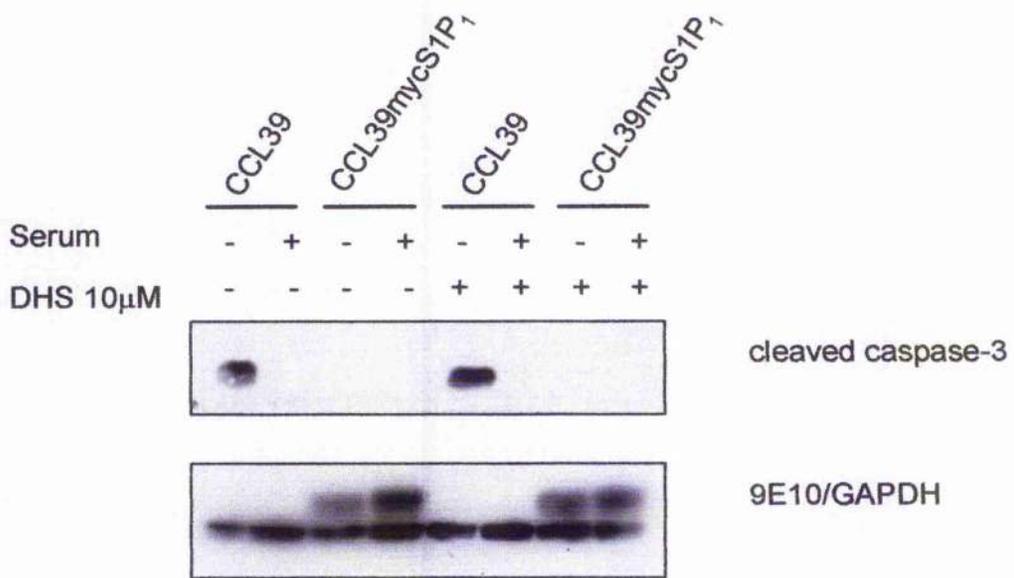


Figure 4.9 The Effect of the Sphingosine kinase Inhibitor DHS on Bim Expression

Detection of Bim by western blot in both CCL39 cells and CCL39mycS1P₁ cells which have been serum-starved and treated concurrently with the sphingosine kinase inhibitor *L-threo*-dihydrosphingosine (DHS) for 16 hours. Receptor levels and GAPDH levels were also detected by western blot and this Figure is representative of three experiments.

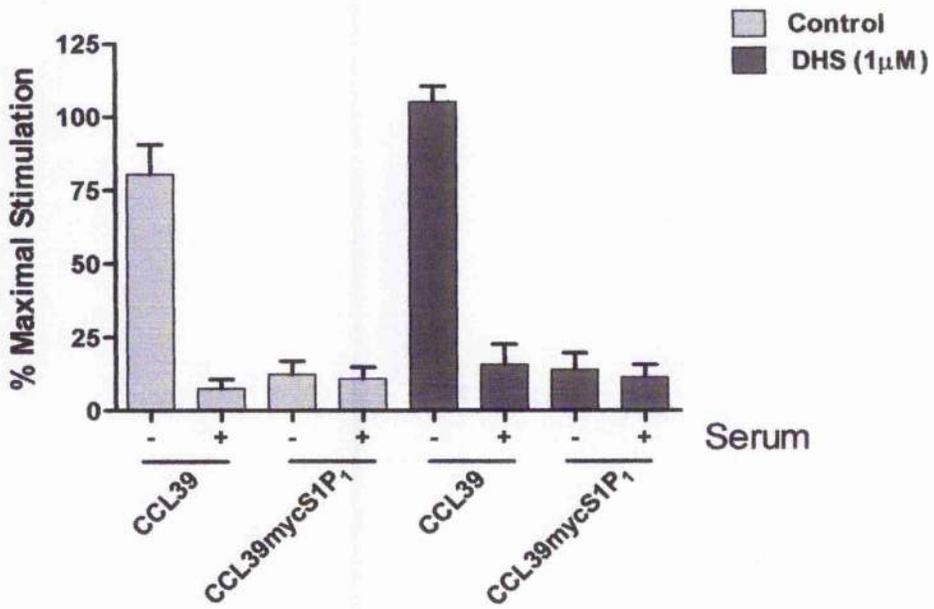
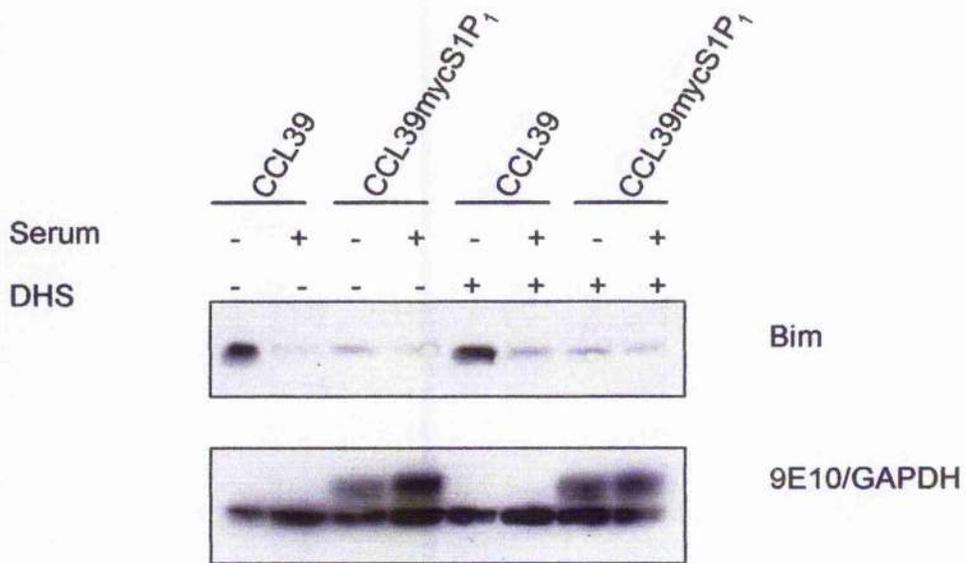


Figure 4.10 The Effect of Pertussis Toxin and U0126 on Caspase-3 Cleavage and Bim Expression

Detection of cleaved caspase-3 and Bim levels by western blot in CCL39mycS1P₁ cells cultured in either complete DMEM or serum-free DMEM for 16 hours. These cells were treated concurrently with the inhibitors U0126 at a concentration of 10 μ M and pertussis toxin (PTX) at a concentration 100ng/ml. Receptor, GAPDH, phospho-ERK (P-ERK) and total ERK levels were also detected by western blot. p values are from a one way ANOVA with a Bonferroni comparisons test, bars marked with * have a $p < 0.05$. This figure is a representative from three experiments.

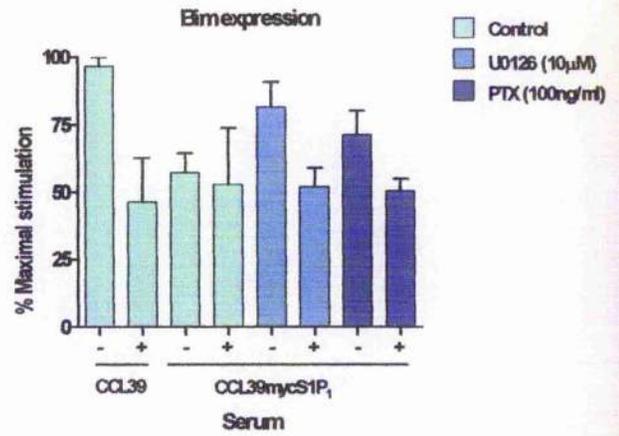
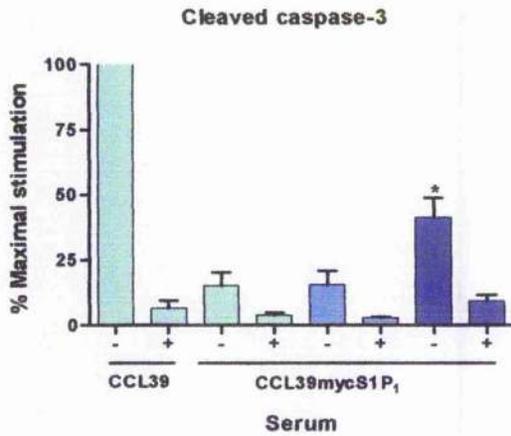
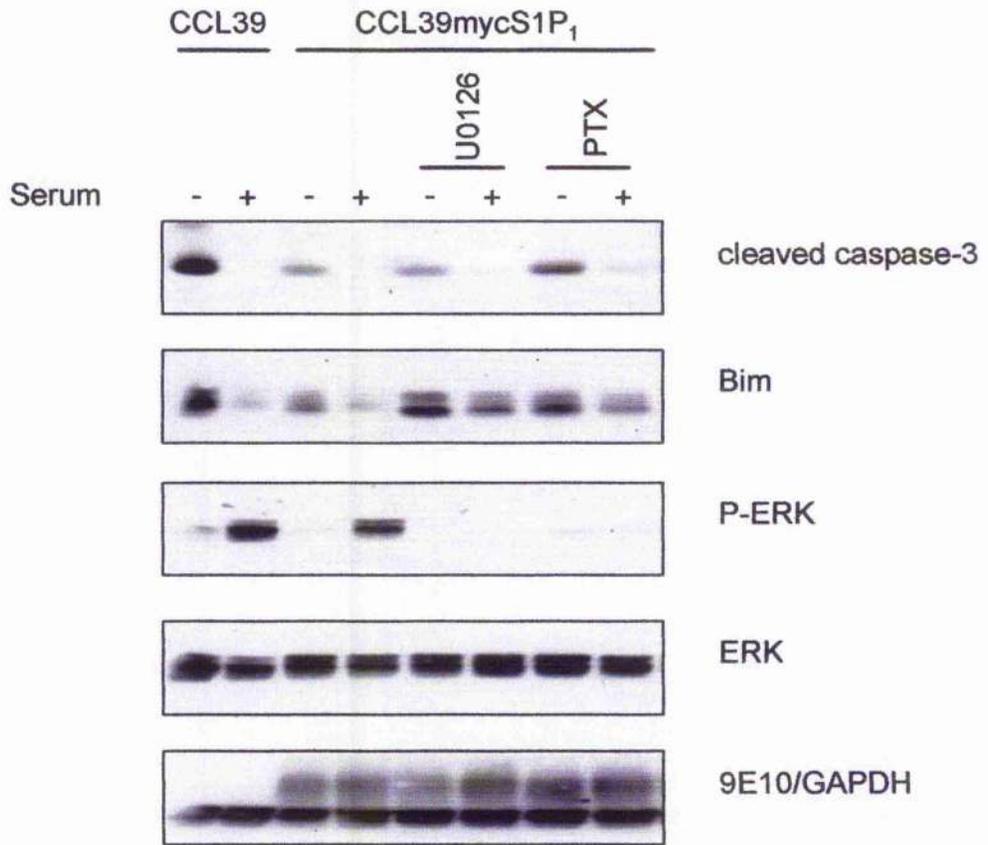


Figure 4.11 The Effect of the Inhibitors LY294002 and GF109203X on Caspase-3 Cleavage

Detection of cleaved caspase-3 and phospho-PKB (P-PKB) by western blot in both CCL39 cells and CCL39mycS1P₁ cells which were cultured in either complete DMEM or serum-free DMEM for 16 hours. CCL39mycS1P₁ cells were treated with either LY294002, GF109203X or both inhibitors together. Receptor and GAPDH levels were also detected by western blot. p values are from a one way ANOVA with a Bonferroni comparisons test, bars marked with *** have a p<0.001. This figure is a representative from three experiments.

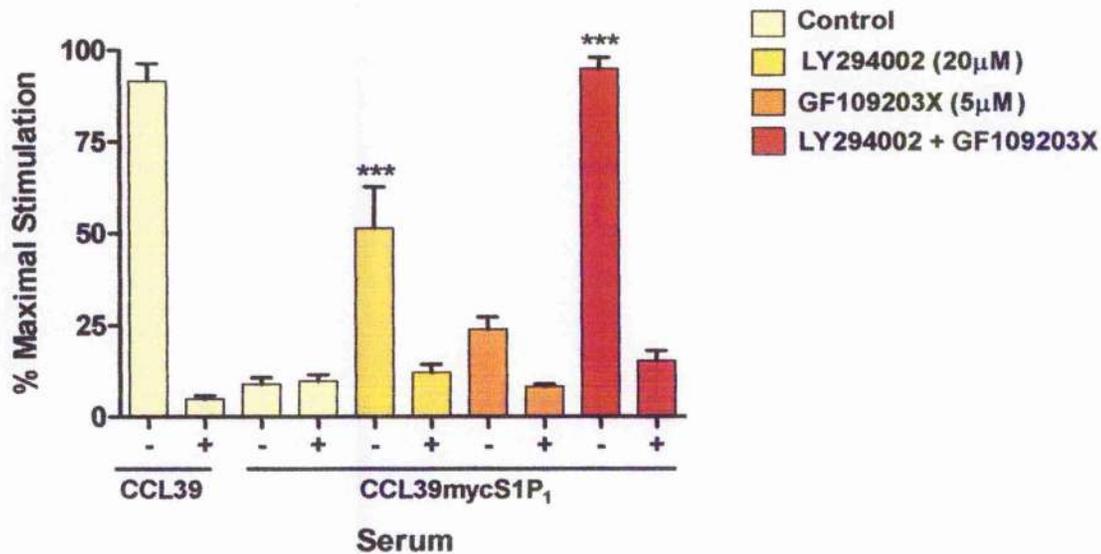
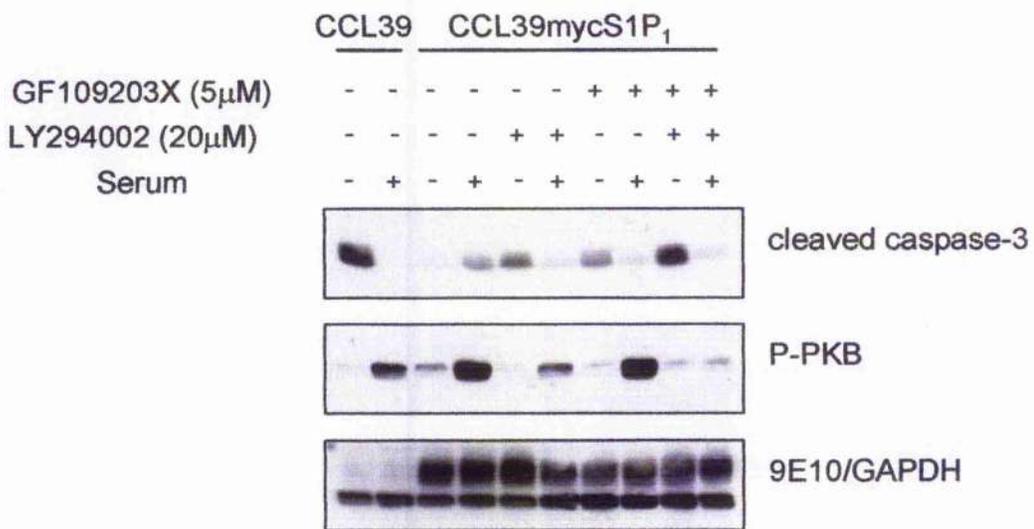


Figure 4.12 The Effect of the Inhibitors LY294002 and GF109203X on Bim Expression Levels

Detection of Bim and phospho-PKB (P-PKB) by western blot in CCL39 cells and CCL39mycS1P₁ cells which were cultured in either complete DMEM or serum-free DMEM for 16 hours. CCL39mycS1P₁ cells were treated with either LY294002, GF109203X or both inhibitors together. Receptor and GAPDH levels were also detected by western blot and this Figure is a representative of three experiments.

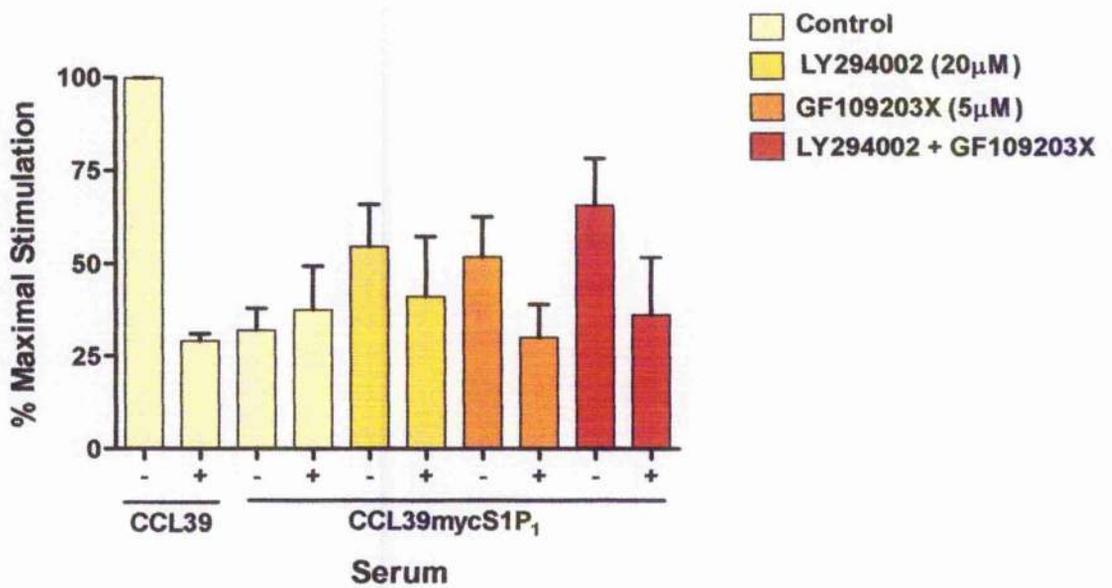
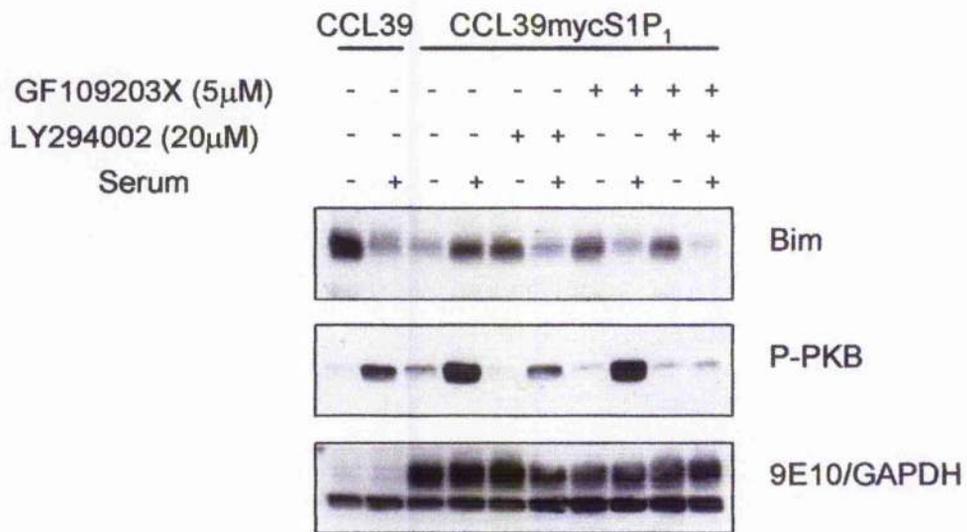
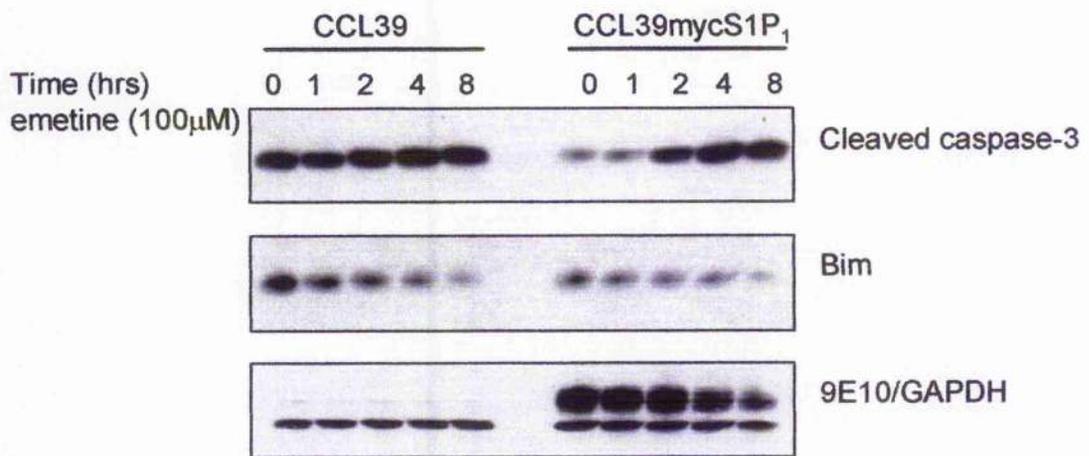
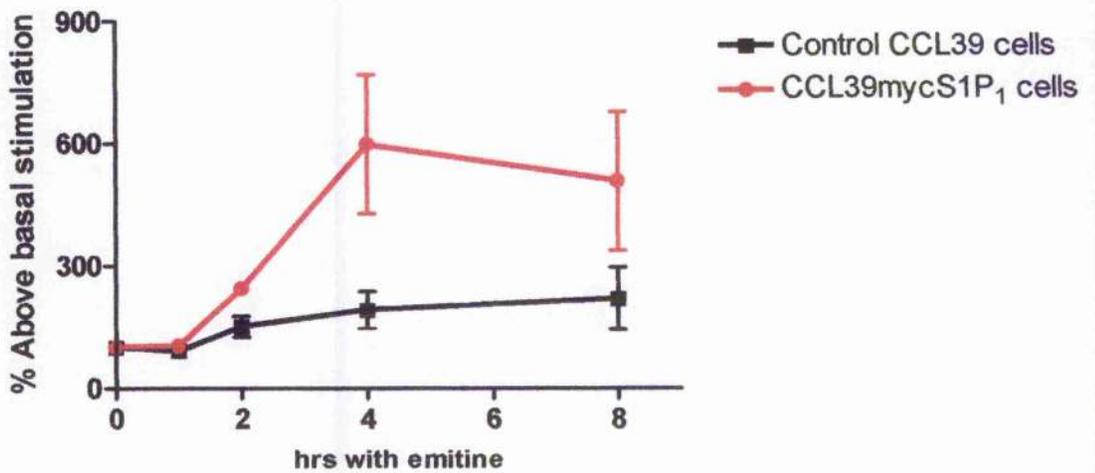


Figure 4.13 The Effect of Emetine Treatment on Caspase-3 Cleavage and Bim Expression

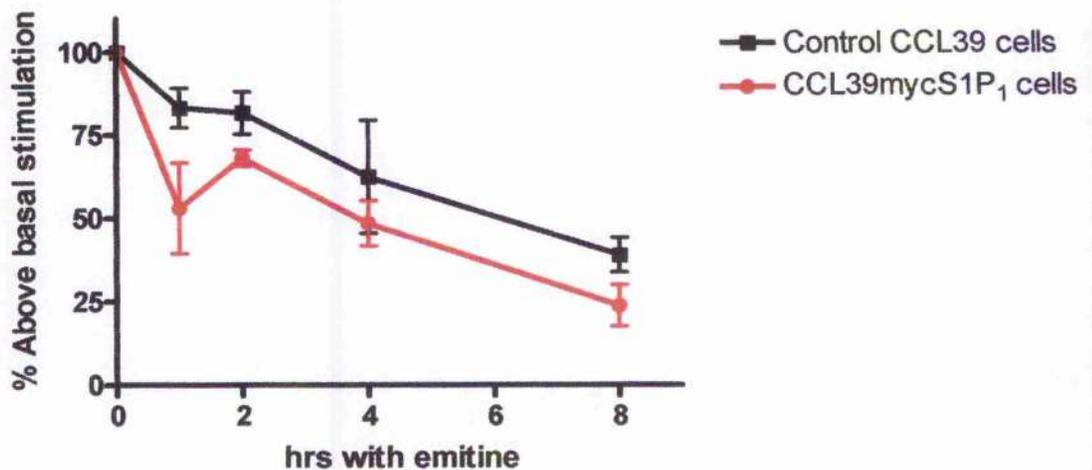
Detection of cleaved caspase-3 and Bim in control CCL39 cells and CCL39mycS1P₁ cells were serum-starved for 16 hours and subjected to treatment with the inhibitor of protein synthesis, emetine (100 μ M) over a time course of 1, 2, 4 and 8 hours. Receptor and GAPDH levels were also detected by western blot and this Figure is a representative of three experiments.



Cleaved caspase-3



Bim Expression



Chapter 5
Final Discussion

Summary

The bioactive lipid S1P has been established as a mediator of growth-related mechanisms such as cytoskeletal dynamics, migration and cell survival [68]. S1P is derived from sphingomyelin, which is a constituent of all eukaryotic membranes and is manufactured within the cell [77]. S1P and its derivatives have been detected in yeast to man and the enzymes controlling its production are also conserved [71]. SphK, perhaps the most important of these, has long been associated with proliferation and cell survival [78, 83] but the mechanism for this was unknown until cell surface receptors for the lipid were identified.

S1P is abundant in activated platelets and once secreted, can mediate its effects through binding and activating five related GPCRs termed S1P₁₋₅ [119]. Extracellular S1P is mainly found associated with HDLs and is present in both serum and plasma [103]. The first cell surface receptor to be identified for S1P and the most studied of the receptors is S1P₁, which was found to be expressed in abundance on endothelial cells [115]. Treatment with S1P was shown to promote capillary-like structures in endothelial cultures suggesting a role in vascular processes such as angiogenesis [76, 116]. S1P₁ can be modified by phosphorylation by intracellular kinases such GRK-2 and PKC which causes the removal of the receptor from the cell surface into vesicles [367].

S1P₁₋₃ are ubiquitously expressed and influence cytoskeletal dynamics and migration *via* the differential regulation of small G proteins. S1P₁ and S1P₃ activate Rac which results in lamellipodial formation and migration whereas S1P₂ inhibits Rac which thereby abolishes lamellipodia and migration [213, 216]. The regulation of Rac has importance in pathological conditions such as cancer. In a melanoma metastasis model use of dominant negative Rac demonstrated a requirement for Rac activation in metastasis and invasion [394].

In Chapter 3 it is shown that the use of inhibitors such as colchicine, cytochalasin B and pertussis toxin did not have any feedback effect on S1P₁ receptor regulation. There was no change in levels of S1P₁ phosphorylation or internalisation. There was no detectable change in levels of GTP-bound Rac or Cdc42. In addition to this, the engagement of integrins which is also

associated with small G protein activation by culturing cells on fibronectin and vitronectin did not induce S1P-mediated Rac or Cdc42 activation. S1P₂, which is known to inhibit Rac was not detected and use of the selective agonist FTY720P which activates every S1P receptor except S1P₂ did not induce Rac or Cdc42 activation. However, S1P treatment did induce the formation of stress fibres which is a Rho-mediated process and inhibitory of Rac [162]. It is possible that these cells lack an essential co-factor required such as the GEF Tiam-1, since the evidence in Chapter 4 indicates PI 3-kinase/PKB pathway, which is well documented to activate Rac, is functional in these cells [395]. It would, therefore, be beneficial to repeat these experiments in cells where S1P₁ is expressed endogenously, such as endothelial cells, rather than in a system where the receptor is over-expressed.

As mentioned previously, S1P has long been associated with cell survival, expression of SphK and subsequent S1P production protects against apoptosis induced by a number of stimuli including the removal of trophic factors [92, 328]. S1P₁ has been demonstrated to activate eNOS through the PI 3-kinase/PKB pathway and there are several mechanisms through which NO mediates its cytoprotective effect (as described in section 1.6.9). Furthermore, it has been demonstrated in CCL39 cells that attachment to the substratum is a requirement for PKB signalling [197].

Regardless of the origin of apoptotic stimuli, each signalling pathway converges at the mitochondria and the release of cytochrome *c*. Removal of trophic factors causes the up-regulation of BH3-only proteins such as Bim. This promotes cytochrome *c* release and initiates the caspase cascade, which ultimately ends in the dismantlement of the cell [283, 336]. Anti-apoptotic Bcl-2 proteins, such as Mcl-1 serve to sequester proapoptotic proteins, therefore, it is often the balance between these classes of protein that will determine cell fate [396]. Caspase-3 is termed an executioner caspase, once activated by cleavage at conserved Asp residues, the cell is committed to the apoptotic pathway, thereby making it a good marker for apoptotic cells [263]. This was supported by findings in Chapter 4 where CCL39 cells subjected to serum deprivation showed elevated levels of

caspase-3 activity in conjunction with the observed elevation of both Bim and caspase-3 levels.

The findings of this thesis indicate that the expression of S1P₁ alone is enough to attenuate the observed increase in caspase-3 cleavage and Bim levels in CCL39 cells. The presence of agonist is not required to see this effect, and inhibition of S1P production does not affect S1P₁-mediated cytoprotection. However, it is likely that this constitutive activity is restricted to pathways controlling survival as it was not accompanied by ERK activation. The protective effect conferred by S1P₁ was attenuated by inhibiting PI 3-kinase (by LY294002) and PKC (by GF203109X). Each of these decreased the level of caspase-3 cleavage but only collectively could they inhibit the S1P₁ receptor's effect. There is further evidence of another level of regulation in the discovery that increased Bim levels do not equate to increased caspase-3 levels. This is compounded by the discovery that inhibition of protein synthesis by emetine treatment attenuates S1P₁-mediated protection from serum withdrawal. This suggests that S1P₁ is the driving force behind the expression of a pro-survival protein which negates the pro-apoptotic effects of Bim expression. An anti-apoptotic Bcl-2 protein such as Mcl-1 seems like a potential candidate as studies in CCL39 cells have demonstrated that treatment with the PI 3-kinase inhibitor LY294002 causes the down-regulation of Mcl-1 in response to serum starvation which results in Bax activation and apoptosis [391]. The next step would be to determine how over-expression of S1P₁ affects Mcl-1 expression.

Although these observations were made in an artificial system, the evidence strongly points to a role for S1P₁ signalling in the up-regulation of one or more anti-apoptotic mediators. Use of selective inhibitors shows that the mechanism by which S1P₁ protects against apoptosis induced by serum withdrawal requires PI 3-kinase and PKC activation.

S1P₁ is an important mediator of growth-related functions of the cell and the vasculature as a whole. Understanding the mechanisms by which it regulates migration, angiogenesis and apoptosis gives valuable insight into treating diseases caused by the dysfunction of these processes such as cancer. S1P₁ signalling is linked to another important mediator of growth-related functions, PDGFR. It is believed that they form a signalling complex

and co-operate as co-mitogens and promoters of growth [384]. There are many PDGF kinase inhibitors that block aberrant PDGF signalling in cancerous cells, these drugs also have potential to prevent restenosis for patients that have undergone angioplasty [386]. Local injection of S1P₁ siRNA has also been demonstrated to reduce tumour growth *in vivo* [232]. Furthermore, the S1P receptor antagonist FTY720 inhibits S1P and VEGF-induced angiogenesis and impedes tumour progression *in vivo* thereby highlighting S1P receptor antagonists as potential anti-cancer therapies [247]. Recent findings indicate that components of the PI 3-kinase/PKB pathway are targets for up-regulation, mutation and translocation more often than any other pathway on human cancers making it a prime therapeutic target [397]. Cancer is a heterogeneous disease and develops due to the erroneous signalling of multiple pathways. Understanding these pathways is vital to produce multi-faceted therapies against tumourigenesis.

References

- [1] R. Fredriksson, M. C. Lagerstrom, L. G. Lundin, and H. B. Schioth, The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints, *Mol Pharmacol* 63 (2003) 1256-1272.
- [2] A. D. Howard, G. McAllister, S. D. Feighner, Q. Liu, R. P. Nargund, L. H. Van der Ploeg, and A. A. Patchett, Orphan G-protein-coupled receptors and natural ligand discovery, *Trends Pharmacol Sci* 22 (2001) 132-140.
- [3] J. M. Stadel, S. Wilson, and D. J. Bergsma, Orphan G protein-coupled receptors: a neglected opportunity for pioneer drug discovery, *Trends Pharmacol Sci* 18 (1997) 430-437.
- [4] D. K. Vassilatis, J. G. Hohmann, H. Zeng, F. Li, J. E. Ranchalis, M. T. Mortrud, A. Brown, S. S. Rodriguez, J. R. Weller, A. C. Wright, J. E. Bergmann, and G. A. Gaitanaris, The G protein-coupled receptor repertoires of human and mouse, *Proc Natl Acad Sci U S A* 100 (2003) 4903-4908.
- [5] S. S. Karnik, C. Gogonea, S. Patil, Y. Saad, and T. Takezako, Activation of G-protein-coupled receptors: a common molecular mechanism, *Trends Endocrinol Metab* 14 (2003) 431-437.
- [6] A. Ulloa-Aguirre, D. Stanislaus, J. A. Janovick, and P. M. Conn, Structure-activity relationships of G protein-coupled receptors, *Arch Med Res* 30 (1999) 420-435.
- [7] J. M. Baldwin, The probable arrangement of the helices in G protein-coupled receptors, *Embo J* 12 (1993) 1693-1703.
- [8] C. D. Strader, I. S. Sigal, R. B. Register, M. R. Candelore, E. Rands, and R. A. Dixon, Identification of residues required for ligand binding to the beta-adrenergic receptor, *Proc Natl Acad Sci U S A* 84 (1987) 4384-4388.
- [9] D. A. Horstman, S. Brandon, A. L. Wilson, C. A. Guyer, E. J. Cragoe, Jr., and L. E. Limbird, An aspartate conserved among G-protein receptors confers allosteric regulation of alpha 2-adrenergic receptors by sodium, *J Biol Chem* 265 (1990) 21590-21595.
- [10] I. Ji, and T. H. Ji, Asp383 in the second transmembrane domain of the lutropin receptor is important for high affinity hormone binding and cAMP production, *J Biol Chem* 266 (1991) 14953-14957.
- [11] J. Wess, Molecular basis of receptor/G-protein-coupling selectivity, *Pharmacol Ther* 80 (1998) 231-264.

- [12] S. S. Ferguson, Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling, *Pharmacol Rev* 53 (2001) 1-24.
- [13] A. L. Parrill, D. A. Wang, D. L. Bautista, J. R. Van Brocklyn, Z. Lorincz, D. J. Fischer, D. L. Baker, K. Liliom, S. Spiegel, and G. Tygyi, Identification of Edg1 receptor residues that recognise sphingosine 1-phosphate, *J Biol Chem* 275 (2000) 39379-39384.
- [14] J. del Castillo, and B. Katz, Interaction at end-plate receptors between different choline derivatives, *Proc Roy Soc Lond B* 146 (1957) 369-381.
- [15] D. Colquhoun, Binding, gating, affinity and efficacy. The interpretation of structure-activity relationships for agonists and the effect of mutating receptors, *Br J Pharmacol* 125 (1998) 923-948.
- [16] L. De Vries, B. Zheng, T. Fischer, E. Elenko, and M. G. Farquhar, The regulator of G protein signaling family, *Annu Rev Pharmacol Toxicol* 40 (2000) 235-271.
- [17] R. A. de Ligt, A. P. Kourounakis, and I. J. AP, Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery, *Br J Pharmacol* 130 (2000) 1-12.
- [18] H. E. Hamm, The many faces of G protein signaling, *J Biol Chem* 273 (1998) 669-672.
- [19] G. Milligan, and E. Kostenis, Heterotrimeric G-proteins: a short history, *Br J Pharmacol* 147 Suppl 1 (2006) S46-55.
- [20] B. E. Hawes, L. M. Luttrell, S. T. Exum, and R. J. Lefkowitz, Inhibition of G protein-coupled receptor signaling by expression of cytoplasmic domains of the receptor, *J Biol Chem* 269 (1994) 15776-15785.
- [21] J. Lameh, M. Philip, Y. K. Sharma, O. Moro, J. Ramachandran, and W. Sadee, Hm1 muscarinic cholinergic receptor internalization requires a domain in the third cytoplasmic loop, *J Biol Chem* 267 (1992) 13406-13412.
- [22] G. B. Downes, and N. Gautam, The G protein subunit gene families, *Genomics* 62 (1999) 544-552.
- [23] D. E. Clapham, and E. J. Neer, G protein beta gamma subunits, *Annu Rev Pharmacol Toxicol* 37 (1997) 167-203.
- [24] J. R. Hepler, Emerging roles for RGS proteins in cell signalling, *Trends Pharmacol Sci* 20 (1999) 376-382.

- [25] C. A. Chen, and D. R. Manning, Regulation of G proteins by covalent modification, *Oncogene* 20 (2001) 1643-1652.
- [26] T. A. Fields, and P. J. Casey, Signalling functions and biochemical properties of pertussis toxin-resistant G-proteins, *Biochem J* 321 (Pt 3) (1997) 561-571.
- [27] N. Vaidehi, W. B. Floriano, R. Trabanino, S. E. Hall, P. Freddolino, E. J. Choi, G. Zamanakos, and W. A. Goddard III, Prediction of structure and function of G protein-coupled receptors, *Proc Natl Acad Sci U S A* 99 (2002) 12622-12627.
- [28] F. Gbahou, A. Rouleau, S. Morisset, R. Parmentier, S. Crochet, J. Lin, Z. Ligneau, J. Tardivel-Lacombe, H. Stark, W. Schunack, C. R. Ganellin, J. Schwartz, and J. Arrang, Protean agonism at histamine H₃ receptors *in vitro* and *in vivo*, *Proc Natl Acad Sci U S A* 100 (2003) 11086-11091.
- [29] R. C. Doss, J. P. Perkins, and T. K. Harden, Recovery of beta-adrenergic receptors following long term exposure of astrocytoma cells to catecholamine. Role of protein synthesis, *J Biol Chem* 256 (1981) 12281-12286.
- [30] R. Jockers, S. Angers, A. Da Silva, P. Benaroch, A. D. Strosberg, M. Bouvier, and S. Marullo, Beta(2)-adrenergic receptor down-regulation. Evidence for a pathway that does not require endocytosis, *J Biol Chem* 274 (1999) 28900-28908.
- [31] J. R. Hadcock, and C. C. Maibon, Down-regulation of beta-adrenergic receptors: agonist-induced reduction in receptor mRNA levels, *Proc Natl Acad Sci U S A* 85 (1988) 5021-5025.
- [32] L. M. Luttrell, and R. J. Lefkowitz, The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals, *J Cell Sci* 115 (2002) 455-465.
- [33] R. J. Lefkowitz, G protein-coupled receptor kinases, *Cell* 74 (1993) 409-412.
- [34] R. T. Premont, J. Inglese, and R. J. Lefkowitz, Protein kinases that phosphorylate activated G protein-coupled receptors, *Faseb J* 9 (1995) 175-182.
- [35] T. Metaye, H. Gibelin, R. Perdrisot, and J. L. Kraimps, Pathophysiological roles of G-protein-coupled receptor kinases, *Cell Signal* 17 (2005) 917-928.

- [36] T. A. Kohout, and R. J. Lefkowitz, Regulation of G protein-coupled Kinases and Arrestin During Receptor desensitisation, *Mol Pharmacol* 63 (2002) 9-18.
- [37] W. E. Miller, and R. J. Lefkowitz, Expanding roles for beta-arrestins as scaffolds and adapters in GPCR signaling and trafficking, *Curr Opin Cell Biol* 13 (2001) 139-145.
- [38] L. M. Luttrell, Y. Daaka, and R. J. Lefkowitz, Regulation of tyrosine kinase cascades by G-protein-coupled receptors, *Curr Opin Cell Biol* 11 (1999) 177-183.
- [39] P. H. McDonald, and R. J. Lefkowitz, Beta-Arrestins: new roles in regulating heptahelical receptors' functions, *Cell Signal* 13 (2001) 683-689.
- [40] A. J. Whitmarsh, and R. J. Davis, Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals, *Trends Biochem Sci* 23 (1998) 481-485.
- [41] D. J. G. Mackay, and A. Hall, Rho GTPases, *The Journal of Biological Chemistry* 273 (1998) 20685-20688.
- [42] P. H. McDonald, C. W. Chow, W. E. Miller, S. A. Laporte, M. E. Field, F. T. Lin, R. J. Davis, and R. J. Lefkowitz, Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3, *Science* 290 (2000) 1574-1577.
- [43] A. N. Pronin, and J. L. Benovic, Regulation of the G protein-coupled receptor kinase GRK5 by protein kinase C, *J Biol Chem* 272 (1997) 3806-3812.
- [44] R. Winstel, S. Freund, C. Krasel, E. Hoppe, and M. J. Lohse, Protein kinase cross-talk: membrane targeting of the beta-adrenergic receptor kinase by protein kinase C, *Proc Natl Acad Sci U S A* 93 (1996) 2105-2109.
- [45] T. T. Chuang, H. LeVine, 3rd, and A. De Blasi, Phosphorylation and activation of beta-adrenergic receptor kinase by protein kinase C, *J Biol Chem* 270 (1995) 18660-18665.
- [46] A. N. Pronin, C. V. Carman, and J. L. Benovic, Structure-function analysis of G protein-coupled receptor kinase-5. Role of the carboxyl terminus in kinase regulation, *J Biol Chem* 273 (1998) 31510-31518.
- [47] M. Cong, S. J. Perry, F. T. Lin, I. D. Fraser, L. A. Hu, W. Chen, J. A. Pitcher, J. D. Scott, and R. J. Lefkowitz, Regulation of membrane targeting of the G protein-coupled receptor kinase 2 by protein kinase A and its anchoring protein AKAP79, *J Biol Chem* 276 (2001) 15192-15199.

- [48] R. J. Lefkowitz, Mechanisms of β -adrenergic receptor desensitisation and resensitisation, *Ad Pharmacol* 42 (1998) 416-420.
- [49] J. A. Koenig, and J. M. Edwardson, Endocytosis and recycling of G protein-coupled receptors, *Trends Pharmacol Sci* 18 (1997) 276-287.
- [50] R. H. Oakley, S. A. Laporte, J. A. Holt, M. G. Caron, and L. S. Barak, Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors, *J Biol Chem* 275 (2000) 17201-17210.
- [51] W. E. Miller, S. Maudsley, S. Ahn, K. D. Khan, L. M. Luttrell, and R. J. Lefkowitz, beta-arrestin1 interacts with the catalytic domain of the tyrosine kinase c-SRC. Role of beta-arrestin1-dependent targeting of c-SRC in receptor endocytosis, *J Biol Chem* 275 (2000) 11312-11319.
- [52] J. Zhang, S. S. Ferguson, L. S. Barak, L. Menard, and M. G. Caron, Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization, *J Biol Chem* 271 (1996) 18302-18305.
- [53] O. B. Goodman, Jr., J. G. Krupnick, V. V. Gurevich, J. L. Benovic, and J. H. Keen, Arrestin/clathrin interaction. Localization of the arrestin binding locus to the clathrin terminal domain, *J Biol Chem* 272 (1997) 15017-15022.
- [54] P. Tsao, T. Cao, and M. von Zastrow, Role of endocytosis in mediating downregulation of G-protein-coupled receptors, *Trends Pharmacol Sci* 22 (2001) 91-96.
- [55] R. J. H. Wojcikiewicz, Regulated ubiquitination of proteins in GPCR-initiated signalling pathways, *Trends Pharmacol Sci* 25 (2004) 35-41.
- [56] S. Collins, M. Bouvier, M. A. Bolanowski, M. G. Caron, and R. J. Lefkowitz, cAMP stimulates transcription of the beta 2-adrenergic receptor gene in response to short-term agonist exposure, *Proc Natl Acad Sci U S A* 86 (1989) 4853-4857.
- [57] B. G. Tholanikunnel, and C. C. Malbon, A 20-nucleotide (A + U)-rich element of beta2-adrenergic receptor (beta2AR) mRNA mediates binding to beta2AR-binding protein and is obligate for agonist-induced destabilization of receptor mRNA, *J Biol Chem* 272 (1997) 11471-11478.
- [58] M. von Zastrow, and B. K. Kobilka, Antagonist-dependent and -independent steps in the mechanism of adrenergic receptor internalization, *J Biol Chem* 269 (1994) 18448-18452.
- [59] V. O. Rybin, X. Xu, M. P. Lisanti, and S. F. Steinberg, Differential targeting of beta -adrenergic receptor subtypes and adenylyl cyclase to

cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway, *J Biol Chem* 275 (2000) 41447-41457.

[60] J. Couet, M. M. Belanger, E. Roussel, and M. C. Drolet, Cell biology of caveolae and caveolin, *Adv Drug Deliv Rev* 49 (2001) 223-235.

[61] R. B. Penn, A. N. Pronin, and J. L. Benovic, Regulation of G protein-coupled receptor kinases, *Trends Cardiovasc Med* 10 (2000) 81-89.

[62] O. Feron, L. Belhassen, L. Kobzik, T. W. Smith, R. A. Kelly, and T. Michel, Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells, *J Biol Chem* 271 (1996) 22810-22814.

[63] S. Nakashima, Protein kinase C α (PKC α): regulation and biological function, *J Biochem* 132 (2002) 669-675.

[64] B. Anliker, and J. Chun, Cell surface receptors in lysophospholipid signaling, *Semin Cell Dev Biol* 15 (2004) 457-465.

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

[66] J. L. W. Thudichum, A treatise on the Chemical Constitution of Brain., Bailliere, Tindall and Cox, London 149 (1884).

[67] T. Hla, Physiological and pathological actions of sphingosine 1-phosphate, *Semin Cell Dev Biol* 15 (2004) 513-520.

[68] S. Spiegel, and S. Milstien, Sphingosine-1-phosphate: an enigmatic signalling lipid, *Nat Rev Mol Cell Biol* 4 (2003) 397-407.

[69] S. Coursol, L. M. Fan, H. Le Stunff, S. Spiegel, S. Gilroy, and S. M. Assmann, Sphingolipid signalling in Arabidopsis guard cells involves heterotrimeric G proteins, *Nature* 423 (2003) 651-654.

[70] C. K. Ng, K. Carr, M. R. McAinsh, B. Powell, and A. M. Hetherington, Drought-induced guard cell signal transduction involves sphingosine-1-phosphate, *Nature* 410 (2001) 596-599.

[71] B. Oskouian, and J. D. Saba, Death and taxis: what non-mammalian models tell us about sphingosine-1-phosphate, *Semin Cell Dev Biol* 15 (2004) 529-540.

- [72] H. Zhang, N. N. Desai, A. Olivera, T. Seki, G. Brooker, and S. Spiegel, Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation, *J Cell Biol* 114 (1991) 155-167.
- [73] O. Cuvillier, G. Pirianov, B. Kleuser, P. G. Vanek, O. A. Coso, J. S. Gutkind, and S. Spiegel, Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate, *Nature* 381 (1996) 800-803.
- [74] T. Hla, and T. J. Macaig, An Abundant Transcript Induced in Differentiating Human Endothelial Cells Encodes a Polypeptide with Structural Similarities to G-protein-coupled Receptors, *J Biol Chem* 265 (1990) 9308-9313.
- [75] M. J. Lee, Sphingosine-1-Phosphate as a Ligand for the G Protein-Coupled Receptor EDG-1, *Science* 279 (1994) 1552-1555.
- [76] M. J. Lee, J. R. Van Brocklyn, S. Thangada, C. H. Liu, A. R. Hand, R. Menzeleev, S. Spiegel, and T. Hla, Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1, *Science* 279 (1998) 1552-1555.
- [77] S. Pyne, and N. Pyne, Sphingosine 1-phosphate signalling via the endothelial differentiation gene family of G-protein-coupled receptors, *Pharmacol Ther* 88 (2000) 115-131.
- [78] M. Maceyka, S. G. Payne, S. Milstien, and S. Spiegel, Sphingosine kinase, sphingosine-1-phosphate, and apoptosis, *Biochim Biophys Acta* 1585 (2002) 193-201.
- [79] C. Ogawa, A. Kihara, M. Gokoh, and Y. Igarashi, Identification and characterization of a novel human sphingosine-1-phosphate phosphohydrolase, hSPP2, *J Biol Chem* 278 (2003) 1268-1272.
- [80] H. Le Stunff, C. Peterson, R. Thornton, S. Milstien, S. M. Mandala, and S. Spiegel, Characterization of murine sphingosine-1-phosphate phosphohydrolase, *J Biol Chem* 277 (2002) 8920-8927.
- [81] P. P. Van Veldhoven, and G. P. Mannaerts, Subcellular localization and membrane topology of sphingosine-1-phosphate lyase in rat liver, *J Biol Chem* 266 (1991) 12502-12507.
- [82] V. A. Sciorra, and A. J. Morris, Roles for lipid phosphate phosphatases in regulation of cellular signaling, *Biochim Biophys Acta* 1582 (2002) 45-51.
- [83] S. M. Pitson, J. D'Andrea R, L. Vandeleur, P. A. Moretti, P. Xia, J. R. Gamble, M. A. Vadas, and B. W. Wattenberg, Human sphingosine kinase: purification, molecular cloning and characterization of the native and recombinant enzymes, *Biochem J* 350 (2000) 429-441.

- [84] M. M. Nagiec, M. Skrzypek, E. E. Nagiec, R. L. Lester, and R. C. Dickson, The LCB4 (YOR171c) and LCB5 (YLR260w) genes of *Saccharomyces* encode sphingoid long chain base kinases, *J Biol Chem* 273 (1998) 19437-19442.
- [85] T. Kohama, A. Olivera, L. Edsall, M. M. Nagiec, R. Dickson, and S. Spiegel, Molecular cloning and functional characterization of murine sphingosine kinase, *J Biol Chem* 273 (1998) 23722-23728.
- [86] H. Liu, M. Sugiura, V. E. Nava, L. C. Edsall, K. Kono, S. Poulton, S. Milstien, T. Kohama, and S. Spiegel, Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform, *J Biol Chem* 275 (2000) 19513-19520.
- [87] D. Meyer zu Heringdorf, H. Lass, I. Kuchar, R. Alemany, Y. Guo, M. Schmidt, and K. H. Jakobs, Role of sphingosine kinase in Ca^{2+} signalling by epidermal growth factor receptor, *FEBS Lett* 461 (1999) 217-222.
- [88] A. Olivera, and S. Spiegel, Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens, *Nature* 365 (1993) 557-560.
- [89] L. Edsall, G. G. Grisha, and S. Spiegel, Involvement of Sphingosine 1-Phosphate in Nerve Growth Factor-mediated Neuronal Survival and Differentiation, *The Journal of Neuroscience* 17 (1997) 6952-6960.
- [90] R. A. Rius, L. C. Edsall, and S. Spiegel, Activation of sphingosine kinase in pheochromocytoma PC12 neuronal cells in response to trophic factors, *FEBS Lett* 417 (1997) 173-176.
- [91] L. C. Edsall, G. G. Pirianov, and S. Spiegel, Involvement of sphingosine 1-phosphate in nerve growth factor-mediated neuronal survival and differentiation, *J Neurosci* 17 (1997) 6952-6960.
- [92] P. Xia, L. Wang, J. R. Gamble, and M. A. Vadas, Activation of sphingosine kinase by tumor necrosis factor-alpha inhibits apoptosis in human endothelial cells, *J Biol Chem* 274 (1999) 34499-34505.
- [93] K. R. Johnson, K. P. Becker, M. M. Facchinetti, Y. A. Hannun, and L. M. Obeid, PKC-dependent activation of sphingosine kinase 1 and translocation to the plasma membrane. Extracellular release of sphingosine-1-phosphate induced by phorbol 12-myristate 13-acetate (PMA), *J Biol Chem* 277 (2002) 35257-35262.
- [94] M. Bektas, P. S. Jolly, C. Muller, J. Eberle, S. Spiegel, and C. C. Geilen, Sphingosine kinase activity counteracts ceramide-mediated cell death in

human melanoma cells: role of Bcl-2 expression, *Oncogene* 24 (2005) 178-187.

[95] N. Igarashi, T. Okada, S. Hayashi, and F. Toshitada, Sphingosine Kinase 2 is a nuclear protein and inhibits DNA synthesis, *The Journal of Biological Chemistry* 278 (2003) 46832-46839.

[96] H. M. Rosenfeldt, J. P. Hobson, M. Maceyka, A. Olivera, V. E. Nava, S. Milstien, and S. Spiegel, EDG-1 links the PDGF receptor to Src and focal adhesion kinase activation leading to lamellipodia formation and cell migration, *Faseb J* 15 (2001) 2649-2659.

[97] L. C. Boujaoude, C. Bradshaw-Wilder, M. Cungai, J. Cohn, B. Ogretmen, Y. A. Hannun, and L. M. Obeid, Cystic Fibrosis Transmembrane Regulator Regulates Uptake of Sphingoid Base Phosphates and Lysophosphatidic Acid, *The Journal of Biological Chemistry* 276 (2001) 35258-35264.

[98] T. Hla, Signaling and biological actions of sphingosine 1-phosphate, *Pharmacol Res* 47 (2003) 401-407.

[99] H. Le Stunff, S. Milstien, and S. Spiegel, Generation and metabolism of bioactive sphingosine-1-phosphate, *J Cell Biochem* 92 (2004) 882-899.

[100] N. Kobayashi, T. Nishi, T. Hirata, A. Kihara, T. Sano, Y. Igarashi, and A. Yamaguchi, Sphingosine 1-phosphate is released from the cytosol of rat platelets in a carrier-mediated manner, *J Lipid Res* 47 (2006) 614-621.

[101] T. S. Panetti, Differential effects of sphingosine 1-phosphate and lysophosphatidic acid on endothelial cells, *Biochim Biophys Acta* 1582 (2002) 190-196.

[102] A. Sachinidis, R. Kettenhofen, S. Seewald, I. Gouni-Berthold, U. Schmitz, C. Seul, Y. Ko, and H. Vetter, Evidence that lipoproteins are carriers of bioactive factors, *Arterioscler Thromb Vasc Biol* 19 (1999) 2412-2421.

[103] W. Siess, Athero- and thrombogenic actions of lysophosphatidic acid and sphingosine-1-phosphate, *Biochim Biophys Acta* 1582 (2002) 204-215.

[104] Y. Yatomi, Y. Igarashi, L. Yang, N. Hisano, R. Qi, N. Asazuma, K. Satoh, Y. Ozaki, and S. Kume, Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum, *J Biochem (Tokyo)* 121 (1997) 969-973.

[105] N. Murata, K. Sato, J. Kon, H. Tomura, and F. Okajima, Quantitative measurement of sphingosine 1-phosphate by radioreceptor-binding assay, *Anal Biochem* 282 (2000) 115-120.

- [106] J. R. Van Brocklyn, Z. Tu, L. C. Edsall, R. R. Schmidt, and S. Spiegel, Sphingosine 1-phosphate-induced cell rounding and neurite retraction are mediated by the G protein-coupled receptor H218, *J Biol Chem* 274 (1999) 4626-4632.
- [107] F. Okajima, Plasma lipoproteins behave as carriers of extracellular sphingosine 1-phosphate: is this an atherogenic mediator or an anti-atherogenic mediator? *Biochim Biophys Acta* 1582 (2002) 132-137.
- [108] B. Cinque, L. Di Marzio, C. Centi, C. Di Rocco, C. Riccardi, and M. Grazia Cifone, Sphingolipids and the immune system, *Pharmacol Res* 47 (2003) 421-437.
- [109] J. Radeff-Huang, T. M. Seasholtz, R. G. Matteo, and J. H. Brown, G protein mediated signaling pathways in lysophospholipid induced cell proliferation and survival, *J Cell Biochem* 92 (2004) 949-966.
- [110] K. R. Watterson, P. H. Ratz, and S. Spiegel, The role of sphingosine-1-phosphate in smooth muscle contraction, *Cell Signal* 17 (2005) 289-298.
- [111] H. Rosen, and E. J. Goetzl, Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network, *Nat Rev Immunol* 5 (2005) 560-570.
- [112] J. H. Hecht, J. A. Weiner, S. R. Post, and J. Chun, Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex, *J Cell Biol* 135 (1996) 1071-1083.
- [113] N. Fukushima, Y. Kimura, and J. Chun, A single receptor encoded by vzg-1/lpA1/edg-2 couples to G proteins and mediates multiple cellular responses to lysophosphatidic acid, *Proc Natl Acad Sci U S A* 95 (1998) 6151-6156.
- [114] S. An, M. A. Dickens, T. Bleu, O. G. Hallmark, and E. J. Goetzl, Molecular cloning of the human Edg2 protein and its identification as a functional cellular receptor for lysophosphatidic acid, *Biochem Biophys Res Commun* 231 (1997) 619-622.
- [115] T. Hla, and T. Maciag, An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors, *J Biol Chem* 265 (1990) 9308-9313.
- [116] G. C. Zondag, F. R. Postma, I. V. Etten, I. Verlaan, and W. H. Moolenaar, Sphingosine 1-phosphate signalling through the G-protein-coupled receptor Edg-1, *Biochem J* 330 (1998) 605-609.

- [117] S. An, T. Bleu, O. G. Hallmark, S. R. Coughlin, and E. J. Goetzl, Identification of cDNAs encoding two G protein-coupled receptors for lysophospholipids, *FEBS Lett* 417 (1997) 279-282.
- [118] S. An, T. Bleu, O. G. Hallmark, and E. J. Goetzl, Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid, *J Biol Chem* 273 (1998) 7906-7910.
- [119] J. Chun, E. J. Goetzl, T. Hla, Y. Igarashi, K. R. Lynch, W. Moolenaar, S. Pyne, and G. Tigyi, International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature, *Pharmacol Rev* 54 (2002) 265-269.
- [120] K. R. Lynch, and D. S. Im, Life on the edge, *Trends Pharmacol Sci* 20 (1999) 473-475.
- [121] S. Pyne, and N. J. Pyne, Sphingosine 1-phosphate signalling and termination at lipid phosphate receptors, *Biochim Biophys Acta* 1582 (2002) 121-131.
- [122] K. W. Young, and S. R. Nahorski, Sphingosine 1-phosphate: a Ca²⁺ release mediator in the balance, *Cell Calcium* 32 (2002) 335-341.
- [123] T. A. Taha, K. M. Argraves, and L. M. Obeid, Sphingosine-1-phosphate receptors: receptor specificity versus functional redundancy, *Biochim Biophys Acta* 1682 (2004) 48-55.
- [124] E. Gonzalez, R. Kou, and T. Michel, Rac1 modulates sphingosine 1-phosphate-mediated activation of phosphoinositide 3-kinase/Akt signaling pathways in vascular endothelial cells, *J Biol Chem* 281 (2006) 3210-3216.
- [125] H. Okamoto, N. Takuwa, K. Gonda, H. Okazaki, K. Chang, Y. Yatomi, H. Shigematsu, and Y. Takuwa, EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to multiple signaling pathways, including phospholipase C activation, Ca²⁺ mobilization, Ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition, *J Biol Chem* 273 (1998) 27104-27110.
- [126] M. J. Lee, S. Thangada, J. H. Paik, G. P. Sapkota, N. Ancellin, S. S. Chae, M. Wu, M. Morales-Ruiz, W. C. Sessa, D. R. Alessi, and T. Hla, Akt-mediated phosphorylation of the G protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis, *Mol Cell* 8 (2001) 693-704.
- [127] R. T. Windh, M. J. Lee, T. Hla, S. An, A. J. Barr, and D. R. Manning, 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 (1999) 27351-27358.

- [128] H. Okamoto, N. Takuwa, Y. Yatomi, K. Gonda, H. Shigematsu, and Y. Takuwa, EDG3 is a functional receptor specific for sphingosine 1-phosphate and sphingosylphosphorylcholine with signaling characteristics distinct from EDG1 and AGR16, *Biochem Biophys Res Commun* 260 (1999) 203-208.
- [129] J. Kon, K. Sato, T. Watanabe, H. Tomura, A. Kuwabara, T. Kimura, K. Tamama, T. Ishizuka, N. Murata, T. Kanda, I. Kobayashi, H. Ohta, M. Ui, and F. Okajima, Comparison of intrinsic activities of the putative sphingosine 1-phosphate receptor subtypes to regulate several signaling pathways in their cDNA-transfected Chinese hamster ovary cells, *J Biol Chem* 274 (1999) 23940-23947.
- [130] K. Gonda, H. Okamoto, N. Takuwa, Y. Yatomi, H. Okazaki, T. Sakurai, S. Kimura, R. Sillard, K. Harii, and Y. Takuwa, The novel sphingosine 1-phosphate receptor AGR16 is coupled via pertussis toxin-sensitive and -insensitive G-proteins to multiple signalling pathways, *Biochem J* 337 (Pt 1) (1999) 67-75.
- [131] J. R. Van Brocklyn, M. H. Graler, G. Bernhardt, J. P. Hobson, M. Lipp, and S. Spiegel, Sphingosine-1-phosphate is a ligand for the G protein-coupled receptor EDG-6, *Blood* 95 (2000) 2624-2629.
- [132] D. S. Im, Characterisation of a Novel Sphingosine 1-Phosphate Receptor, Edg-8, *The Journal of Biological Chemistry* 275 (2000) 14281-14286.
- [133] R. L. Malek, R. E. Toman, L. C. Edsall, S. Wong, J. Chiu, C. A. Letterle, J. R. Van Brocklyn, S. Milstien, S. Spiegel, and N. H. Lee, Nrg-1 belongs to the endothelial differentiation gene family of G protein-coupled sphingosine-1-phosphate receptors, *J Biol Chem* 276 (2001) 5692-5699.
- [134] M. H. Graler, G. Bernhardt, and M. Lipp, EDG6, a novel G-protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue, *Genomics* 53 (1998) 164-169.
- [135] Y. Yamazaki, J. Kon, K. Sato, H. Tomura, M. Sato, T. Yoneya, H. Okazaki, F. Okajima, and H. Ohta, Edg-6 as a putative sphingosine 1-phosphate receptor coupling to Ca(2+) signaling pathway, *Biochem Biophys Res Commun* 268 (2000) 583-589.
- [136] T. Kohno, H. Matsuyuki, Y. Inagaki, and Y. Igarashi, Sphingosine 1-phosphate promotes cell migration through the activation of Cdc42 in Edg-6/S1P4-expressing cells, *Genes Cells* 8 (2003) 685-697.
- [137] K. Uhlenbrock, H. Gassenhuber, and E. Kostenis, Sphingosine 1-phosphate is a ligand of the human gpr3, gpr6 and gpr12 family of

constitutively active G protein-coupled receptors, *Cell Signal* 14 (2002) 941-953.

[138] A. Niedernberg, A. Blaukat, T. Schoneberg, and E. Kostenis, Regulated and constitutive activation of specific signalling pathways by the human S1P5 receptor, *Br J Pharmacol* 138 (2003) 481-493.

[139] A. Marchese, M. Sawzdargo, T. Nguyen, R. Cheng, H. H. Heng, T. Nowak, D. S. Im, K. R. Lynch, S. R. George, and F. O'Dowd B, Discovery of three novel orphan G-protein-coupled receptors, *Genomics* 56 (1999) 12-21.

[140] Y. Kawasawa, K. Kume, T. Izumi, and T. Shimizu, Mammalian PSP24s (alpha and beta isoforms) are not responsive to lysophosphatidic acid in mammalian expression systems, *Biochem Biophys Res Commun* 276 (2000) 957-964.

[141] Y. Xu, K. Zhu, G. Hong, W. Wu, L. M. Baudhuin, Y. Xiao, and D. S. Damron, Sphingosylphosphorylcholine is a ligand for ovarian cancer G-protein-coupled receptor 1, *Nat Cell Biol* 2 (2000) 261-267.

[142] D. S. Im, Discovery of new G protein-coupled receptors for lipid mediators, *J Lipid Res* 45 (2004) 410-418.

[143] E. Kostenis, Novel clusters of receptors for sphingosine-1-phosphate, sphingosylphosphorylcholine, and (lyso)-phosphatidic acid: new receptors for "old" ligands, *J Cell Biochem* 92 (2004) 923-936.

[144] T. Y. Shih, D. R. Williams, M. O. Weeks, J. M. Maryak, W. C. Vass, and E. M. Scolnick, Comparison of the genomic organization of Kirsten and Harvey sarcoma viruses, *J Virol* 27 (1978) 45-55.

[145] U. H. Chien, M. Lai, T. Y. Shih, I. M. Verma, E. M. Scolnick, P. Roy-Burman, and N. Davidson, Heteroduplex analysis of the sequence relationships between the genomes of Kirsten and Harvey sarcoma viruses, their respective parental murine leukemia viruses, and the rat endogenous 30S RNA, *J Virol* 31 (1979) 752-760.

[146] D. J. Capon, P. H. Seeburg, J. P. McGrath, J. S. Hayflick, U. Edman, A. D. Levinson, and D. V. Goeddel, Activation of Ki-ras2 gene in human colon and lung carcinomas by two different point mutations, *Nature* 304 (1983) 507-513.

[147] J. R. Feramisco, M. Gross, T. Kamata, M. Rosenberg, and R. W. Sweet, Microinjection of the oncogene form of the human H-ras (T-24) protein results in rapid proliferation of quiescent cells, *Cell* 38 (1984) 109-117.

- [148] D. W. Stacey, and H. Kung, Transformation of NIH 3T3 cells by microinjection of Ha-ras p21 protein, *Nature* 319 (1984) 508-511.
- [149] R. Brown, C. J. Marshall, S. G. Penhale, and A. Hall, Mechanism of activation of an N-ras gene in the human fibrosarcoma cell line HT1080, *Embo J* 3 (1984).
- [150] Y. Takai, T. Sasaki, and T. Matozaki, Small GTP-binding proteins, *Physiol Rev* 81 (2001) 153-208.
- [151] T. Magee, and C. Marshall, New insights into the interaction of Ras with the plasma membrane, *Cell* 98 (1999) 9-12.
- [152] Y. Zheng, Dbl family guanine nucleotide exchange factors, *Trends Biochem Sci* 26 (2001) 724-732.
- [153] S. Y. Moon, and Y. Zheng, Rho GTPase-activating proteins in cell regulation, *Trends Cell Biol* 13 (2003) 13-22.
- [154] A. Sekine, M. Fujiwara, and S. Narumiya, Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase, *J Biol Chem* 264 (1989) 8602-8605.
- [155] P. Chardin, P. Boquet, P. Madaule, M. R. Popoff, E. J. Rubin, and D. M. Gill, The mammalian G protein rhoC is ADP-ribosylated by Clostridium botulinum exoenzyme C3 and affects actin microfilaments in Vero cells, *Embo J* 8 (1989) 1087-1092.
- [156] H. F. Paterson, A. J. Self, M. D. Garrett, I. Just, K. Aktories, and A. Hall, Microinjection of recombinant p21rho induces rapid changes in cell morphology, *J Cell Biol* 111 (1990) 1001-1007.
- [157] Y. Miura, A. Kikuchi, T. Musha, S. Kuroda, H. Yaku, T. Sasaki, and Y. Takai, Regulation of morphology by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI) in Swiss 3T3 cells, *J Biol Chem* 268 (1993) 510-515.
- [158] A. J. Ridley, and A. Hall, Signal transduction pathways regulating Rho-mediated stress fibre formation: requirement for a tyrosine kinase, *Embo J* 13 (1994) 2600-2610.
- [159] A. J. Ridley, H. F. Paterson, C. L. Johnston, D. Diekmann, and A. Hall, The small GTP-binding protein rac regulates growth factor-induced membrane ruffling, *Cell* 70 (1992) 401-410.

- [160] R. Kozma, S. Ahmed, A. Best, and L. Lim, The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts, *Mol Cell Biol* 15 (1995) 1942-1952.
- [161] C. D. Nobes, and A. Hall, Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia, *Cell* 81 (1995) 53-62.
- [162] M. Raftopoulou, and A. Hall, Cell migration: Rho GTPases lead the way, *Dev Biol* 265 (2004) 23-32.
- [163] A. L. Bishop, and A. Hall, Rho GTPases and their effector proteins, *Biochem J* 348 Pt 2 (2000) 241-255.
- [164] F. Michiels, J. C. Stam, P. L. Hordijk, R. A. van der Kammen, L. Ruuls-Van Stalle, C. A. Feltkamp, and J. G. Collard, Regulated membrane localization of Tiam1, mediated by the NH2-terminal pleckstrin homology domain, is required for Rac-dependent membrane ruffling and C-Jun NH2-terminal kinase activation, *J Cell Biol* 137 (1997) 387-398.
- [165] A. E. Mertens, R. C. Roovers, and J. G. Collard, Regulation of Tiam1-Rac signalling, *FEBS Lett* 546 (2003) 11-16.
- [166] A. Hall, Rho GTPases and the control of cell behaviour, *Biochem Soc Trans* 33 (2005) 891-895.
- [167] L. Stephens, C. Ellson, and P. Hawkins, Roles of PI3Ks in leukocyte chemotaxis and phagocytosis, *Curr Opin Cell Biol* 14 (2002) 203-213.
- [168] J. Han, K. Luby-Phelps, B. Das, X. Shu, Y. Xia, R. D. Mosteller, U. M. Krishna, J. R. Falck, M. A. White, and D. Broek, Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav, *Science* 279 (1998) 558-560.
- [169] A. S. Nimnual, B. A. Yatsula, and D. Bar-Sagi, Coupling of Ras and Rac guanosine triphosphatases through the Ras exchanger Sos, *Science* 279 (1998) 560-563.
- [170] K. Reif, C. D. Nobes, G. Thomas, A. Hall, and D. A. Cantrell, Phosphatidylinositol 3-kinase signals activate a selective subset of Rac/Rho-dependent effector pathways, *Curr Biol* 6 (1996) 1445-1455.
- [171] G. M. Bokoch, C. J. Vlahos, Y. Wang, U. G. Knaus, and A. E. Traynor-Kaplan, Rac GTPase interacts specifically with phosphatidylinositol 3-kinase, *Biochem J* 315 (Pt 3) (1996) 775-779.

[172] S. Bagrodia, and R. A. Cerione, Pak to the future, *Trends Cell Biol* 9 (1999) 350-355.

[173] S. Arber, F. A. Barbayannis, H. Hanser, C. Schneider, C. A. Stanyon, O. Bernard, and P. Caroni, Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase, *Nature* 393 (1998) 805-809.

[174] M. Maekawa, T. Ishizaki, S. Boku, N. Watanabe, A. Fujita, A. Iwamatsu, T. Obinata, K. Ohashi, K. Mizuno, and S. Narumiya, Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase, *Science* 285 (1999) 895-898.

[175] Y. Kawano, Y. Fukata, N. Oshiro, M. Amano, T. Nakamura, M. Ito, F. Matsumura, M. Inagaki, and K. Kaibuchi, Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo, *J Cell Biol* 147 (1999) 1023-1038.

[176] T. J. Mitchison, and L. P. Cramer, Actin-based motility and locomotion, *Cell* (1996) 371-379.

[177] N. Watanabe, T. Kato, A. Fujita, T. Ishizaki, and S. Narumiya, Cooperation between mDia1 and ROCK in Rho-induced actin reorganization, *Nat Cell Biol* 1 (1999) 136-143.

[178] L. M. Machesky, and R. H. Insall, Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex, *Curr Biol* 8 (1998) 1347-1356.

[179] T. H. Millard, S. J. Sharp, and L. M. Machesky, Signalling to actin assembly via the WASP (Wiskott-Aldrich syndrome protein)-family proteins and the Arp2/3 complex, *Biochem J* 380 (2004) 1-17.

[180] S. Eden, R. Rohatgi, A. V. Podtelejnikov, M. Mann, and M. W. Kirschner, Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck, *Nature* 418 (2002) 790-793.

[181] S. H. Zigmond, How WASP regulates actin polymerization, *J Cell Biol* 150 (2000) F117-120.

[182] B. P. Liu, M. Chrzanowska-Wodnicka, and K. Burridge, Microtubule depolymerization induces stress fibers, focal adhesions, and DNA synthesis via the GTP-binding protein Rho, *Cell Adhes Commun* 5 (1998) 249-255.

[183] T. Ishizaki, Y. Morishima, M. Okamoto, T. Furuyashiki, T. Kato, and S. Narumiya, Coordination of microtubules and the actin cytoskeleton by the Rho effector mDia1, *Nat Cell Biol* 3 (2001) 8-14.

- [184] H. Daub, K. Gevaert, J. Vandekerckhove, A. Sobel, and A. Hall, Rac/Cdc42 and p65PAK regulate the microtubule-destabilizing protein stathmin through phosphorylation at serine 16, *J Biol Chem* 276 (2001) 1677-1680.
- [185] C. Lamaze, T. H. Chuang, L. J. Terlecky, G. M. Bokoch, and S. L. Schmid, Regulation of receptor-mediated endocytosis by Rho and Rac, *Nature* 382 (1996) 177-179.
- [186] K. F. Talias, J. H. Hartwig, H. Ishihara, Y. Shibasaki, L. C. Cantley, and C. L. Carpenter, Type I alpha phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly, *Curr Biol* 10 (2000) 153-156.
- [187] W. J. Wu, J. W. Erickson, R. Lin, and R. A. Cerione, The gamma-subunit of the coatamer complex binds Cdc42 to mediate transformation, *Nature* 405 (2000) 800-804.
- [188] R. Kroschewski, A. Hall, and I. Mellman, Cdc42 controls secretory and endocytic transport to the basolateral plasma membrane of MDCK cells, *Nat Cell Biol* 1 (1999) 8-13.
- [189] P. A. Michaelis, C. Mineo, Y. S. Ying, and R. G. Anderson, Polarized distribution of endogenous Rac1 and RhoA at the cell surface, *J Biol Chem* 274 (1999) 21430-21436.
- [190] F. Castellano, P. Montcourrier, J. C. Guillemot, E. Gouin, L. Machesky, P. Cossart, and P. Chavrier, Inducible recruitment of Cdc42 or WASP to a cell-surface receptor triggers actin polymerization and filopodium formation, *Curr Biol* 9 (1999) 351-360.
- [191] F. Castellano, P. Montcourrier, and P. Chavrier, Membrane recruitment of Rac1 triggers phagocytosis, *J Cell Sci* 113 (Pt 17) (2000) 2955-2961.
- [192] S. Sabharanjak, P. Sharma, R. G. Parton, and S. Mayor, GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway, *Dev Cell* 2 (2002) 411-423.
- [193] A. E. Aplin, and R. L. Juliano, Integrin and cytoskeletal regulation of growth factor signaling to the MAP kinase pathway, *J Cell Sci* 112 (Pt 5) (1999) 695-706.
- [194] M. A. Schwartz, and S. J. Shattil, Signaling networks linking integrins and rho family GTPases, *Trends Biochem Sci* 25 (2000) 388-391.
- [195] K. A. DeMali, K. Wennerberg, and K. Burridge, Integrin signaling to the actin cytoskeleton, *Curr Opin Cell Biol* 15 (2003) 572-582.

- [196] E. H. Danen, and K. M. Yamada, Fibronectin, integrins, and growth control, *J Cell Physiol* 189 (2001) 1-13.
- [197] M. Le Gall, J. C. Chambard, D. Grall, and E. Van Obberghen-Schilling, Adhesion-dependent control of Akt/protein kinase B occurs at multiple levels, *J Cell Physiol* 196 (2003) 98-104.
- [198] D. D. Schlaepfer, and T. Hunter, Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src, *J Biol Chem* 272 (1997) 13189-13195.
- [199] J. W. Smith, The structural basis of integrin-ligand (RGD) interaction, New York Academic Press (1994) 1-32.
- [200] M. A. del Pozo, L. S. Price, N. B. Alderson, X. D. Ren, and M. A. Schwartz, Adhesion to the extracellular matrix regulates the coupling of the small GTPase Rac to its effector PAK, *Embo J* 19 (2000) 2008-2014.
- [201] L. S. Price, J. Leng, M. A. Schwartz, and G. M. Bokoch, Activation of Rac and Cdc42 by integrins mediates cell spreading, *Mol Biol Cell* 9 (1998) 1863-1871.
- [202] C. K. Miranti, L. Leng, P. Maschberger, J. S. Brugge, and S. J. Shattil, Identification of a novel integrin signaling pathway involving the kinase Syk and the guanine nucleotide exchange factor Vav1, *Curr Biol* 8 (1998) 1289-1299.
- [203] Q. Zhang, O. Peyruchaud, K. J. French, M. K. Magnusson, and D. F. Mosher, Sphingosine 1-phosphate stimulates fibronectin matrix assembly through a Rho-dependent signal pathway, *Blood* 93 (1999) 2984-2990.
- [204] D. Q. Zheng, A. S. Woodard, G. Tallini, and L. R. Languino, Substrate specificity of alpha(v)beta(3) integrin-mediated cell migration and phosphatidylinositol 3-kinase/AKT pathway activation, *J Biol Chem* 275 (2000) 24565-24574.
- [205] M. L. Matter, and E. Ruoslahti, A signaling pathway from the alpha5beta1 and alpha(v)beta3 integrins that elevates bcl-2 transcription, *J Biol Chem* 276 (2001) 27757-27763.
- [206] D. R. Alessi, F. B. Caudwell, M. Andjelkovic, B. A. Hemmings, and P. Cohen, Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase, *FEBS Lett* 399 (1996) 333-338.

- [207] D. Fulton, J. P. Gratton, T. J. McCabe, J. Fontana, Y. Fujio, K. Walsh, T. F. Franke, A. Papapetropoulos, and W. C. Sessa, Regulation of endothelium-derived nitric oxide production by the protein kinase Akt, *Nature* 399 (1999) 597-601.
- [208] D. A. Cross, D. R. Alessi, P. Cohen, M. Andjelkovich, and B. A. Hemmings, Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B, *Nature* 378 (1995) 785-789.
- [209] S. R. Datta, H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, and M. E. Greenberg, Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery, *Cell* 91 (1997) 231-241.
- [210] J. Downward, Mechanisms and consequences of activation of protein kinase B/Akt, *Curr Opin Cell Biol* 10 (1998) 262-267.
- [211] T. S. Panetti, J. Nowlen, and D. F. Mosher, Sphingosine-1-phosphate and lysophosphatidic acid stimulate endothelial cell migration, *Arterioscler Thromb Vasc Biol* 20 (2000) 1013-1019.
- [212] T. S. Panetti, D. F. Hannah, C. Avraamides, J. P. Gaughan, C. Marcinkiewicz, A. Huttenlocher, and D. F. Mosher, Extracellular matrix molecules regulate endothelial cell migration stimulated by lysophosphatidic acid, *J Thromb Haemost* 2 (2004) 1645-1656.
- [213] M. J. Kluk, and T. Hla, Role of the sphingosine 1-phosphate receptor EDG-1 in vascular smooth muscle cell proliferation and migration, *Circ Res* 89 (2001) 496-502.
- [214] M. Essler, M. Retzer, H. Ilchmann, S. Linder, and P. C. Weber, Sphingosine 1-phosphate dynamically regulates myosin light chain phosphatase activity in human endothelial cells, *Cell Signal* 14 (2002) 607-613.
- [215] J. H. Paik, S. Chae, M. J. Lee, S. Thangada, and T. Hla, Sphingosine 1-phosphate-induced endothelial cell migration requires the expression of EDG-1 and EDG-3 receptors and Rho-dependent activation of alpha vbeta3- and beta1-containing integrins, *J Biol Chem* 276 (2001) 11830-11837.
- [216] H. Okamoto, N. Takuwa, T. Yokomizo, N. Sugimoto, S. Sakurada, H. Shigematsu, and Y. Takuwa, 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 (2000) 9247-9261.
- [217] Y. Ryu, N. Takuwa, N. Sugimoto, S. Sakurada, S. Usui, H. Okamoto, O. Matsui, and Y. Takuwa, Sphingosine-1-phosphate, a platelet-derived

lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells, *Circ Res* 90 (2002) 325-332.

[218] K. Tamama, J. Kon, K. Sato, H. Tomura, A. Kuwabara, T. Kimura, T. Kanda, H. Ohta, M. Ui, I. Kobayashi, and F. Okajima, Extracellular mechanism through the Edg family of receptors might be responsible for sphingosine-1-phosphate-induced regulation of DNA synthesis and migration of rat aortic smooth-muscle cells, *Biochem J* 353 (2001) 139-146.

[219] D. Lepley, J. H. Paik, T. Hla, and F. Ferrer, The G protein-coupled receptor S1P2 regulates Rho/Rho kinase pathway to inhibit tumor cell migration, *Cancer Res* 65 (2005) 3788-3795.

[220] T. Sanchez, S. Thangada, M. T. Wu, C. D. Kontos, D. Wu, H. Wu, and T. Hla, PTEN as an effector in the signaling of antimigratory G protein-coupled receptor, *Proc Natl Acad Sci U S A* 102 (2005) 4312-4317.

[221] S. K. Goparaju, P. S. Jolly, K. R. Watterson, M. Bektas, S. Alvarez, S. Sarkar, L. Mel, I. Ishii, J. Chun, S. Milstien, and S. Spiegel, The S1P2 receptor negatively regulates platelet-derived growth factor-induced motility and proliferation, *Mol Cell Biol* 25 (2005) 4237-4249.

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

[223] Y. Shikata, K. G. Birukov, and J. G. Garcia, S1P induces FA remodeling in human pulmonary endothelial cells: role of Rac, GIT1, FAK, and paxillin, *J Appl Physiol* 94 (2003) 1193-1203.

[224] J. Folkman, and P. A. D'Amore, Blood vessel formation: what is its molecular basis? *Cell* 87 (1996) 1153-1155.

[225] R. D. Rudic, E. G. Shesely, N. Maeda, O. Smithies, S. S. Segal, and W. C. Sessa, Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling, *J Clin Invest* 101 (1998) 731-736.

[226] Y. Rikitake, K. Hirata, S. Kawashima, M. Ozaki, T. Takahashi, W. Ogawa, N. Inoue, and M. Yokoyama, Involvement of endothelial nitric oxide in sphingosine-1-phosphate-induced angiogenesis, *Arterioscler Thromb Vasc Biol* 22 (2002) 108-114.

[227] M. J. Lee, S. Thangada, K. P. Claffey, N. Ancellin, C. H. Liu, M. Kluk, M. Volpi, R. I. Sha'afi, and T. Hla, Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate, *Cell* 99 (1999) 301-312.

[228] F. Wang, J. R. Van Brocklyn, J. P. Hobson, S. Movafagh, Z. Zukowska-Grojec, S. Milstien, and S. Spiegel, Sphingosine 1-phosphate stimulates cell migration through a G(i)-coupled cell surface receptor. Potential involvement in angiogenesis, *J Biol Chem* 274 (1999) 35343-35350.

[229] J. G. Garcia, F. Liu, A. D. Verin, A. Birukova, M. A. Dechert, W. T. Gerthoffer, J. R. Bamberg, and D. English, Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement, *J Clin Invest* 108 (2001) 689-701.

[230] Y. Liu, R. Wada, T. Yamashita, Y. Mi, C. X. Deng, J. P. Hobson, H. M. Rosenfeldt, V. E. Nava, S. S. Chae, M. J. Lee, C. H. Liu, T. Hla, S. Spiegel, and R. L. Proia, Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation, *J Clin Invest* 106 (2000) 951-961.

[231] M. L. Allende, T. Yamashita, and R. L. Proia, G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation, *Blood* 102 (2003) 3665-3667.

[232] S. S. Chae, J. H. Paik, H. Furneaux, and T. Hla, Requirement for sphingosine 1-phosphate receptor-1 in tumor angiogenesis demonstrated by in vivo RNA interference, *J Clin Invest* 114 (2004) 1082-1089.

[233] P. Lindahl, H. Bostrom, L. Karlsson, M. Hellstrom, M. Kalen, and C. Betsholtz, Role of platelet-derived growth factors in angiogenesis and alveogenesis, *Curr Top Pathol* 93 (1999) 27-33.

[234] M. Hellstrom, M. Kalen, P. Lindahl, A. Abramsson, and C. Betsholtz, Role of PDGF-B and PDGF- β in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse, *Development* 126 (1999) 3047-3055.

[235] I. Ishii, B. Friedman, X. Ye, S. Kawamura, C. McGiffert, J. J. Contos, M. A. Kingsbury, G. Zhang, J. H. Brown, and J. Chun, Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3, *J Biol Chem* 276 (2001) 33697-33704.

[236] I. Ishii, X. Ye, B. Friedman, S. Kawamura, J. J. Contos, M. A. Kingsbury, A. H. Yang, G. Zhang, J. H. Brown, and J. Chun, Marked perinatal lethality and cellular signaling deficits in mice null for the two sphingosine 1-phosphate (S1P) receptors, S1P(2)/LP(B2)/EDG-5 and S1P(3)/LP(B3)/EDG-3, *J Biol Chem* 277 (2002) 25152-25159.

- [237] J. H. Paik, A. Skoura, S. S. Chae, A. E. Cowan, D. K. Han, R. L. Proia, and T. Hla, Sphingosine 1-phosphate receptor regulation of N-cadherin mediates vascular stabilization, *Genes Dev* 18 (2004) 2392-2403.
- [238] Y. Li, T. Uruno, C. Haudenschild, S. M. Dudek, J. G. Garcia, and X. Zhan, Interaction of cortactin and Arp2/3 complex is required for sphingosine-1-phosphate-induced endothelial cell remodeling, *Exp Cell Res* 298 (2004) 107-121.
- [239] K. J. Bayless, and G. E. Davis, Sphingosine-1-phosphate markedly induces matrix metalloproteinase and integrin-dependent human endothelial cell invasion and lumen formation in three-dimensional collagen and fibrin matrices, *Biochem Biophys Res Commun* 312 (2003) 903-913.
- [240] H. Tedesco-Silva, G. Mourad, B. D. Kahan, J. G. Boira, W. Weimar, S. Mulgaonkar, B. Nashan, S. Madsen, B. Charpentier, P. Pellet, and Y. Vanrenterghem, FTY720, a novel immunomodulator: efficacy and safety results from the first phase 2A study in de novo renal transplantation, *Transplantation* 79 (2005) 1553-1560.
- [241] Q. Zhang, Y. Chen, R. L. Fairchild, P. S. Heeger, and A. Valujskikh, Lymphoid sequestration of alloreactive memory CD4 T cells promotes cardiac allograft survival, *J Immunol* 176 (2006) 770-777.
- [242] D. D. Pinschewer, A. F. Ochsenbein, B. Odermatt, V. Brinkmann, H. Hengartner, and R. M. Zinkernagel, FTY720 immunosuppression impairs effector T cell peripheral homing without affecting induction, expansion, and memory, *J Immunol* 164 (2000) 5761-5770.
- [243] V. Brinkmann, S. Chen, L. Feng, D. Pinschewer, Z. Nikolova, and R. Hof, FTY720 alters lymphocyte homing and protects allografts without inducing general immunosuppression, *Transplant Proc* 33 (2001) 530-531.
- [244] M. Kiuchi, K. Adachi, A. Tomatsu, M. Chino, S. Takeda, Y. Tanaka, Y. Maeda, N. Sato, N. Mitsutomi, K. Sugahara, and K. Chiba, Asymmetric synthesis and biological evaluation of the enantiomeric isomers of the immunosuppressive FTY720-phosphate, *Bioorg Med Chem* 13 (2005) 425-432.
- [245] J. Butler, D. Lana, O. Round, and K. LaMontagne, Functional characterization of sphingosine 1-phosphate receptor agonist in human endothelial cells, *Prostaglandins Other Lipid Mediat* 73 (2004) 29-45.
- [246] M. H. Graler, and E. J. Goetzl, The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors, *Faseb J* 18 (2004) 551-553.

[247] K. LaMontagne, A. Littlewood-Evans, C. Schnell, T. O'Reilly, L. Wyder, T. Sanchez, B. Probst, J. Butler, A. Wood, G. Liao, E. Billy, A. Theuer, T. Hla, and J. Wood, Antagonism of sphingosine-1-phosphate receptors by FTY720 inhibits angiogenesis and tumor vascularization, *Cancer Res* 66 (2006) 221-231.

[248] G. C. Shore, and J. Viallet, Modulating the bcl-2 family of apoptosis suppressors for potential therapeutic benefit in cancer, *Hematology (Am Soc Hematol Educ Program)* (2005) 226-230.

[249] K. Burridge, and K. Wennerberg, Rho and Rac take center stage, *Cell* 116 (2004) 167-179.

[250] H. Rosen, and J. Liao, Sphingosine 1-phosphate pathway therapeutics: a lipid ligand-receptor paradigm, *Curr Opin Chem Biol* 7 (2003) 461-468.

[251] A. G. Renehan, C. Booth, and C. S. Potten, What is apoptosis, and why is it important? *Bmj* 322 (2001) 1536-1538.

[252] L. F. Barros, T. Hermosilla, and J. Castro, Necrotic volume increase and the early physiology of necrosis, *Comp Biochem Physiol A Mol Integr Physiol* 130 (2001) 401-409.

[253] M. J. Berridge, P. Lipp, and M. D. Bootman, The versatility and universality of calcium signalling, *Nat Rev Mol Cell Biol* 1 (2000) 11-21.

[254] S. L. Fink, and B. T. Cookson, Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells, *Infect Immun* 73 (2005) 1907-1916.

[255] J. F. Kerr, A. H. Wyllie, and A. R. Currie, Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics, *Br J Cancer* 26 (1972) 239-257.

[256] B. Fadeel, and S. Orrenius, Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease, *J Intern Med* 258 (2005) 479-517.

[257] G. M. Cohen, X. M. Sun, H. Fearnhead, M. MacFarlane, D. G. Brown, R. T. Snowden, and D. Dinsdale, Formation of large molecular weight fragments of DNA is a key committed step of apoptosis in thymocytes, *J Immunol* 153 (1994) 507-516.

[258] S. J. Martin, C. P. Reutelingsperger, A. J. McGahon, J. A. Rader, R. C. van Schie, D. M. LaFace, and D. R. Green, Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of

the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl, *J Exp Med* 182 (1995) 1545-1556.

[259] S. J. Martin, and D. R. Green, Protease activation during apoptosis: death by a thousand cuts? *Cell* 82 (1995) 349-352.

[260] B. B. Wolf, and D. R. Green, Suicidal tendencies: apoptotic cell death by caspase family proteinases, *J Biol Chem* 274 (1999) 20049-20052.

[261] Y. Tsujimoto, J. Cossman, E. Jaffe, and C. M. Croce, Involvement of the bcl-2 gene in human follicular lymphoma, *Science* 228 (1985) 1440-1443.

[262] M. O. Hengartner, and H. R. Horvitz, *C. elegans* cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2, *Cell* 76 (1994) 665-676.

[263] K. C. Zimmermann, C. Bonzon, and D. R. Green, The machinery of programmed cell death, *Pharmacol Ther* 92 (2001) 57-70.

[264] N. N. Danial, and S. J. Korsmeyer, Cell death: critical control points, *Cell* 116 (2004) 205-219.

[265] E. H. Cheng, M. C. Wei, S. Weiler, R. A. Flavell, T. W. Mak, T. Lindsten, and S. J. Korsmeyer, BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis, *Mol Cell* 8 (2001) 705-711.

[266] J. I. Fletcher, and D. C. Huang, BH3-only proteins: orchestrating cell death, *Cell Death Differ* 13 (2006) 1268-1271.

[267] R. Ley, K. E. Ewings, K. Hadfield, and S. J. Cook, Regulatory phosphorylation of Bim: sorting out the ERK from the JNK, *Cell Death Differ* 12 (2005) 1008-1014.

[268] H. Puthalakath, and A. Strasser, Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins, *Cell Death Differ* 9 (2002) 505-512.

[269] M. Adachi, X. Zhao, and K. Imai, Nomenclature of dynein light chain-linked BH3-only protein Bim isoforms, *Cell Death Differ* 12 (2005) 192-193.

[270] L. O'Connor, A. Strasser, L. A. O'Reilly, G. Hausmann, J. M. Adams, S. Cory, and D. C. Huang, Bim: a novel member of the Bcl-2 family that promotes apoptosis, *Embo J* 17 (1998) 384-395.

- [271] P. Bouillet, D. Metcalf, D. C. Huang, D. M. Tarlinton, T. W. Kay, F. Kontgen, J. M. Adams, and A. Strasser, Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity, *Science* 286 (1999) 1735-1738.
- [272] J. Whitfield, S. J. Neame, L. Paquet, O. Bernard, and J. Ham, Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release, *Neuron* 29 (2001) 629-643.
- [273] T. Akiyama, P. Bouillet, T. Miyazaki, Y. Kadono, H. Chikuda, U. I. Chung, A. Fukuda, A. Hikita, H. Seto, T. Okada, T. Inaba, A. Sanjay, R. Baron, H. Kawaguchi, H. Oda, K. Nakamura, A. Strasser, and S. Tanaka, Regulation of osteoclast apoptosis by ubiquitylation of proapoptotic BH3-only Bcl-2 family member Bim, *Embo J* 22 (2003) 6653-6664.
- [274] C. R. Weston, K. Balmanno, C. Chalmers, K. Hadfield, S. A. Molton, R. Ley, E. F. Wagner, and S. J. Cook, Activation of ERK1/2 by deltaRaf-1:ER* represses Bim expression independently of the JNK or PI3K pathways, *Oncogene* 22 (2003) 1281-1293.
- [275] R. Ley, K. Balmanno, K. Hadfield, C. Weston, and S. J. Cook, Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim, *J Biol Chem* 278 (2003) 18811-18816.
- [276] H. Harada, B. Quearry, A. Ruiz-Vela, and S. J. Korsmeyer, Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity, *Proc Natl Acad Sci U S A* 101 (2004) 15313-15317.
- [277] H. Puthalakath, D. C. Huang, L. A. O'Reilly, S. M. King, and A. Strasser, The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex, *Mol Cell* 3 (1999) 287-296.
- [278] K. Lei, and R. J. Davis, JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis, *Proc Natl Acad Sci U S A* 100 (2003) 2432-2437.
- [279] D. W. Nicholson, and N. A. Thornberry, Caspases: killer proteases, *Trends Biochem Sci* 22 (1997) 299-306.
- [280] M. M. Metzstein, G. M. Stanfield, and H. R. Horvitz, Genetics of programmed cell death in *C. elegans*: past, present and future, *Trends Genet* 14 (1998) 410-416.

- [281] J. Yuan, S. Shaham, S. Ledoux, H. M. Ellis, and H. R. Horvitz, The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 beta-converting enzyme, *Cell* 75 (1993) 641-652.
- [282] D. Xue, S. Shaham, and H. R. Horvitz, The *Caenorhabditis elegans* cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease, *Genes Dev* 10 (1996) 1073-1083.
- [283] E. A. Slee, C. Adrain, and S. J. Martin, Serial killers: ordering caspase activation events in apoptosis, *Cell Death Differ* 6 (1999) 1067-1074.
- [284] Q. L. Deveraux, R. Takahashi, G. S. Salvesen, and J. C. Reed, X-linked IAP is a direct inhibitor of cell-death proteases, *Nature* 388 (1997) 300-304.
- [285] N. Roy, Q. L. Deveraux, R. Takahashi, G. S. Salvesen, and J. C. Reed, The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases, *Embo J* 16 (1997) 6914-6925.
- [286] N. E. Crook, R. J. Clem, and L. K. Miller, An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif, *J Virol* 67 (1993) 2168-2174.
- [287] R. Takahashi, Q. Deveraux, I. Tamm, K. Welsh, N. Assa-Munt, G. S. Salvesen, and J. C. Reed, A single BIR domain of XIAP sufficient for inhibiting caspases, *J Biol Chem* 273 (1998) 7787-7790.
- [288] Y. Yang, S. Fang, J. P. Jenson, A. M. Weissman, and J. D. Ashwell, Ubiquitin Protein Ligase Activity of IAPs and Their Degradation in Proteasomes in Response to Apoptotic Stimuli, *Science* 288 (2000) 874-877.
- [289] D. L. Vaux, and J. Silke, IAPs, RINGs and ubiquitylation, *Nat Rev Mol Cell Biol* 6 (2005) 287-297.
- [290] Q. L. Deveraux, and J. C. Reed, IAP family proteins--suppressors of apoptosis, *Genes Dev* 13 (1999) 239-252.
- [291] M. Muzio, A. M. Chinnaiyan, F. C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J. D. Bretz, M. Zhang, R. Gentz, M. Mann, P. H. Krammer, M. E. Peter, and V. M. Dixit, FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex, *Cell* 85 (1996) 817-827.
- [292] M. E. Peter, The flip side of FLIP, *Biochem J* 382 (2004) e1-3.
- [293] A. Ashkenazi, and V. M. Dixit, Apoptosis control by death and decoy receptors, *Curr Opin Cell Biol* 11 (1999) 255-260.

[294] S. G. Cho, and E. J. Choi, Apoptotic signaling pathways: caspases and stress-activated protein kinases, *J Biochem Mol Biol* 35 (2002) 24-27.

[295] M. C. Wei, W. X. Zong, E. H. Cheng, T. Lindsten, V. Panoutsakopoulou, A. J. Ross, K. A. Roth, G. R. MacGregor, C. B. Thompson, and S. J. Korsmeyer, Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death, *Science* 292 (2001) 727-730.

[296] S. R. Wiley, K. Schooley, P. J. Smolak, W. S. Din, C. P. Huang, J. K. Nicholl, G. R. Sutherland, T. D. Smith, C. Rauch, C. A. Smith, and et al., Identification and characterization of a new member of the TNF family that induces apoptosis, *Immunity* 3 (1995) 673-682.

[297] P. Schneider, J. L. Bodmer, M. Thome, K. Hofmann, N. Holler, and J. Tschopp, Characterization of two receptors for TRAIL, *FEBS Lett* 416 (1997) 329-334.

[298] J. Han, L. A. Goldstein, B. R. Gastman, and H. Rabinowich, Interrelated roles for Mcl-1 and BIM in regulation of TRAIL-mediated mitochondrial apoptosis, *J Biol Chem* 281 (2006) 10153-10163.

[299] F. L. Kiechle, and X. Zhang, Apoptosis: biochemical aspects and clinical implications, *Clin Chim Acta* 326 (2002) 27-45.

[300] T. Rich, R. L. Allen, and A. H. Wyllie, Defying death after DNA damage, *Nature* 407 (2000) 777-783.

[301] A. Gross, J. Jockel, M. C. Wei, and S. J. Korsmeyer, Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis, *Embo J* 17 (1998) 3878-3885.

[302] W. P. Roos, and B. Kaina, DNA damage-induced cell death by apoptosis, *Trends Mol Med* (2006).

[303] J. Zha, H. Harada, E. Yang, J. Jockel, and S. J. Korsmeyer, Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L), *Cell* 87 (1996) 619-628.

[304] F. Condorelli, P. Salomoni, S. Cotteret, V. Cesi, S. M. Srinivasula, E. S. Alnemri, and B. Calabretta, Caspase cleavage enhances the apoptosis-inducing effects of BAD, *Mol Cell Biol* 21 (2001) 3025-3036.

[305] C. Du, M. Fang, Y. Li, L. Li, and X. Wang, Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition, *Cell* 102 (2000) 33-42.

- [306] A. M. Verhagen, P. G. Ekert, M. Pakusch, J. Silke, L. M. Connolly, G. E. Reid, R. L. Moritz, R. J. Simpson, and D. L. Vaux, Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins, *Cell* 102 (2000) 43-53.
- [307] Z. Liu, C. Sun, E. T. Olejniczak, R. P. Meadows, S. F. Betz, T. Oost, J. Herrmann, J. C. Wu, and S. W. Fesik, Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain, *Nature* 408 (2000) 1004-1008.
- [308] G. Wu, J. Chai, T. L. Suber, J. W. Wu, C. Du, X. Wang, and Y. Shi, Structural basis of IAP recognition by Smac/DIABLO, *Nature* 408 (2000) 1008-1012.
- [309] H. Zou, W. J. Henzel, X. Liu, A. Lutschg, and X. Wang, Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3, *Cell* 90 (1997) 405-413.
- [310] D. Acehan, X. Jiang, D. G. Morgan, J. E. Heuser, X. Wang, and C. W. Akey, Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation, *Mol Cell* 9 (2002) 423-432.
- [311] S. M. Srinivasula, M. Ahmad, T. Fernandes-Alnemri, and E. S. Alnemri, Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization, *Mol Cell* 1 (1998) 949-957.
- [312] P. Li, D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri, and X. Wang, Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade, *Cell* 91 (1997) 479-489.
- [313] E. A. Slee, M. T. Harte, R. M. Kluck, B. B. Wolf, C. A. Casiano, D. D. Newmeyer, H. G. Wang, J. C. Reed, D. W. Nicholson, E. S. Alnemri, D. R. Green, and S. J. Martin, Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner, *J Cell Biol* 144 (1999) 281-292.
- [314] D. G. Kirsch, A. Doseff, B. N. Chau, D. Lim, N. C. de Souza-Pinto, R. Hansford, M. B. Kastan, Y. A. Lazebnik, and J. M. Hardwick, Caspase-3-dependent Cleavage of Bcl-2 Promotes Release of Cytochrome c, *The Journal of Biological Chemistry* 274 (1999) 21155-21161.
- [315] M. Enari, H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata, A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD, *Nature* 391 (1998) 43-50.
- [316] A. H. Boulares, A. G. Yakovlev, V. Ivanova, B. A. Stoica, G. Wang, S. Iyer, and M. Smulson, Role of poly(ADP-ribose) polymerase (PARP)

cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells, *J Biol Chem* 274 (1999) 22932-22940.

[317] M. Tewari, L. T. Quan, K. O'Rourke, S. Desnoyers, Z. Zeng, D. R. Beidler, G. G. Poirier, G. S. Salvesen, and V. M. Dixit, Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase, *Cell* 81 (1995) 801-809.

[318] B. Buendia, A. Santa-Maria, and J. C. Courvalin, Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis, *J Cell Sci* 112 (Pt 11) (1999) 1743-1753.

[319] K. Orth, A. M. Chinnaiyan, M. Garg, C. J. Froelich, and V. M. Dixit, The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A, *J Biol Chem* 271 (1996) 16443-16446.

[320] L. Rao, D. Perez, and E. White, Lamin proteolysis facilitates nuclear events during apoptosis, *J Cell Biol* 135 (1996) 1441-1455.

[321] D. M. Vanags, M. I. Porn-Ares, S. Coppola, D. H. Burgess, and S. Orrenius, Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis, *J Biol Chem* 271 (1996) 31075-31085.

[322] S. Kothakota, T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T. J. McGarry, M. W. Kirschner, K. Kohts, D. J. Kwiatkowski, and L. T. Williams, Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis, *Science* 278 (1997) 294-298.

[323] B. Levkau, B. Herren, H. Koyama, R. Ross, and E. W. Raines, Caspase-mediated cleavage of focal adhesion kinase pp125FAK and disassembly of focal adhesions in human endothelial cell apoptosis, *J Exp Med* 187 (1998) 579-586.

[324] T. Rudel, F. T. Zenke, T. H. Chuang, and G. M. Bokoch, p21-activated kinase (PAK) is required for Fas-induced JNK activation in Jurkat cells, *J Immunol* 160 (1998) 7-11.

[325] T. Rudel, and G. M. Bokoch, Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2, *Science* 276 (1997) 1571-1574.

[326] M. Maceyka, H. Sankala, N. C. Hait, H. Le Stunff, H. Liu, R. Toman, C. Collier, M. Zhang, L. S. Satin, A. H. Merrill, Jr., S. Milstien, and S. Spiegel, SphK1 and SphK2, sphingosine kinase isoenzymes with opposing functions in sphingolipid metabolism, *J Biol Chem* 280 (2005) 37118-37129.

- [327] J. Davaille, L. Li, A. Mallat, and S. Lotersztajn, Sphingosine 1-phosphate triggers both apoptotic and survival signals for human hepatic myofibroblasts, *J Biol Chem* 277 (2002) 37323-37330.
- [328] A. Olivera, T. Kohama, L. Edsall, V. Nava, O. Cuvillier, S. Poulton, and S. Spiegel, Sphingosine kinase expression increases intracellular sphingosine-1-phosphate and promotes cell growth and survival, *J Cell Biol* 147 (1999) 545-558.
- [329] P. Xia, L. Wang, P. A. Moretti, N. Albanese, F. Chai, S. M. Pitson, R. J. D'Andrea, J. R. Gamble, and M. A. Vadas, Sphingosine kinase interacts with TRAF2 and dissects tumor necrosis factor-alpha signaling, *J Biol Chem* 277 (2002) 7996-8003.
- [330] Y. Su, D. Rosenthal, M. Smulson, and S. Spiegel, Sphingosine 1-phosphate, a novel signaling molecule, stimulates DNA binding activity of AP-1 in quiescent Swiss 3T3 fibroblasts, *J Biol Chem* 269 (1994) 16512-16517.
- [331] S. Betito, and O. Cuvillier, Regulation by sphingosine 1-phosphate of Bax and Bad activities during apoptosis in a MEK-dependent manner, *Biochem Biophys Res Commun* 340 (2006) 1273-1277.
- [332] J. R. Van Brocklyn, M. J. Lee, R. Menzeleev, A. Olivera, L. Edsall, O. Cuvillier, D. M. Thomas, P. J. Coopman, S. Thangada, C. H. Liu, T. Hla, and S. Spiegel, Dual actions of sphingosine-1-phosphate: extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival, *J Cell Biol* 142 (1998) 229-240.
- [333] V. Limaye, X. Li, C. Hahn, P. Xia, M. C. Berndt, M. A. Vadas, and J. R. Gamble, Sphingosine kinase-1 enhances endothelial cell survival through a PECAM-1-dependent activation of PI-3K/Akt and regulation of Bcl-2 family members, *Blood* 105 (2005) 3169-3177.
- [334] N. Igarashi, T. Okada, S. Hayashi, T. Fujita, S. Jahangeer, and S. Nakamura, Sphingosine kinase 2 is a nuclear protein and inhibits DNA synthesis, *J Biol Chem* 278 (2003) 46832-46839.
- [335] H. Liu, R. E. Toman, S. K. Goparaju, M. Maceyka, V. E. Nava, H. Sankala, S. G. Payne, M. Bektas, I. Ishii, J. Chun, S. Milstien, and S. Spiegel, Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis, *J Biol Chem* 278 (2003) 40330-40336.
- [336] D. C. Huang, and A. Strasser, BH3-Only proteins-essential initiators of apoptotic cell death, *Cell* 103 (2000) 839-842.

[337] L. C. Edsall, O. Cuvillier, S. Twitty, S. Spiegel, and S. Milstien, Sphingosine kinase expression regulates apoptosis and caspase activation in PC12 cells, *J Neurochem* 76 (2001) 1573-1584.

[338] N. Hisano, Y. Yatomi, K. Satoh, S. Akimoto, M. Mitsumata, M. A. Fujino, and Y. Ozaki, Induction and suppression of endothelial cell apoptosis by sphingolipids: a possible in vitro model for cell-cell interactions between platelets and endothelial cells, *Blood* 93 (1999) 4293-4299.

[339] N. Murata, K. Sato, J. Kon, H. Tomura, M. Yanagita, A. Kuwabara, M. Ui, and F. Okajima, Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions, *Biochem J* 352 (2000) 809-815.

[340] R. Ross, Atherosclerosis--an inflammatory disease, *N Engl J Med* 340 (1999) 115-126.

[341] B. Zhang, H. Tomura, A. Kuwabara, T. Kimura, S. Miura, K. Noda, F. Okajima, and K. Saku, Correlation of high density lipoprotein (HDL)-associated sphingosine 1-phosphate with serum levels of HDL-cholesterol and apolipoproteins, *Atherosclerosis* 178 (2005) 199-205.

[342] T. Kimura, K. Sato, A. Kuwabara, H. Tomura, M. Ishiwara, I. Kobayashi, M. Ui, and F. Okajima, Sphingosine 1-phosphate may be a major component of plasma lipoproteins responsible for the cytoprotective actions in human umbilical vein endothelial cells, *J Biol Chem* 276 (2001) 31780-31785.

[343] I. Suc, I. Escargueil-Blanc, M. Trolly, R. Salvayre, and A. Negre-Salvayre, HDL and ApoA prevent cell death of endothelial cells induced by oxidized LDL, *Arterioscler Thromb Vasc Biol* 17 (1997) 2158-2166.

[344] Y. G. Kwon, J. K. Min, K. M. Kim, D. J. Lee, T. R. Billiar, and Y. M. Kim, Sphingosine 1-phosphate protects human umbilical vein endothelial cells from serum-deprived apoptosis by nitric oxide production, *J Biol Chem* 276 (2001) 10627-10633.

[345] J. R. Nofer, and G. Assmann, Atheroprotective effects of high-density lipoprotein-associated lysosphingolipids, *Trends Cardiovasc Med* 15 (2005) 265-271.

[346] C. Mineo, I. S. Yuhanna, M. J. Quon, and P. W. Shaul, High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases, *J Biol Chem* 278 (2003) 9142-9149.

[347] I. S. Yuhanna, Y. Zhu, B. E. Cox, L. D. Hahner, S. Osborne-Lawrence, P. Lu, Y. L. Marcel, R. G. Anderson, M. E. Mendelsohn, H. H. Hobbs, and P.

W. Shaul, High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase, *Nat Med* 7 (2001) 853-857.

[348] J. R. Nofer, M. van der Giet, M. Tolle, I. Wolinska, K. von Wnuck Lipinski, H. A. Baba, U. J. Tietge, A. Godecke, I. Ishii, B. Kleuser, M. Schafers, M. Fobker, W. Zidek, G. Assmann, J. Chun, and B. Levkau, HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3, *J Clin Invest* 113 (2004) 569-581.

[349] H. T. Chung, H. O. Pae, B. M. Choi, T. R. Billiar, and Y. M. Kim, Nitric oxide as a bioregulator of apoptosis, *Biochem Biophys Res Commun* 282 (2001) 1075-1079.

[350] G. A. Blaise, D. Gauvin, M. Gangal, and S. Authier, Nitric oxide, cell signaling and cell death, *Toxicology* 208 (2005) 177-192.

[351] R. Barsacchi, C. Perrotta, P. Sestili, O. Cantoni, S. Moncada, and E. Clementi, Cyclic GMP-dependent inhibition of acid sphingomyelinase by nitric oxide: an early step in protection against apoptosis, *Cell Death Differ* 9 (2002) 1248-1255.

[352] T. Andoh, P. B. Chock, and C. C. Chiueh, Preconditioning-mediated neuroprotection: role of nitric oxide, cGMP, and new protein expression, *Ann N Y Acad Sci* 962 (2002) 1-7.

[353] J. Li, T. R. Billiar, R. V. Talanian, and Y. M. Kim, Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation, *Biochem Biophys Res Commun* 240 (1997) 419-424.

[354] S. Dimmeler, J. Haendeler, M. Nehls, and A. M. Zeiher, Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases, *J Exp Med* 185 (1997) 601-607.

[355] P. R. Dash, J. E. Cartwright, P. N. Baker, A. P. Johnstone, and G. S. Whitley, Nitric oxide protects human extravillous trophoblast cells from apoptosis by a cyclic GMP-dependent mechanism and independently of caspase 3 nitrosylation, *Exp Cell Res* 287 (2003) 314-324.

[356] B. Zech, R. Kohl, A. von Knethen, and B. Brune, Nitric oxide donors inhibit formation of the Apaf-1/caspase-9 apoptosome and activation of caspases, *Biochem J* 371 (2003) 1055-1064.

[357] Y. Taniyama, and K. K. Griendling, Reactive oxygen species in the vasculature, *Hypertension* 42 (2003) 1075-1081.

[358] T. Matsunaga, S. Kotamraju, S. V. Kalivendi, A. Dhanasekaran, J. Joseph, and B. Kalyanaraman, Ceramide-induced intracellular oxidant formation, iron signaling, and apoptosis in endothelial cells: protective role of endogenous nitric oxide, *J Biol Chem* 279 (2004) 28614-28624.

[359] J. Igarashi, and T. Michel, Agonist-modulated targeting of the EDG-1 receptor to plasmalemmal caveolae. eNOS activation by sphingosine 1-phosphate and the role of caveolin-1 in sphingolipid signal transduction, *J Biol Chem* 275 (2000) 32363-32370.

[360] D. G. Hemmings, Signal transduction underlying the vascular effects of sphingosine 1-phosphate and sphingosylphosphorylcholine, *Naunyn Schmiedebergs Arch Pharmacol* 373 (2006) 18-29.

[361] M. Morales-Ruiz, M. J. Lee, S. Zollner, J. P. Gratton, R. Scotland, I. Shiojima, K. Walsh, T. Hla, and W. C. Sessa, 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 (2001) 19672-19677.

[362] T. Tanimoto, Z. G. Jin, and B. C. Berk, Transactivation of vascular endothelial growth factor (VEGF) receptor Flk-1/KDR is involved in sphingosine 1-phosphate-stimulated phosphorylation of Akt and endothelial nitric-oxide synthase (eNOS), *J Biol Chem* 277 (2002) 42997-43001.

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

[364] S. Siehler, Y. Wang, X. Fan, R. T. Windh, and D. R. Manning, 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 (2001) 48733-48739.

[365] S. Shishodia, and B. B. Aggarwal, Nuclear factor-kappaB activation: a question of life or death, *J Biochem Mol Biol* 35 (2002) 28-40.

[366] Y. Osawa, H. Uchinami, J. Bielawski, R. F. Schwabe, Y. A. Hannun, and D. A. Brenner, Roles for C16-ceramide and sphingosine 1-phosphate in regulating hepatocyte apoptosis in response to tumor necrosis factor-alpha, *J Biol Chem* 280 (2005) 27879-27887.

[367] K. R. Watterson, E. Johnston, C. Chalmers, A. Pronin, S. J. Cook, J. L. Benovic, and T. M. Palmer, Dual regulation of EDG1/S1P(1) receptor phosphorylation and internalization by protein kinase C and G-protein-coupled receptor kinase 2, *J Biol Chem* 277 (2002) 5767-5777.

- [368] H. Ogita, and Y. Takai, Activation of Rap1, Cdc42, and rac by nectin adhesion system, *Methods Enzymol* 406 (2006) 415-424.
- [369] J. M. Haugh, F. Codazzi, M. Teruel, and T. Meyer, Spatial sensing in fibroblasts mediated by 3' phosphoinositides, *J Cell Biol* 151 (2000) 1269-1279.
- [370] S. Zhang, J. Han, M. A. Sellis, J. Chernoff, U. G. Knaus, R. J. Ulevitch, and G. M. Bokoch, Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1, *J Biol Chem* 270 (1995) 23934-23936.
- [371] Y. Sogabe, M. Abe, Y. Yokoyama, and O. Ishikawa, Basic fibroblast growth factor stimulates human keratinocyte motility by Rac activation, *Wound Repair Regen* 14 (2006) 457-462.
- [372] S. N. Willis, L. Chen, G. Dewson, A. Wei, E. Naik, J. I. Fletcher, J. M. Adams, and D. C. Huang, Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins, *Genes Dev* 19 (2005) 1294-1305.
- [373] P. J. Reddig, and R. L. Juliano, Clinging to life: cell to matrix adhesion and cell survival, *Cancer Metastasis Rev* 24 (2005) 425-439.
- [374] M. J. Reginato, K. R. Mills, J. K. Paulus, D. K. Lynch, D. C. Sgroi, J. Debnath, S. K. Muthuswamy, and J. S. Brugge, Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis, *Nat Cell Biol* 5 (2003) 733-740.
- [375] A. Sunters, S. Fernandez de Mattos, M. Stahl, J. J. Brosens, G. Zoumpoulidou, C. A. Saunders, P. J. Coffey, R. H. Medema, R. C. Coombes, and E. W. Lam, FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines, *J Biol Chem* 278 (2003) 49795-49805.
- [376] M. Engstrom, R. Karlsson, and J. I. Jonsson, Inactivation of the forkhead transcription factor FoxO3 is essential for PKB-mediated survival of hematopoietic progenitor cells by kit ligand, *Exp Hematol* 31 (2003) 316-323.
- [377] A. Brunet, A. Bonni, M. J. Zigmund, M. Z. Lin, P. Juo, L. S. Hu, M. J. Anderson, K. C. Arden, J. Blenis, and M. E. Greenberg, Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor, *Cell* 96 (1999) 857-868.
- [378] S. Guo, G. Rena, S. Cichy, X. He, P. Cohen, and T. Unterman, Phosphorylation of serine 256 by protein kinase B disrupts transactivation by FKHR and mediates effects of insulin on insulin-like growth factor-binding

protein-1 promoter activity through a conserved insulin response sequence, *J Biol Chem* 274 (1999) 17184-17192.

[379] C. J. Chalmers, K. Balmanno, K. Hadfield, R. Ley, and S. J. Cook, Thrombin inhibits Bim (Bcl-2-interacting mediator of cell death) expression and prevents serum-withdrawal-induced apoptosis via protease-activated receptor 1, *Biochem J* 375 (2003) 99-109.

[380] S. A. Molton, C. Weston, K. Balmanno, C. Newson, D. E. Todd, A. P. Garner, and S. J. Cook, The conditional kinase DeltaMEKK1:ER* selectively activates the JNK pathway and protects against serum withdrawal-induced cell death, *Cell Signal* 17 (2005) 1412-1422.

[381] S. Bu, M. Yamanaka, H. Pei, A. Bielawska, J. Bielawski, Y. A. Hannun, L. Obeid, and M. Trojanowska, Dihydrosphingosine 1-phosphate stimulates MMP1 gene expression via activation of ERK1/2-Ets1 pathway in human fibroblasts, *Faseb J* 20 (2006) 184-186.

[382] G. Milligan, G. D. Kim, I. Mullaney, and E. J. Aadle, Regulation of cellular G_s alpha levels and basal adenylyl cyclase activity by expression of the beta 2-adrenoceptor in neuroblastoma cell lines, *Mol Cell Biochem* 149 (1995) 213-216.

[383] C. Waters, B. Sambhi, K. C. Kong, D. Thompson, S. M. Pitson, S. Pyne, and N. J. Pyne, Sphingosine 1-phosphate and platelet-derived growth factor (PDGF) act via PDGF beta receptor-sphingosine 1-phosphate receptor complexes in airway smooth muscle cells, *J Biol Chem* 278 (2003) 6282-6290.

[384] F. Alderton, S. Rakhit, K. C. Kong, T. Palmer, B. Sambhi, S. Pyne, and N. J. Pyne, Tethering of the platelet-derived growth factor beta receptor to G-protein-coupled receptors. A novel platform for integrative signaling by these receptor classes in mammalian cells, *J Biol Chem* 276 (2001) 28578-28585.

[385] C. M. Waters, J. Long, I. Gorshkova, Y. Fujiwara, M. Connell, K. E. Belmonte, G. Tigyi, V. Natarajan, S. Pyne, and N. J. Pyne, Cell migration activated by platelet-derived growth factor receptor is blocked by an inverse agonist of the sphingosine 1-phosphate receptor-1, *Faseb J* 20 (2006) 509-511.

[386] A. Levitzki, PDGF receptor kinase inhibitors for the treatment of PDGF driven diseases, *Cytokine Growth Factor Rev* 15 (2004) 229-235.

[387] H. M. El-Shewy, K. R. Johnson, M. H. Lee, A. A. Jaffa, L. M. Obeid, and L. M. Luttrell, Insulin-like growth factors mediate heterotrimeric G protein-dependent ERK1/2 activation by transactivating sphingosine-1-phosphate receptors, *J Biol Chem* (2006).

- [388] L. A. Sitailo, S. S. Tibudan, and M. F. Denning, The protein kinase C delta catalytic fragment targets MCL-1 for degradation to trigger apoptosis, *J Biol Chem* (2006).
- [389] T. Yang, H. L. Buchan, K. J. Townsend, and R. W. Craig, MCL-1, a member of the BCL-2 family, is induced rapidly in response to signals for cell differentiation or death, but not to signals for cell proliferation, *J Cell Physiol* 166 (1996) 523-536.
- [390] U. Maurer, C. Charvet, A. S. Wagman, E. Dejardin, and D. R. Green, Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1, *Mol Cell* 21 (2006) 749-760.
- [391] S. A. Molton, D. E. Todd, and S. J. Cook, Selective activation of the c-Jun N-terminal kinase (JNK) pathway fails to elicit Bax activation or apoptosis unless the phosphoinositide 3'-kinase (PI3K) pathway is inhibited, *Oncogene* 22 (2003) 4690-4701.
- [392] A. M. Domina, J. A. Vrana, M. A. Gregory, S. R. Hann, and R. W. Craig, MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol, *Oncogene* 23 (2004) 5301-5315.
- [393] J. Zhuang, and H. J. Brady, Emerging role of Mcl-1 in actively counteracting BH3-only proteins in apoptosis, *Cell Death Differ* 13 (2006) 1263-1267.
- [394] H. Yamaguchi, J. Kitayama, N. Takuwa, K. Arikawa, I. Inoki, K. Takehara, H. Nagawa, and Y. Takuwa, Sphingosine-1-phosphate receptor subtype-specific positive and negative regulation of Rac and haematogenous metastasis of melanoma cells, *Biochem J* 374 (2003) 715-722.
- [395] H. C. Welch, W. J. Coadwell, L. R. Stephens, and P. T. Hawkins, Phosphoinositide 3-kinase-dependent activation of Rac, *FEBS Lett* 546 (2003) 93-97.
- [396] S. N. Willis, and J. M. Adams, Life in the balance: how BH3-only proteins induce apoptosis, *Curr Opin Cell Biol* 17 (2005) 617-625.
- [397] B. T. Hennessy, D. L. Smith, P. T. Ram, Y. Lu, and G. B. Mills, Exploiting the PI3K/AKT pathway for cancer drug discovery, *Nat Rev Drug Discov* 4 (2005) 988-1004.