The Effect of Palmitoylation on the Function of the Alpha Subunits of the Guanine Nucleotide Binding Proteins G_{11} and G_o.

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For Danny & Etta

And for Jacqueline:
"You have made a place in my heart where
I thought there was no room for anything else.
You have made flowers grow where I cultivated
dust and stones."

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List of Abbreviations.

A:	Amps
ADP:	adenosine 5'-diphosphate
ATP:	adenosine 5'-triphosphate
A_X:	absorbance at xnm
βARK:	β adrenergic receptor kinase
bp:	base pairs
Bromophenol blue:	3',3",5',5"-tetrabromophenolsulphophthalein
BSA:	bovine serum albumin
cAMP:	adenosine 3':5'-cyclic monophosphate
cDNA	complementary DNA
cGMP:	guanosine 3':5'-cyclic monophosphate
CIAP:	calf intestinal alkaline phosphatase
CoA:	coenzyme A
C terminus:	carboxyl terminus
dATP:	deoxyribo adenosine 5'-triphosphate
dCTP:	deoxyribo cytosine 5'-triphosphate
dGTP:	deoxyribo guanosine 5'-triphosphate
DMEM:	Dulbecco's Modified Eagle's Medium
dNTP:	equimolar solution of dATP, dCTP, dGTP and dTTP
dsDNA:	double stranded DNA
DTT:	dithiothreitol
dTTP:	deoxyribo thymidine 5'-triphosphate
EDTA:	ethylenediamin-N,N,N',N'-tetra-acetic acid
EF-Tu:	elongation factor Tu
Forskolin:	7β-1α,6β,9α-trihydroxy-8,13-epoxy-labd-14-en-11-one

GPI:	glycosylphosphatidyl inositol
GppNHp:	guanylyl 5'-imidodiphosphate
GRK:	G protein linked receptor kinase
GTP:	guanosine 5'-triphosphate
GTPγS:	guanosine 5'-[3-*O*-thio]triphosphate
HAT:	hypoxanthine aminopterin thymidine
HEK 293:	human embryonic kidney 293 cells
Ig:	Immunoglobulin
kb:	kilobase pairs
kDa:	kilodaltons
mA:	milliamps
MAP kinase:	mitogen activated protein kinase
MgOAc:	magnesium acetate
Mr:	relative mobility
mRNA:	messenger RNA
NAD^+:	nicotinamide adenine dinucleotide
NBS:	newborn bovine serum
NMT:	myristoyl-CoA:protein *N*-myristoyl transferase
NP-40:	nonidet P-40
N terminus:	amino terminus
OD_X:	optical density at xnm
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PH domain:	Pleckstrin homology domain
PMSF:	phenylmethylsulphonyl fluoride
SDS:	sodium dodecyl sulphate
TCA:	trichloroacetic acid

TEMED:	N,N,N',N'-tetramethylethylenediamine
Tris:	tris(hydroxymethyl)aminomethane
UV:	ultraviolet
V:	volts
W:	watts

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Abstract

Heterotrimeric guanine nucleotide binding proteins function to couple seven transmembrane spanning serpentine receptors to effector systems governing the regulation of intracellular second messengers. The basic molecular mechanism by which these G proteins operate has been known for several years, but the precise details of their functions, specificities and regulation is at present unresolved. These G proteins are characterised by being composed of three non-identical subunits termed α, β and γ. Molecular cloning techniques have revealed the presence of 17α, 4β and 10γ genes encoding these subunits. The α subunits have been classed into 4 groups termed the G_s, G_i, G_q and G_{12} families.

Upon agonist occupation of a receptor, the G protein undergoes a conformational change causing GDP, which is bound to the α subunit in its inactive state, to be released and allowing GTP to enter the nucleotide binding pocket, causing release from the receptor and dissociation of the G protein into a free α subunit and a βγ dimer. These moieties then interact with their target effector systems before the intrinsic GTPase activity of the α subunit hydrolyses the GTP to GDP, allowing heterotrimer reformation and receptor interaction, returning the system to its resting state. It was originally thought that the α subunit solely contained the sites for interaction with both the receptor and the effector. It is now apparent however that the βγ dimer plays an important role in governing the specificity of G protein-receptor interaction, and can affect second messenger generating systems such as adenylyl cyclase, phosphoinositidase C and certain ion channels.

G proteins are membrane associated proteins. The initial theory that the role of the βγ complex was to anchor the α subunit to the membrane, via prenylated residues at the C terminus of the γ subunit is largely outdated. The presence of the 14 carbon saturated fatty acid, myristic acid, at the N terminus of some G_i family members was thought to hold the key to this dilemma. Myristoylated peptides however do not have sufficient free energy to interact with the membrane. Also, not all myristoylated proteins are membrane associated and all of the other G proteins lack the necessary consensus sequence for N-myristoylation. Another mechanism must exist to account for the membrane interactions of G protein α subunits.

Recently it has been demonstrated that the 16 carbon fatty acid, palmitic acid, is also present in the N terminal region of the majority of G protein α subunits, as well as several other proteins involved in intracellular signalling, notably Src family members. This lipidation is thought to be a post translational, reversible modification, generally occurring via labile thio-ester bonds. This is in contrast to N-myristoylation, which is co-translational and generally irreversible.

This thesis aimed to determine the effect of mutating the target palmitoylation sites of rat $G_{o1}\alpha$ and murine $G_{11}\alpha$ on their ability to interact with the plasma membrane when transfected into heterologous cell systems. To do this, it was necessary to develop a system in which it was possible to unambiguosly detect the relevant $G_{o1}\alpha$ or $G_{11}\alpha$ polypeptide by western blotting. It was found that acrylamide gels containing urea in the resolving gel could sufficiently resolve the polypeptides to enable detection by nonspecific antisera against $G_o\alpha$ isoforms and $G_{q/11}\alpha$.

Use was made of a 4M-8M urea gradient in a 12.5% acrylamide/0.0625% bisacrylamide SDS-PAGE gel system to resolve isoforms of $G_o\alpha$ from rat brain membranes and from membranes of the neuroblastoma x glioma hybrid cell line NG108-15. Following separation of membranes of Rat-1 cells transfected with wild type and palmitate negative mutants of rat $G_{o1}\alpha$, immunoblots with an antiserum directed against $G_o\alpha$ were able to detect an immunoreactive band which corresponded to a band in rat brain and NG108-15 membranes, which had previously been identified as $G_{o1}\alpha$.

To detect murine $G_{11}\alpha$ unambiguously, a 10% acrylamide/0.24% bisacrylamide SDS-PAGE gel containing 6M urea was employed to separate species variants of this α subunit. This system has been reported to separate murine $G_{11}\alpha$ from the simian form of the polypeptide, such that the murine form would migrate more rapidly through the gel matrix. Thus when the cDNA encoding murine $G_{11}\alpha$ was transfected into simian COS-1 cells, it could be detected using an antiserum which recognises $G_q\alpha$ and $G_{11}\alpha$.

It has been reported in the literature that introduction of the cysteine-to-serine mutation at position 3 (C3S) into rat $G_{o1}\alpha$ would abolish palmitoylation and reduce the ability of this polypeptide to interact with the plasma membrane of transiently transfected COS-1 cells. In this thesis it was decided to assess the membrane association of this C3S $G_{o1}\alpha$ polypeptide in comparison to the wild type protein in a stably transfected rat cell line. It was found that approximately 30% of the immunoreactive C3S $G_{o1}\alpha$ was present in the cytosolic fraction of these cells, following homogenisation and separation into membrane and cytosolic fractions, while all the wild type protein was present in the membrane. These figures agreed well with prior experiments in which membranes of NG108-15 cells were chemically depalmitoylated with 1M

hydroxylamine and immunoblotted for $G_o\alpha$. Both the wild type and palmitate negative mutant of $G_{o1}\alpha$ were substrates for pertussis toxin catalysed ADP-ribosylation, indicating $\alpha\beta\gamma$ heterotrimer formation. Evidence was also found that C3S $G_{o1}\alpha$ was cycling between a membrane compartment and the cytosol of the cell.

$G_{11}\alpha$ does not contain a cysteine at position 3 but does possess two cysteines at positions 9 and 10 which align with the sites of palmitoylation of other G proteins. To determine whether these cysteine residues were the sites of palmitoylation of murine $G_{11}\alpha$, mutants of the protein were created by site-directed mutagenesis in which cys9, cys10 or both were mutated to serine. Upon transient transfection into simian COS-1 cells, all three mutants showed a similar membrane:cytosol distribution; approximately 30% of immunoreactive murine $G_{11}\alpha$ was found in the cytosol, which again agreed well with chemical depalmitoylation experiments.

When [^{3}H] palmitate incorporation studies were carried out on these mutants following transient transfection it was found that no palmitate was incorporated into C9SC10S $G_{11}\alpha$. The C9S and C10S single mutants of $G_{11}\alpha$ only incorporated palmitate to some 20% of that found on wild type $G_{11}\alpha$. This indicated that both cysteine residues at positions 9 and 10 were targets for palmitoylation and suggested the possibility of cooperativity in the transfer of palmitic acid to $G_{11}\alpha$.

These studies have demonstrated the role that palmitoylation plays in the membrane association of these two polypeptides and may aid the elucidation of the wider issue of how palmitoylation affects the function of G proteins in general. It may be that palmitoylation could affect the interaction of α

subunits with receptors or effectors or it could be a regulatory signal for uncoupling of the G protein from the signalling cascade.

Chapter 1.

Introduction.

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1. 1. Historical Perspective.

It is now apparent that a family of highly homologous guanine nucleotide binding proteins (G proteins) play a pivotal role in coupling agonist liganded receptors to the alteration of a corresponding, effector mediated, change in the levels of an intracellular second messenger.

The hypothesis that hormones could produce changes in second messenger levels by regulating the enzyme responsible for its production was first postulated following a series of experiments demonstrating that the actions of adrenaline on canine liver could be mimicked by cAMP [Rall *et al.*, 1957; Sutherland *et al.*, 1962]. Sutherland proposed that adrenaline bound to specific cell surface receptors and, once liganded, these receptors could cause a conformational change in the catalytic moiety of adenylyl cyclase resulting in an activation of the enzyme.

The involvement of GTP in this signalling event was demonstrated in 1971 when Rodbell and co-workers noted the requirement of GTP in glucagon mediated activation of adenylyl cyclase [Rodbell *et al.*, 1971b] and that GTP enhanced the dissociation of glucagon from its adenylyl cyclase coupled receptor [Rodbell *et al.*, 1971a; Harwood *et al.*, 1973]. Non-hydrolysable analogues of GTP (GTPγS and GppNHp) were shown to irreversibly activate adenylyl cyclase in the absence of agonist [Schramm &

Rodbell, 1975], the presence of which increased the rate of formation of the irreversibly activated complex [Cautrecasas *et al.*, 1975], suggesting that the role of liganded receptor was to facilitate the guanine nucleotide mediated activation of the enzyme.

The role of GTP in regulating the receptor-effector interaction was shown by Cassel & Selinger who demonstrated an increase in high affinity GTPase activity associated with β-adrenergic stimulation of adenylyl cyclase [Cassel & Selinger, 1976]. The ability to reconstitute this activity into membranes of the murine S49 lymphoma mutant, cyc⁻, lead to the purification of this entity, known as G_s for its stimulatory effect on adenylyl cyclase.

The cycle by which these G proteins turn on and off the signal from an activated receptor was first postulated by Gilman [1984]. He suggested that in the resting state, the receptor was coupled to a GDP liganded G protein, and that hormonal activation of the receptor caused a conformational change in both proteins leading to the exchange of GDP for GTP. This guanine nucleotide exchange promoted dissociation of the G protein from its trimeric form into a free active α subunit and a βγ dimer. The α subunit, which bound the guanine nucleotide, could then interact with the effector system before the α subunits' intrinsic GTPase activity hydrolysed the GTP to GDP promoting reformation of the holomeric protein, re-association with the receptor and turning off of the signal [Gilman, 1984; Gilman 1987] .

Since this groundbreaking work, much effort has been expended in studying the role the G protein plays in regulating transmembrane signalling. Since the advent of molecular cloning techniques many variants of

the three subunits of G proteins have been identified. The control of these distinct, yet highly homologous, proteins is evidently crucial to how the cell collects and correlates extracellular stimuli.

The G protein cycle postulated by Gilman [Gilman, 1984; 1987] indicates a purely sedentary role for the βγ dimer. Work over recent years has demonstrated that this is not the case and a functional role for this dimer in interacting with a wide range of effector systems including adenylyl cyclase, phosphoinositidase C, phospholipase A_2 and the MAP kinase cascade has been shown [Clapham & Neer, 1993; Sternweis, 1994].

These heterotrimeric G proteins are, however, members of a larger superfamily of guanine nucleotide binding proteins of which elongation factor Tu (EF-Tu) and the $p21^{ras}$ proto-oncogene product are notable members. This Introduction will concentrate mainly on the role of the heterotrimeric G proteins. However, the small molecular weight G proteins have many similarities to the heterotrimeric ones and reference to them will be made.

1. 2. The structure and function of heterotrimeric G proteins.

All the heterotrimeric G proteins involved in hormonal signalling share a basic structure consisting of one α subunit, one β subunit and one γ subunit, in order of decreasing molecular mass [Gilman, 1987]. These polypeptides range in mass from 39-52kDa for α subunits, 35-36kDa for β subunits and 8-10kDa for γ subunits and couple serpentine receptors to

one or more intracellular second messenger generating systems such as adenylyl cyclase, phosphoinositidase C, phospholipase A2 and various ion channels and pumps.

Since the advent of molecular cloning techniques, the family of heterotrimeric G protein genes has expanded, and to date there are now 17 separate genes encoding α subunits [Simon *et al.*, 1991; McLaughlin *et al.*, 1992], 4 genes encoding β subunits and 10 genes encoding γ subunits. To create even more diversity, $G_s\alpha$ and $G_o\alpha$ undergo alternative splicing of the primary transcript, and there is evidence to suggest a functional role for these splice variants. The presence of these different gene products indicates a complexity in terms of cellular signalling that is only now beginning to be understood.

1. 2. 1. Gα subunits.

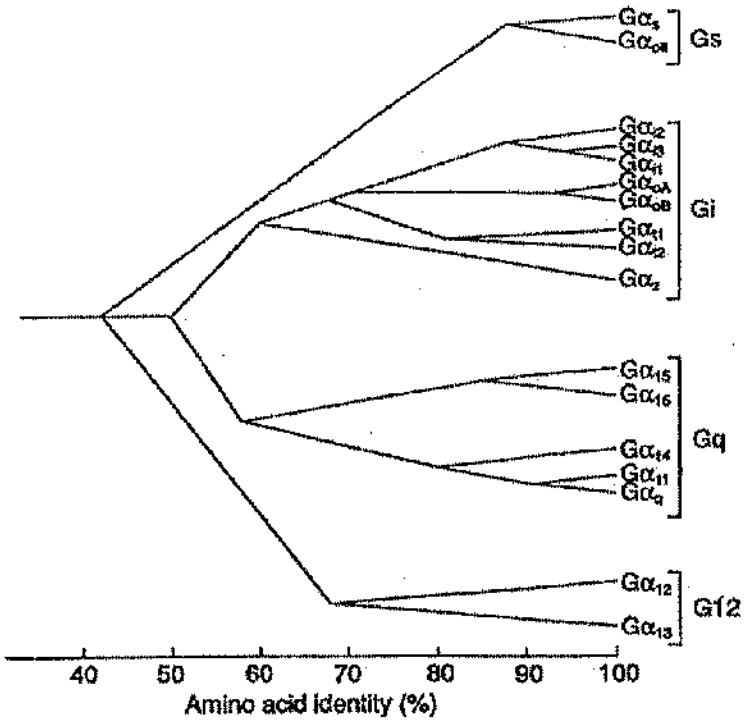
All α subunits share sequence homology to a greater or lesser degree, which reflects the similar function of these proteins. Simon *et al.* [1991] have grouped 16 α subunits, according to amino acid sequence homology, into 4 subfamilies (**figure 1. 1.**) termed the G_s , G_i, G_q and G_{12} families. The subsequent discovery of gustducin, an α subunit related to transducin, indicated that it belonged to the G_i family [McLaughlin *et al.*, 1992].

The homology between the α subunits is not uniform over the length of the polypeptide but regions of high and low identity exist between all α subunits. The four areas of highest identity represent regions critical for

Figure 1. 1. Amino acid identity of the cloned G protein α subunits.

The Gα subunits so far identified have been grouped into 4 families based on their amino acid identity as shown. The subsequent discovery of gustducin placed this α subunit in the G_i family. The two splice variants of $G_o\alpha$ are also shown. All other α subunits shown are products of specific genes. This figure was taken from Simon *et al.*, [1991].

Figure 1.1


Gαs
Gαolf
Gs
Gαi2
Gαi3
Gαi1
GαoA
GαoB
Gαt1
Gαt2
Gαz
Gi
Gα15
Gα16
Gα14
Gα11
Gαq
Gq
Gα12
Gα13
G12
40
50
60
70
80
90
100
Amino acid identity (%)

binding of guanine nucleotides and site the GTPase activity of the α subunit. Areas of greatest diversity may represent areas of functional differences in coupling of the α subunits to βγ subunits, receptors or effectors [Simon *et al.*, 1991]. The areas of the α subunits which are responsible for the particular functions of the proteins will be discussed later. What follows is a consideration of the functions and properties of the various subunits.

1. 2. 1. 1. Members of the G_S family.

$G_S\alpha$: The most studied of the two members of this family, and perhaps the most studied α subunit of all is $G_S\alpha$. So called for its stimulatory effect on adenylyl cyclase, this α subunit was first identified by Cassel & Selinger [1976] who demonstrated the requirement of a hormone sensitive GTPase in the stimulation of adenylyl cyclase and that the hydrolysis of GTP served as the turn-off mechanism behind the stimulation [Cassel & Selinger, 1978]. Ross & Gilman [1977] demonstrated that the disruption of hormone stimulated adenylyl cyclase in the cyc^- mutant of S49 lymphoma cells was due to the absence of a GTP-binding protein. By reconstitution of β adrenergic stimulated adenylyl cyclase activity into membranes of the cyc^- cells, Northup *et al.* [1980] were able to purify the protein, now termed $G_S\alpha$. The protein was found to be a trimer of α, β, and γ subunits and has been shown to be a mixture of two oligomers made up of two distinct α subunits and indistinguishable βγ dimers. The α subunits, of apparent Mr 52 and 45kDa respectively, vary in relative concentrations between cell types although any functional differences between the two have yet to be identified.

cDNAs corresponding to $G_s\alpha$ have been obtained from various sources. Deduced amino acid sequences for these cDNAs indicate few amino acid differences between human and murine forms of the protein [Simon *et al.*, 1991]. This high degree of cross species conservation is evident in all α subunits. The first cDNA isolated encoded a protein of 394 amino acids, with a predicted molecular weight of 46kDa, but upon expression in either mammalian COS-M6 cells [Robishaw *et al.*, 1986a] or prokaryotic *E.coli* [Gilman, 1987] the cDNA directed the synthesis of the 52kDa form of the protein, indicating that the mobility of the protein in SDS-PAGE is anomalous [Gilman, 1987]. The cDNA for the second form of $G_s\alpha$ encodes a polypeptide with a predicted molecular weight of 44.5 kDa and directed the synthesis of the 45kDa form in both COS-M6 cells and *E.coli* [Robishaw *et al.*, 1986b; Gilman, 1987].

Four distinct cDNAs encoding $G_s\alpha$ proteins have now been isolated [Bray *et al.*, 1986]. The isolation of the $G_s\alpha$ gene has shown the presence of 13 exons and 12 introns covering about 20kb of DNA [Kozasa *et al.*, 1988]. The four cDNAs encoding $G_s\alpha$ are produced by alternate splicing of this single gene. $G_s\alpha$-1 and $G_s\alpha$-2 are identical except for a single 45 nucleotide stretch absent in $G_s\alpha$-3 and $G_s\alpha$-4. $G_s\alpha$-2 and $G_s\alpha$-4 have an additional 3 nucleotides located 3' to the above 45 nucleotides. This is due to the alternate splicing of exon 3 of the gene such that $G_s\alpha$-1 and -2 have exon 3 and $G_s\alpha$-3 and -4 do not [Bray *et al.*, 1986; Kozasa *et al.*, 1988; Kaziro *et al.*, 1990]. The production of an additional serine residue in $G_s\alpha$-2 and -4 by this splicing may represent an potential phosphorylation site on $G_s\alpha$ for protein kinase C. $G_s\alpha$ has been shown to be the target of phosphorylation by this kinase *in vitro* [Pyne *et al.*, 1992]

The function of $G_s\alpha$ is, as has already been stated, to stimulate the enzyme adenylyl cyclase to produce cAMP from ATP. The enzyme itself is a single transmembrane spanning polypeptide with both N- and C- termini located intracellularly. The protein spans the membrane twelve times and contains two large intracellular domains one between transmembrane helices 6 and 7 and another located at the C-terminus [Taussig & Gilman, 1995]. These regions are both thought to form catalytic sites in the protein (termed C). This molecular architecture resembles that of ATP driven pumps such as the P glycoprotein [Cooper *et al.*, 1995]. Indeed the adenylyl cyclase of *Paramecium* has been shown to be a K^+ pump as well [Schultz *et al.*, 1992]. It is entirely possible that adenylyl cyclases of other species also have ion conductance properties. $G_s\alpha$ is also known to regulate the plasma membrane Ca^{2+} ATPase, presumably due to its topological resemblance to adenylyl cyclase.

Two substances are commonly used to affect the interaction of $G_s\alpha$ with adenylyl cyclase. The diterpene forskolin stabilises the interaction with $G_s\alpha$ and C causing the constitutive activation of adenylyl cyclase [Gilman, 1987]. An exotoxin from *Vibrio choleræ*, termed cholera toxin, also causes the persistent activation of the adenylyl cyclase system. It does so by the enzymatic transfer of ADP ribose from NAD^+ to an arginine residue in $G_s\alpha$ close to the site which houses the GTPase activity of the protein. The α subunit is locked in its GTP bound, active state causing constitutive activation of adenylyl cyclase [Gilman, 1987]. Both these effects give rise to chronically high levels of cAMP inside the cell which has a variety of effects on intracellular proteins.

$G_s\alpha$ is ubiquitously expressed in all cells although the relative levels of expression of the splice variants vary from cell type to cell type and from tissue to tissue. The exact purpose of this differential expression is unclear at present as there has been no evidence of any functional difference between the splice variants.

$G_{olf}\alpha$: This α subunit was isolated from a cDNA library from rat olfactory neuroepithelium [Jones & Reed, 1989]. Its expression was originally thought to be restricted to olfactory tissues [Jones & Reed, 1989] but is now known to be found in other areas of the CNS and is particularly prevalent in striatum [Hervé *et al.*, 1993]. Its function, to stimulate the production of cAMP by adenylyl cyclase, is the same as $G_s\alpha$, to which it shares 88% amino acid identity [Simon *et al.*, 1991]. An isozyme of adenylyl cyclase (type III) has been identified that is particularly rich in olfactory tissues. This may represent a specific G protein/effector subsystem.

1. 2. 1. 2. Members of the G_i family.

Originally described as the G protein involved in the hormonal inhibition of adenylyl cyclase, $G_i\alpha$ has subsequently proved to be a mixture of 3 α subunits termed $G_{i1}\alpha$, $G_{i2}\alpha$ and $G_{i3}\alpha$ [Jones & Reed, 1987].

$G_{i1}\alpha$: The largest member of the trio, at 41kDa, is $G_{i1}\alpha$ and like most of the other members of the family this protein is subject to modification by an exotoxin from the causative agent of Whooping Cough, *Bordetella pertussis*. This adds an ADP-ribose group, donated by NAD^+, to a cysteine residue 4 amino acids from the C-terminus of the α subunit. This transfer occurs most

efficiently when the G protein is in its holomeric, heterotrimeric form, complexed to its receptor [Gilman, 1987; Milligan 1988]. This ADP-ribosylation causes a stabilisation of the trimeric form of the protein and causes the release of the inactive G protein from the receptor preventing transduction of the hormone signal to the appropriate effector system. This releases the tonic inhibition of effector systems regulated by G_i proteins, causing their constitutive activation.

$G_{i1}\alpha$ demonstrates a more limited tissue distribution compared to $G_{i2}\alpha$ and $G_{i3}\alpha$, being concentrated in neuronal tissues and certain other tissues. This may indicate a role in regulating ion channels as opposed to adenylyl cyclase.

$G_{i2}\alpha$: This α subunit is the smallest of the three G_i proteins. It too is a substrate for pertussis toxin catalysed ADP-ribosylation and shares around 88% amino acid identity with $G_{i1}\alpha$. Although other members of the G_i family of α subunits have been shown to cause inhibition of adenylyl cyclase [Taussig & Gilman, 1995], there is more evidence to suggest that $G_{i2}\alpha$ is the physiological regulator of this process. Using antibodies against $G_{i2}\alpha$ to selectively uncouple $G_{i2}\alpha$ from its adenylyl cyclase, McKenzie & Milligan [1990b] demonstrated that $G_{i2}\alpha$ was responsible for the inhibition of forskolin stimulated adenylyl cyclase activity in NG108-15 neuroblastoma x glioma hybrid cells.

Another system which $G_{i2}\alpha$ has been reported to interact with is the MAP kinase cascade [van Corven *et al.*, 1993; Albas *et al.*, 1993], a series of protein kinases ultimately leading to the phosphorylation and

activation of Mitogen Activated Protein kinase, an enzyme which catalyses the phosphorylation of a variety of cellular proteins to cause, among other things, entry into the cell cycle and stimulation of transcription. It is probable however that this stimulation of MAP kinase is mediated by βγ [Crespo *et al.*, 1994].

$G_{i3}\alpha$: The third of the G_i family of α subunits is thought to stimulate certain ion channels in the plasma membrane. Work showed a G_i protein purified from human erythrocyte membranes to be capable of stimulating receptor activated K^+ channels in atrial and pituitary membrane patches [Yatani *et al*, 1987]. This protein was subsequently found to be G_{i3}. Work in recent years has identified another ion channel under the control of $G_{i3}\alpha$. The renal epithelial cell line A6 contains an amiloride sensitive Na^+ channel which can be activated via $G_{i3}\alpha$ [Cantiello *et al.*, 1989] apparently via an activation of phospholipase A_2 [Cantiello *et al.*, 1990]. The α subunit was also present in a 700kDa complex which also contained the Na^+ channel [Ausiello *et al.*, 1992].

$G_{i3}\alpha$ has also been demonstrated to be involved in trafficking of proteins from the Golgi to the plasma membrane. Overexpression of $G_{i3}\alpha$ under the control of an inducible promoter inhibited the secretion of heparin sulphate proteoglycan from LLC-PK_1 epithelial cells [Stow *et al.*, 1991]. In the same study, these investigators found that in both these cells and NRK epithelial cells, $G_{i3}\alpha$ was localised to Golgi membranes and that the $G_{i3}\alpha$ found on isolated rat liver Golgi membranes was a substrate for pertussis toxin catalysed ADP ribosylation. Pertussis toxin treatment reversed the inhibition of secretion of heparin sulphate proteoglycan indicating a role for $G_{i3}\alpha$ in Golgi trafficking of a constitutively secreted protein.

$G_o\alpha$: Originally identified as a 39kDa pertussis toxin substrate of unknown function purified from bovine brain and termed G_o for 'G other' [Sternweis & Robishaw, 1984; Neer *et al.*, 1984; Milligan & Klee, 1985], this α subunit has been the focus of intense research due to its limited tissue distribution; $G_o\alpha$ is present mainly in neuronal and electrically excitable cells. Although the function of $G_o\alpha$ was initially a mystery, an elegant series of experiments by Hescheler *et al.* [1990] demonstrated that activated $G_o\alpha$ could inhibit the opening of a voltage sensitive Ca^{2+} channel, which was subsequently shown to be an N-type Ca^{2+} channel. $G_o\alpha$ has also been shown to gate K^+ channels [van Dongen *et al.*, 1988].

The cDNA for $G_o\alpha$ was initially isolated from a number of sources, both mammalian and insect. The gene encoding the protein is large and covers about 90kb in humans [Kaziro, 1990] and contains 10 exons. Alternate splicing of the gene was first demonstrated in 1990 by two groups who isolated cDNAs encoding the original clone, which they termed $G_{o1}\alpha$ or $G_{oA}\alpha$, and a second splice variant termed $G_{o2}\alpha$ or $G_{oB}\alpha$ [Hsu *et al.*, 1990; Strathmann *et al.*, 1990]. These two splice variants are identical at their N terminus but show considerable variation at their C terminus. Overall, these two splice variants share approximately 90% amino acid identity [Simon *et al.*, 1991]. Another form of $G_o\alpha$, termed $G_o{}^*\alpha$, has been purified from bovine brain [Goldsmith *et al.*, 1988] although a cDNA for this form has never been isolated and no functional data has been reported for it. It does not migrate with either $G_{o1}\alpha$ or $G_{o2}\alpha$ under SDS-PAGE conditions developed to resolve them [Mullaney & Milligan, 1990]. Recently a third splice variant has been purified from both porcine and bovine brain membranes [Nurnberg *et al.*,

1994], named $G_{o3}\alpha$. This form is not $G_o{}^*\alpha$ either as its electrophoretic mobility is closer to that of $G_{o1}\alpha$.

Like the splice variants of $G_s\alpha$, the $G_o\alpha$ splice variants show a differential tissue distribution. Asano *et al.* [1992] described the distribution of $G_{o1}\alpha$ as being restricted primarily to neuronal cells while $G_{o2}\alpha$ was found in peripheral tissues such as pituitary, lung and testis.

Two groups have demonstrated the differential regulation of $G_{o1}\alpha$ and $G_{o2}\alpha$ during differentiation. Upon treatment with agents which raise intracellular cAMP, Mullaney & Milligan [1990] found that the pattern of $G_o\alpha$ isoform expression in NG108-15 neuroblastoma x glioma hybrid cells changed. Using a 2D gel system, these workers found that differentiation of NG108-15 cells caused an increase in the more acidic isoform; the expression of the more basic form remained unchanged. Brabet *et al.* [1990] described a similar situation in the neuroblastoma cell line N1E-115 where upon differentiation, these cells began to express more of the acidic form of $G_o\alpha$. Subsequent use of isoform specific antisera indicated that the more acidic form of $G_o\alpha$ was in fact $G_{o1}\alpha$ [Rouot *et al.*, 1991]. The physiological relevance of this was demonstrated by Rouot *et al.* [1991] who showed the progressive decline of $G_{o2}\alpha$ and the concurrent increase in expression of $G_{o1}\alpha$ during the development of mouse brains from the 15th day of gestation to 15 days post partum. This regulation was shown to occur as a result of a change in the degradation rates of the two isoforms [Brabet *et al.*, 1991].

Unlike the splice variants of $G_s\alpha$, those of $G_o\alpha$ have been shown to have distinct functional differences. Using an antisense DNA

approach to selectively eliminate the splice variants in GH_3 cells, Kleuss *et al.* [1991] demonstrated that while $G_{o1}\alpha$ could couple to a muscarinic acetylcholine receptor, $G_{o2}\alpha$ could not and conversely, that while $G_{o2}\alpha$ could couple to a somatostatin receptor, $G_{o1}\alpha$ could not. Similarly, by microinjection of purified $G_{o1}\alpha$ and $G_{o2}\alpha$ into identified neurones of the pond snail *Helisoma trivolvis*, Man-Son-Hing *et al.*, [1992] demonstrated that only $G_{o2}\alpha$ could inhibit the voltage sensitive Ca^{2+} current in these neurones, perhaps indicating a role for $G_{o1}\alpha$ in regulating the other described role of G_o, that of gating K^+ currents. In their study identifying $G_{o3}\alpha$, Nurnberg *et al.* [1994] also indicated a distinct role for $G_{o3}\alpha$ in intracellular signalling, as the purified protein could not reconstitute the carbachol mediated inhibition of the voltage sensitive Ca^{2+} current in pertussis toxin treated SH-SY5Y cells, while both purified $G_{o1}\alpha$ and $G_{o2}\alpha$ could fulfil this function.

$G_t\alpha$: Transducin, or $G_t\alpha$, was originally identified as the entity which "transduced" the activation state of rhodopsin to the phosphodiesterase reponsible for the lowering of levels of cGMP, influx of Na from the outside of the cell, depolarisation and thus initiation of the nerve impulse to the brain. The protein was purified around the same time as G_s and found to consist of three distinct subunits, like G_s [Gilman, 1987]. Subsequent cloning of $G_t\alpha$ by four groups produced two separate clones, named $G_{t1}\alpha$ and $G_{t2}\alpha$ [Lochric *et al*, 1985; Tanabe *et al.*, 1985; Yatsunami & Khorana, 1985; Medynski *et al.*, 1985]. Subsequent studies showed that $G_{t1}\alpha$ was restricted to retinal rod membranes while $G_{t2}\alpha$ was found only in retinal cone membranes [Grunwald *et al.*, 1986; Lerea *et al.*, 1986]. $G_{t1}\alpha$ and $G_{t2}\alpha$ share approximately 80% amino acid identity, bind to rhodopsin and cone opsin respectively and activate a cGMP specific phosphodiesterase. Recently $G_{t1}\alpha$ has been found in non-

retinal taste tissue [Ruiz-Avila *et al.*, 1995], where it functions to couple the bitter taste receptor to a phosphodiesterase.

The transducins are unique among the hetrotrimeric G proteins in that they can be washed from the retinal membrane in the absence of detergent [Spiegel, 1990]. This is not due to any known differences in the amino acid composition of $G_{t1}\alpha$ or $G_{t2}\alpha$, as will be elaborated upon later in this chapter, but is instead due to specific differences in the lipid environment of the retina causing laurylation of the α subunit, and the expression of a specific retinal form of the γ subunit, now termed γ_1, which is prenylated with a farnesyl moiety. Both of these lipid moeities are less hydrophobic than the more common myristate and geranylgeranyl.

Gustducin α: The newest α subunit to be isolated was obtained from a taste tissue cDNA library [McLaughlin *et al.*, 1992]. Analysis of the primary amino acid sequence of gustducin α suggested that this α subunit belongs to the G_i family as it contained a consensus sequence for pertussis toxin catalysed ADP-ribosylation. It showed particular similarity to the transducins. Rat gustducin demonstrates 81% and 90% identity to bovine $G_{t1}\alpha$ and $G_{t2}\alpha$, respectively [McLaughlin *et al.*, 1992] and 79% identity to rat $G_{t1}\alpha$ [Ruiz-Avila *et al.*, 1995].

Analysis of the expression of gustducin α indicated that it is only expressed in taste tissue. No message corresponding to gustducin was found in non-taste lingual tissue, olfactory epithelium, retina, brain, liver, heart or kidney. Gustducin was found in all three taste papillae, that is circumvallate, foliate and fungiform [McLaughlin *et al.*, 1992]. These workers postulated

that, by analogy to the transducins, gustducin activation would also lead to the activation of a phosphodiesterase, although a peptide derived from gustducin α could not activate a taste cell phosphodiesterase when the corresponding peptide from $G_{t1}\alpha$ could [Ruiz-Avila *et al.*, 1995]. Baculovirus expressed gustducin α could interact with bovine rhodopsin and bovine retinal cGMP phosphodiesterase and both bovine brain and retinal $\beta\gamma$ [Hoon *et al.*, 1995].

$G_z\alpha$: The most divergent of the G_i family of G proteins, sharing only 62% amino acid homology with other members of the family, $G_z\alpha$ is unique as it is the only G_i family member that lacks the cysteine residue which acts the acceptor for the ADP ribose group transferred from NAD^+ by pertussis toxin [Casey *et al.*, 1990]. It was cloned from human brain and retina simultaneously [Matsuoka *et al.*, 1988; Fong *et al.*, 1988]

$G_z\alpha$ also has several properties that distinguish it from other α subunits. It has both a very slow guanine nucleotide release rate and a slow intrinsic GTPase activity [Hinton *et al.*, 1990]. It also has a limited tissue distribution, being mainly restricted to neurones, particularly those with long axonal processes, and platelets [Hinton *et al.*, 1990]. Like most other members of the G_i family it is, however, dually acylated by myristic acid on gly2 and palmitic acid on cys3. This will be elaborated upon in the following section and in subsequent chapters. As yet, however, $G_z\alpha$ has no known signalling function assigned specifically, although it may inhibit type I and type V adenylyl cyclases [Taussig & Gilman, 1995] and has been reported to allow pertussis toxin insensitive inhibition of adenylyl cyclase.

1. 2. 1. 3. Members of the G_q family.

$G_q\alpha$: The cloning of two distinct G protein α subunits by Strathmann & Simon [1990] opened a whole new era in the study of G proteins. The first of these, which they termed $G_q\alpha$, was predicted to encode a protein of 359 amino acids, equivalent to approximately 42kDa under SDS-PAGE. It lacked the cysteine residue required for pertussis toxin catalysed ADP-ribosylation. The protein was found ubiquitously, both in terms of tissue specificity and in organism specificity, from mammals and other vertebrates to invertebrates such as *Drosophila*. This alluded to a universal function for $G_q\alpha$ and it subsequently transpired that it was the long sought after G protein regulator of phosphoinositidase C [Smrcka *et al.*, 1991].

Phosphoinositidase C is in fact a variety of isozymes which belong to a gene superfamily. All the isozymes function in the same manner, to hydrolyse phosphatidylinositol-4,5-bisphosphate (PIP_2) yielding the second messenger molecules *sn*-1,2-diradylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). This bifurcation of the signal leads to the activation of two distinct pathways. The lipid molecule DAG causes the activation of a series of isozymes termed protein kinase C (PKC). PKC isozymes are serine/threonine protein kinases which affect many cellular processes including the activation of the Raf kinase, which is then able to activate another series of protein kinases ending in the phosphorylation and activation of MAP kinase. IP_3, being a polar molecule, is released from the membrane to bind to specific receptors, located on the endoplasmic reticulum. This causes the release of Ca^{2+} from stores in the ER, again leading to effects on cellular systems.

The phosphoinositidase C isozymes differ in their regulation, however, and only those belonging to the β class are under direct G protein control [Rhee & Choi, 1992] although phosphoinositidase C δ_1 may be stimulated by βγ [Park *et al.*, 1993]. Those of the γ class are activated through their Src Homology 2 (SH2) domains by binding phosphotyrosine residues of growth factor receptors. The β isozymes are activated both by the α subunit of G_q family members and the βγ dimer (see later), which can be generated from any class of α subunit. This dual regulation gives rise to both the pertussis toxin insensitive activation of PLCβs, mediated by the α subunit of G_q family members, and their pertussis toxin sensitive activation, caused by the release of βγ from activated α subunits of G_i family members. The sites of action on PLCβ for G protein α subunits and βγ dimers are apparently distinct, however [Smrcka & Sternweis, 1993; Lee *et al.*, 1993], and the efficacies of their activation differ between the different, known, members of the PLCβ family, termed PLCβ1-4 (see **table 1. 1.**).

$G_{11}\alpha$: In the same paper that announced the cloning of $G_q\alpha$, Strathmann & Simon [1990] reported the presence of another α subunit, which they termed $G_{11}\alpha$. This protein was very similar to $G_q\alpha$, sharing approximately 90% identity at the amino acid level [Simon *et al.*, 1991] and also contained 359 amino acids. This protein too was not a substrate for pertussis toxin and to date little if any functional difference has been reported between $G_q\alpha$ and $G_{11}\alpha$. Purified $G_{q/11}$ from brain can activate PLCβ1 in a reconstitution assay and the two α subunits may have identical abilities to activate PLCβ1 [Blank *et al*, 1991].

Table 1. 1. Comparison of the regulation of phosphoinositidase C by G protein α and βγ subunits.

The β isoforms of phosphoinositidase C (PIC) are regulated by G proteins. To date all are known to be activated by α and βγ subunits but to different degrees. This is displayed in this table such that the stimulations are of the order ++++>+++>++>+>+/-. This table was constructed by data from Smrcka & Sternweis [1993] and Lee *et al.* [1993].

Table 1. 1.

PLCβ Isoform	Activation by $G_{q/11}\alpha$	Activation by Gβγ
β1	+++	+
β2	+/-	++
β3	++++	+++
β4	?	?

Despite the apparently similar function of the two proteins, the tissue distribution of $G_q\alpha$ and $G_{11}\alpha$ shows some variation. Although both proteins are found almost ubiquitously, the exception being an apparent lack of $G_{11}\alpha$ in human haematopoietic cells [Milligan *et al.*, 1993], the relative amounts of the two proteins differ in both brain regions [Milligan, 1992] and peripheral tissues [Milligan *et al.*, 1993].

Chronic activation of receptors linked to these α subunits, such as the M1 muscarinic acetylcholine receptor [Mitchell *et al.*, 1993] or gonadotrophin releasing hormone (GnRH) receptor [Shah & Milligan, 1994], led to the downregulation of both $G_q\alpha$ and $G_{11}\alpha$ equally and non-selectively without affecting the G protein α subunits $G_s\alpha$ and $G_{i2}\alpha$. This would indicate an apparent lack of specificity in signalling through $G_q\alpha$ and $G_{11}\alpha$. This begs the question, why does a receptor apparently activate two different G proteins to fulfil the same function? The answer to this question is unclear at present. The differences in primary structure between $G_q\alpha$ and $G_{11}\alpha$ are mostly clustered near the N terminus of the proteins in regions concerned with $\beta\gamma$ binding and the relative rate of guanine nucleotide exchange and hydrolysis [Simon *et al.*, 1991], perhaps indicating that $G_q\alpha$ and $G_{11}\alpha$ couple differently to different combinations of β and γ.

$G_{14}\alpha$: The third member of this family is also a PLCβ activating enzyme. Analysis of the distribution of mRNA encoding $G_{14}\alpha$ indicates a more restricted pattern of expression, and is found primarily in stromal and epithelial cells [Wilkie *et al.*, 1991]. Another groups who also reported the cloning of $G_{14}\alpha$, found mRNA corresponding to the α subunit in liver, lung and kidney and none in bovine cerebral cortex and atrium [Nakamura *et al.*,

1991]. $G_{14}\alpha$ shares approximately 80% identity with $G_q\alpha$ and $G_{11}\alpha$ at the amino acid level.

$G_{15}\alpha$: This α subunit has an even more restricted tissue distribution, being found only in cells of a haematopoietic lineage; it is found in murine B cells and myeloid cells and shares a 57% amino acid homology with $G_q\alpha$, $G_{11}\alpha$ and $G_{14}\alpha$ [Simon *et al.*, 1991; Amatruda *et al.*, 1991].

$G_{16}\alpha$: The final member of this class of G proteins is a human homologue of $G_{15}\alpha$ and shares approximately 83% amino acid homology to it. Its tissue distribution is restricted to haematopoietic cells such as T cells and myeloid cells [Amatruda *et al.*, 1991]. It contains structural differences from other α subunits, namely two inserts near the C terminus, in the region proposed to house the effector contact site for α subunits, and a divergent N terminus. The first insert (amino acids 317-323) is similar to the corresponding region in $G_s\alpha$ while the second insert (amino acids 331-337) contains several charged residues not found in other α subunits [Amatruda *et al.*, 1991]. It also contains a proline residue at position 50, in the "A" site (see later), a region involved in GTP binding and hydrolysis, where other α subunits contain alanine (G_s or G_i) or threonine (G_q). This may indicate an impaired capacity to bind guanine nucleotides and hydrolyse GTP as α subunits with divergent amino acids at this point ($G_z\alpha$, $G_q\alpha$ and $G_{11}\alpha$) [Simon *et al.*, 1991] have reduced abilities to fulfil these functions [Pang & Sternweis, 1990; Casey *et al.*, 1990].

1. 2. 1. 4. Members of the G_{12} family.

$G_{12}\alpha$ and $G_{13}\alpha$:

The final class of G protein α subunits contains two known members, $G_{12}\alpha$ and $G_{13}\alpha$ [Strathmann & Simon, 1991]. These polypeptides share relatively little primary sequence identity with the other α subunits (approximately 45%) and have less than 70% identity with each other [Strathmann & Simon, 1991]. They are both expressed ubiquitously [Strathmann & Simon, 1991].

Comparatively little is known about either subunit and it has only been recently that the role of these two α subunits has become clearer. Using constitutively activated mutants of $G_{12}\alpha$ and $G_{13}\alpha$ [Xu *et al.*, 1993] demonstrated an activation of arachidonic acid metabolism and Offermanns *et al.* [1994] have shown using photoreactive azidoanilide analogues of GTP that both $G_{12}\alpha$ and $G_{13}\alpha$ are activated by the thrombin and thromboxane A_2 receptors in platelet membranes. Several recent reports have suggested a role for $G_{12}\alpha$ and $G_{13}\alpha$ in regulating Na+/H+ exchange [Voyno-Yasenetskaya *et al.*, 1994; Dhanasekaran *et al.*, 1994]. Like the activated mutant of $G_{i2}\alpha$ in Rat 1a cells [Gupta *et al.*, 1992], activated mutants of $G_{12}\alpha$ and $G_{13}\alpha$ have been shown to be focus forming in NIH-3T3 cells [Xu *et al.*, 1993; Jiang *et al.*, 1993].

Like G_q family members and $G_z\alpha$ [Pang & Sternweis, 1990; Casey *et al.*, 1990; Wange *et al.*, 1991] $G_{12}\alpha$ and $G_{13}\alpha$ have a slower basal guanine nucleotide exchange rate than $G_s\alpha$ and most G_i family members [Offermanns *et al.*, 1994].

1. 2. 2. The structure and function of the βγ dimer.

Under non-reducing conditions, the β and γ subunits of heterotrimeric G proteins are found exclusively, at the membrane, as a tightly associated complex. To date there exists four known β subunits ranging in size from 35 to 36 kDa and at least 10 γ subunits of approximately 8 to 10kDa. The β subunits share approximately 80% amino acid identity with the differences spread throughout the sequence [Simon *et al.*, 1991; Gao *et al.*, 1987; von Weizsacker *et al.*, 1992]. The γ subunits on the other hand share relatively little sequence identity, γ_1 is only 38% identical to γ_2 and is similarly different from other γ subunits [Clapham & Neer, 1993].

These subunits do not randomly form in any combination. Only certain allowed associations are found in cells. For example, the γ_1 subunit, whose expression is restricted to the retina, will only associate with the β_1 subunit. On the other hand, the β_1 subunit can complex to γ_1, γ_2, γ_3, γ_5 and γ_7. β_2 can associate with any of γ_2, γ_3, γ_5 or γ_7. β3 associates with neither γ_1 or γ_2 [Pronin & Gautam, 1992; Schmidt *et al.*, 1992; Iñiguez-Lluhi *et al.*, 1992; Ueda *et al.*, 1994]. These reports indicated that certain combinations of β and γ subunits were either not formed or were unstable.

Perhaps one of the most interesting and revealing discoveries of recent years has been the active role of the βγ dimer in signalling events. Originally, βγ was thought to play a purely sedentary role in signal transduction, by acting as an anchor for the more active α subunit. The first

evidence of an active role for βγ was presented by Jelsema & Axelrod [1987] who demonstrated an activation of phospholipase A_2 by βγ in outer rod segments of bovine retina. Around the same time came a report of the activation of a cardiac K^+ channel, the $I_{K.ACh}$ channel, which is activated by the muscarinic acetylcholine receptor. The channel can be activated, in patch clamp experiments by either the α subunit or the βγ complex, and this activation by one of the two proteins was not conditional upon preactivation with the other [Logothetis *et al.*, 1987; Codina *et al.*, 1987; Logothetis *et al.*, 1988]. The βγ dimer activates the $I_{K.ACh}$ channel more successfully than the α subunit, while Gα alone can activate another K^+ channel the $I_{K.ATP}$ channel in the same patch [Ito *et al.*, 1992]. It is unclear whether the activation of the K^+ channel is direct or whether it is via the prior activation of phospholipase A_2 [Kim *et al.*, 1989; Clapham & Neer, 1993].

Since these pioneering studies, it has become apparent that βγ carries out a number of diverse functions. Perhaps one of the best documented is the activation of the β isoforms of phosphoinositidase C. This was first demonstrated by Camps *et al.* [1992] using purified or partially purified components. These workers demonstrated the stimulation of phosphoinositidase C from HL-60 cell cytosol by bovine retinal βγ and showed that this stimulation was blocked by GDP liganded $G_t\alpha$, was additive to the stimulation by GTPγS, was not dependent on prior actiavtion of phospholipase A_2 and was specific for one isoform of phosphoinositidase C.

The intricacies of this regulation are still being worked out. It is apparent, however, that the degree of stimulation of the various β isoforms of phosphoinositidase C by the βγ dimer are not the same (see **table 1. 1.**). It has

been suggested that the sites of interaction on phosphoinositidase C for α and $\beta\gamma$ differ [Smrcka & Sternweis, 1993; Lee *et al.*, 1993] and it has been shown that while the α subunit interacts with a site at the C terminal region of phosphoinositidase Cβ_2 and that this region is probably distinct from the $\beta\gamma$ contact site [Lee *et al.*, 1993]. Wu *et al.* [1993] demonstrated that the $\beta\gamma$ contact site was found in the N terminal part of phosphoinositidase Cβ_2 while the α contact site was in the C terminal portion of the enzyme.

Wu *et al.* [1993] also demonstrated that different combinations of β and γ could activate phosphoinositidase Cβ_2 to different degrees, thus $\beta_1\gamma_1 > \beta_1\gamma_5 > \beta_2\gamma_5$ ($\beta_1\gamma_1$ caused a 10 fold stimulation of inositol phosphate production while $\beta_1\gamma_5$ caused an 8 fold stimulation and $\beta_2\gamma_5$ caused a 4 fold stimulation) while $\beta_2\gamma_1$ did not cause activation (presumably because this dimer cannot form [Pronin & Gautam, 1992]). Ueda *et al.* [1994] demonstrated that $\beta_1\gamma_2$, $\beta_1\gamma_3$, $\beta_1\gamma_5$, $\beta_1\gamma_7$, $\beta_2\gamma_2$, $\beta_2\gamma_3$, $\beta_2\gamma_5$ or $\beta_2\gamma_7$ were equally effective in activating phosphoinositidase Cβ_3 while $\beta_1\gamma_1$ was less effective.

Adenylyl cyclase has also been shown to be regulated by the $\beta\gamma$ complex. This regulation is not as all encompassing as that for the β isoforms of phosphoinositidase C but resembles more the type specific regulation of cardiac K^+ channels by $\beta\gamma$. This regulation adds yet another dimension to the regulation of adenylyl cyclase (see **table 1. 2.**). The $\beta\gamma$ complex can not only activate certain isoforms of adenylyl cyclase but can also cause the inhibition of certain other isoforms. In insect Sf9 cells transfected with recombinant baculoviruses encoding adenylyl cyclase isoforms, Tang & Gilman [1991]

Table 1. 2. The regulation of adenylyl cyclase.

Adenylyl cyclase is present as many different isoforms, each the product of distinct genes, sometimes with many isoforms being present in a single cell type. The regulation of these isoforms is complex involving Ca^{2+} and calmodulin (Ca^{2+}/Calmod), protein kinase C (PKC) as well as $\ddot{G}$ protein subunits. This is displayed here for the type I-VIII isoforms. No data was available for more recently isolated clones. This table was taken from Cooper *et al.* [1995].

Table 1. 2.

Adenylyl Cyclase	**$G_s\alpha$ Stimulation**	**$\beta\gamma$ Effect**	**Ca^{2+}/Calmod. Effect**	**PKC Stimulation**
I	Mild	Inhibition	Stimulation	No
II	Yes	Stimulation	No	Yes
III	Yes	?	Stimulation	No
IV	Yes	Stimulation	No	No
V	Yes	No	Inhibition	No
VI	Yes	No	Inhibition	No
VII	Yes	?	No	Yes
VIII	Mild	?	Stimulation	No

demonstrated that βγ purified from bovine brain caused inhibition of type I adenylyl cyclase while causing activation of type II adenylyl cyclase. This inhibition of type I adenylyl cyclase was demonstrated to be due to a direct interaction of βγ with the enzyme [Taussig *et al.*, 1993] as opposed to via sequestration of calmodulin by βγ as had been suggested previously [Katada *et al.*, 1987]. It has also been demonstrated that while the stimulation of type II [Tang & Gilman, 1991] and type IV [Gao & Gilman, 1991] isoforms of adenylyl cyclases by βγ is only modest and requires large concentrations of the dimer, this stimulation can be significantly enhanced in the presence of activated $G_s\alpha$ [Tang & Gilman, 1991; Gao & Gilman, 1991]. Experiments with chimeric adenylyl cyclase indicated that the site of interaction with βγ on adenylyl cyclase was present at the C terminal region of the enzyme [Tang & Gilman, 1991;Clapham & Neer, 1993]. It has also been shown that specific dimer compositions can differentially regulate isoforms of adenylyl cyclase $\beta_1\gamma_1$ is only approximately 10% as effective as $\beta_1\gamma_2$, $\beta_1\gamma_3$, $\beta_1\gamma_5$, $\beta_1\gamma_7$, $\beta_2\gamma_2$, $\beta_2\gamma_3$, $\beta_2\gamma_5$ or $\beta_2\gamma_7$ [Iñiguez-Lluhi *et al.*, 1992; Ueda *et al.*, 1994].

The βγ dimer also has a role in the regulation of the transduction of the signal from a serpentine receptor to certain members of the growing family of G protein linked receptor kinases (GRKs). These kinases have an important role in the desensitisation of serpentine receptors by phosphorylating the receptors and preventing their interaction with, and activation of, G proteins. In this case the βγ dimer acts to recruit the GRK (for example βARK) from the cytosol to the plasma membrane and this binding of βγ to βARK increases the catalytic activity of the enzyme [Pitcher *et al.*, 1992; Kameyama *et al.*, 1993]. The binding of βγ to βARK may be facilitated via the Pleckstrin homology (PH) domain of βARK [Touhara *et al.*, 1994]. The PH

domain of βARK is thought to lie in or near the βγ binding site of βARK [Musacchio *et al.*, 1993; Shaw, 1993], which has been mapped to a 125 amino acid region near the C terminus of βARK [Pitcher *et al.*, 1992; Koch *et al.*, 1993]. The βγ complex has been shown to bind several PH domains when they were expressed as fusion proteins with glutathione-S-transferase [Touhara *et al.*, 1994]. This mechanism is not universal, however, as some of the GRKs have lipid moieties attached to them in order to target them to the plasma membrane. Rhodopsin kinase is modified by a farnesyl moiety at its C terminus [Inglese *et al.*, 1992] and GRK5 is palmitoylated [Stoffel *et al.*, 1994]. This role for βγ points to the complex series of feedback mechanisms which exist to "fine tune" the signals produced by serpentine receptors.

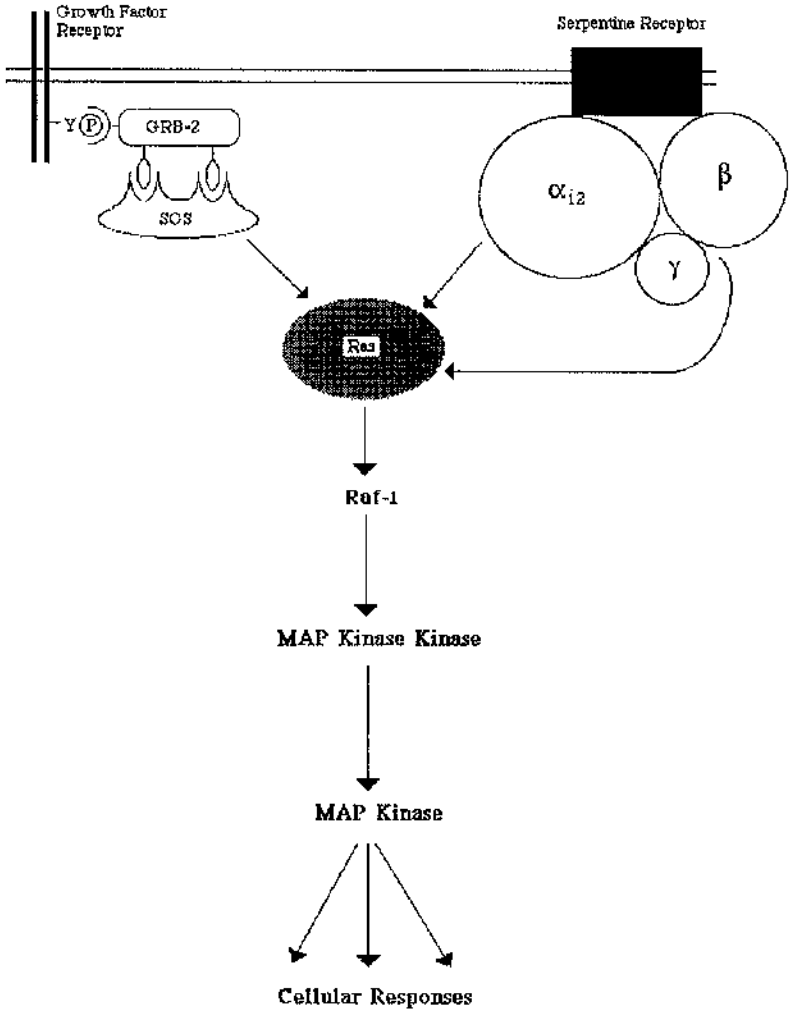
A recent report has also shown the activation of the MAP kinase cascade by βγ. Crespo *et al.* [1994] found that βγ caused a $p21^{ras}$ dependent phosphorylation of myelin basic protein, a substrate for MAP kinase. The basic cascade is displayed in **figure 1. 2.** They hypothesised that this may be due to interaction of βγ with $p21^{ras}$, the direct upstream activator of Raf [Van Aelst *et al.*, 1993; Vojtek *et al.*, 1993], the first kinase in the MAP kinase cascade, although recently the direct interaction of βγ with Raf-1 has been demonstrated [Pumiglia *et al.*, 1995].

The precise mechanism by which one single complex can regulate so many apparently diverse proteins is unclear. The exact combination of β and γ may dictate which proteins the complex will interact with, although there is little evidence to suggest this is so, with the exception of the $\beta_1\gamma_1$ combination. One system where this has become evident is the modulation of the N-type Ca^{2+} channel of GH3 rat pituitary tumour cells. Using an antisense

Figure 1. 2. The MAP kinase cascade.

The classical method of activation of MAP kinase involved the binding of the adaptor molecule GRB-2 to phosphotyrosine residues of activated growth factor receptors, via its Src homology-2 domain. This in turn recruited SOS, which bound to the Src homology-3 domains of GRB-2, which in turn activated Ras, an immediate upstream regulator of Raf-1. This kinase then phosphorylates and activates another enzyme, MAP kinase kinase, the immediate upstream activator of MAP kinase. This causes a myriad of cellular responses. Recently it has been shown that activation of G_{i2} could also activate the MAP kinase cascade, although this activation may in fact be due to the release of $\beta\gamma$, which then may act upon Ras. However, $\beta\gamma$ has recently been shown to interact with Raf-1 and it may be this interaction which causes the activation of the cascade. Please note that Src homology-2 and Src homology-3 domains are regions, first identified in the Src non-receptor tyrosine kinase which have been shown to bind to phosphotyrosine residues and short polyproline regions, respectively, of may proteins involved in cellular signalling and the cytoskeleton.

Figure 1. 2.


Growth Factor Receptor
Serpentine Receptor
Y
P
GRB-2
SOS
α_{i2}
β
γ
Ras
Raf-1
MAP Kinase Kinase
MAP Kinase
Cellular Responses

oligonucleotide approach to specifically target G protein subunits, Kleuss *et al.* [1991] demonstrated that $G_{o1}\alpha$ could only mediate muscarinic acetylcholine receptor mediated inhibition of the Ca^{2+} channel while the somatostatin receptor used only $G_{o2}\alpha$. In a further extension of this, Kleuss *et al.* [1992; 1993] demonstrated that only $G_{o1}\alpha\beta_3\gamma_4$ caused the muscarinic receptor mediated inhibition of Ca^{2+} currents, while the somatostatin receptor used only $G_{o2}\alpha\beta_1\gamma_3$ to achieve this. It has been proposed that, due to the high degree of sequence identity found in the β subunits, it is the γ subunits, with a lower identity, which dictates the majority of the specificity in coupling to receptors and effector systems [Clapham & Neer, 1993], and so regulates the different effects of βγ in signalling systems.

1. 2. 2. Structural determinants in G protein function.

Much of the early work done on the structure of G protein α subunits and how it correlated to function was by extension of work on the small molecular weight G proteins $p21^{ras}$ and Ef-Tu, 3 dimensional structures for which were known [Pai *et al.*, 1989; 1990; Milburn *et al.*, 1990; Schlichting *et al.*, 1990], such that a 3D structural model for G protein α subunits could be inferred [Holbrook & Kim, 1989; Berlot & Bourne, 1992; Thomas *et al.*, 1993]. It has only been recently that the crystal structures of GTPγS and GDP liganded $G_t\alpha$ have been available [Noel *et al.*, 1993; Lambright *et al.*, 1994].

Upon activation of the receptor, the heterotrimeric G protein undergoes a series of steps leading to interaction with effector systems, known

as the G protein cycle (**figure 1. 3.**). Activated receptor causes a conformational change in the G protein α subunit such that the bound GDP is released and GTP enters the nucleotide binding site. Binding of GTP triggers two events; the G protein has a lower affinity for receptor in its GTP bound state and thus is released from it, and the trimer dissociates into free α subunit and the βγ complex. These entities can then interact with their effector systems before the intrinsic GTPase activity of the α subunit hydrolyses the GTP to GDP. In the GDP bound state, the α subunit has a lower affinity for effector and is released to bind once more to βγ and subsequently interact with receptor ready to be activated once more [Gilman, 1987].

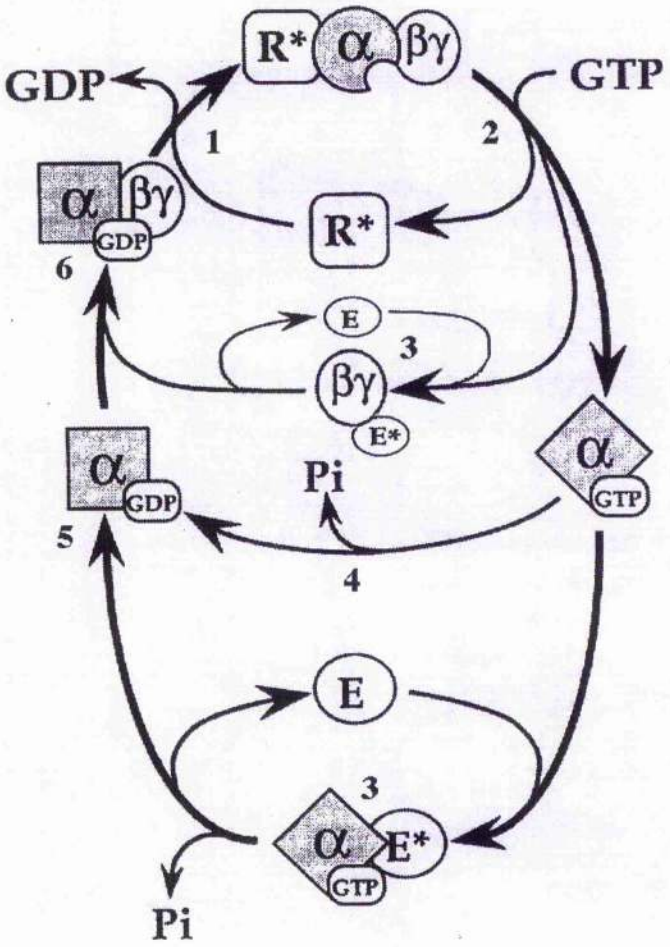
The guanine nucleotide binding site on the α subunit is composed of 5 separate stretches of amino acid sequence spread throughout the protein which come together to form a pocket in 3 dimensions. These 5 regions, termed "G" regions share significant homology to the equivalent regions of $p21^{ras}$ and other members of the GTPase superfamily [Conklin & Bourne, 1993]. The C terminal region of the α subunit, which is thought to be the contact site for the receptor (see later), was thought to form a mobile region that prevents release of bound GDP. Upon agonist activation of receptors, it was proposed that this region may move aside to allow GDP to leave the binding site and allow GTP to enter [Denker *et al.*, 1992b].

The recent publication of the crystal structure of GTPγS liganded $G_t\alpha$ indicated that this was indeed correct. Noel *et al.* [1993] demonstrated that two distinct domains of $G_t\alpha$, one of which acts as the nucleotide binding domain while the other restricts movement into and out of the binding pocket, co-operated to form a "lid" which the activated receptor

Figure 1. 3. The G protein cycle.

Receptor activation (R*) by ligand induces the release of GDP from the G protein in its holomeric from (1). GTP can then enter the nucleotide binding site causing activation of the G protein. This then dissociates into free α and $\beta\gamma$ subunits (2). The activated α subunit and the $\beta\gamma$ dimer can then interact with their effector systems (3) before the intrinsic GTPase activity of the α subunit hydrolyses the GTP to GDP (4) causing re-association of the $\alpha\beta\gamma$ heterotrimer (5) which is then ready to be reactivated by the receptor. This figure was adapted from Conklin & Bourne [1993].

Figure 1.3



must pry apart to allow guanine nucleotide exchange. The structure also validated much of the work implied by the $p21^{ras}$ and Ef-Tu structures and that done using bacterial toxins, antibodies, peptides and mutagenic strategies; the surfaces which contacted receptors and effectors were in the predicted locations. Following the structure of GTPγS complexed $G_t\alpha$, the same group of workers determined the structure of the GDP liganded α subunit [Lambright *et al.*, 1994]. This gave an insight into the way which the α subunit transmits its activation status to receptors, effectors and βγ. This will be discussed later.

The N terminal region is thought to be important in βγ interactions. Limited tryptic cleavage of $G_o\alpha$, such that the first 21 amino acids of the protein were removed, prevented βγ binding to $G_o\alpha$ [Fung & Nash, 1983; Neer *et al.*, 1988]. Monoclonal antibodies against, and a myristoylated peptide corresponding to, the N terminus of $G_{t1}\alpha$ compete for βγ binding [Mazzoni & Hamm, 1989; Mazzoni *et al.*, 1991; Kokame *et al.*, 1992]. Denker *et al.* [1992a] demonstrated that amino acids 7-10 of $G_o\alpha$ were important for βγ binding. A second site in the α subunit is also thought to contact βγ. A mutation of $G_s\alpha$, G226A, which prevented GTP induced conformational change [Miller *et al.*, 1988], also prevented GTP from triggering βγ dissociation [Miller *et al.*, 1988; Lee *et al.*, 1992]. This region of $G_s\alpha$ is the most highly conserved area in G protein α subunits [Conklin & Bourne, 1993] suggesting a crucial role in the function of the α subunit. Thomas *et al.* [1993] demonstrated, using a chemical crosslinking approach, that this region of $G_o\alpha$ was in close proximity to βγ. Although the direct interaction of α and β subunits has only been demonstrated by crosslinking experiments [Bubis & Khorana, 1990], the direct interaction of α and γ has been experimentally demonstrated by Rahmatullah & Robishaw [1994] using

an affinity agarose column chromatography based strategy. These workers demonstrated the interaction of $G_o\alpha$ with γ_2, and postulated that this interaction was mediated via the N terminal region of $G_o\alpha$, due to the trypsinolysis protection effect of γ_2 on the α subunit, and the C terminal region of the γ subunit, due to the requirement for prenylation of γ_2 [Rahmatullah & Robishaw, 1994].

Several areas of the α subunit are thought to contact the serpentine receptor. One of the earliest to be identified was the C terminal tail. The *Bordetella pertussis* exotoxin pertussis toxin, which is known to specifically catalyse the transfer of ADP-ribose to a cysteine residue 4 amino acids from the C terminus of most members of the G_i family of α subunits [Gilman, 1987], causes the G protein to become dissociated from the receptor [West *et al.*, 1985]. Mutations in the C terminal tail and antibodies directed against it can also cause uncoupling of the receptor G protein complex [Sullivan *et al.*, 1987; Simonds *et al.*, 1989b; Gutowski *et al.*, 1991; Hirsch *et al.*, 1991; Shenker *et al.*, 1991]. An elegant set of experiments by Conklin *et al.* [1993] changed the receptor specificity of $G_q\alpha$ to that of $G_{i2}\alpha$ by substitution of 3 amino acids at the C terminal end. The presence of a glycine 3 amino acids from the C terminus was thought to be important in defining this specificity [Conklin *et al.*, 1993] and this residue in $G_{t1}\alpha$ is thought to form part of a β turn which directly contacts rhodopsin [Hamm, 1991]. It is thought therefore that these β turns, which are important for protein-protein interactions [Bansal & Gierasch, 1991], provide signals for recognition by specific receptors [Conklin & Bourne, 1993].

The second site on the α subunit which may contact the receptor is the N terminal region [Conklin & Bourne, 1993]. The N and C termini are thought to be in close proximity in 3 dimensional space [Conklin & Bourne, 1993]. A N terminal $G_{t1}\alpha$ peptide inhibited the interactions of G_t with rhodopsin while apparently not affecting interaction of $G_{t1}\alpha$ with βγ [Hamm *et al.*, 1988; Conklin & Bourne, 1993]. Furthermore, mastoparan, a peptide from wasp venom which can mimic the receptor causing activation of G_i family proteins, can be cross-linked to a cysteine residue near the N terminus of $G_o\alpha$ [Higashijima & Ross, 1991]. The binding of receptor to an N terminal region of the α subunit would suggest that the βγ complex may also contact receptor. Receptors can cause GDP release from the αβγ trimer much more efficiently than from the dissociated α subunit [Fung, 1983; Fung & Nash, 1983; Florio & Sternweis, 1989] probably due to the direct interaction of receptor and βγ [Kelleher & Johnson, 1988; Phillips & Cerione, 1992; Phillips *et al.*, 1992].

The third site for receptor-α interaction is a site near the C terminus represented by amino acids 311-328 of $G_{t1}\alpha$. A peptide corresponding to these residues blocked the activation of G_t by rhodopsin. It also acted synergystically with the extreme C terminal peptide of $G_{t1}\alpha$ [Hamm *et al.*, 1988; Hamm, 1991].

To identify areas of the α subunit which may interact with effector proteins, Rarick *et al.* [1992] used peptides to mimic the activation of the retinal cGMP-PDE by $G_{t1}\alpha$. One peptide representing amino acids 293-314 stimulated the PDE with an efficacy close to that of GTP liganded $G_{t1}\alpha$. This peptide corresponded to the effector activating residues of $G_s\alpha$ identified

by Berlot & Bourne [1992]. An elegant set of experiments by Berlot & Bourne [1992] demonstrated an effector contact site on $G_s\alpha$, part of which was thought to undergo GTP induced conformational changes. These workers extended work by Osawa *et al.* [1990] by creating chimeric proteins of $G_{i2}\alpha$ in which residues in the region 236-356 were replaced by cognate areas of $G_s\alpha$. By this approach Berlot & Bourne identified 4 areas of $G_s\alpha$, corresponding to amino acids 236-240, 261-262, 277-285 and 349-356, which when mutated resulted in a protein which did not stimulate adenylyl cyclase. Of these the first three areas did not affect the expression or GTP induced conformational change of the chimeras. These areas were thought to form a surface in 3 dimensions, based on the 3D structure of $p21^{ras}$, part of which was thought to undergo a GTP induced conformational change [Berlot & Bourne, 1992]. This presumption has been confirmed by the crystal structures of GDP and GTPγS complexed $G_t\alpha$. Binding of GTP caused the α subunit to protrude slightly such that areas which contact βγ and effectors bulge. These areas overlap indicating that in order to interact with effectors, the α subunit must first release βγ [Lambright *et al.*, 1993; Noel *et al.*, 1994; Bourne, 1994].

1. 3. G protein linked receptors.

Although this Introduction focuses mainly on G proteins, it may be prudent to consider the receptors to which they couple. All the receptors which couple to G proteins are thought to exibit a distinct molecular morphology consisting of seven transmembrane spanning α helices, and hence are referred to as serpentine receptors. The sole known exception to this general rule is the insulin-like growth factor II receptor which has an intrinsic

tyrosine kinase activity but can also activate G proteins through an amphipathic α helix [Higashijima *et al.*, 1988; Higashijima *et al.*, 1990].

The present understanding of the structure of serpentine receptors is based on the structure of the photoactivated proton pump of *Halobacterium halobium*. This protein, termed bacteriorhodopsin, is folded in such a way that the N terminus of the protein is located on the extracellular surface of the cell and the C terminus is intracellular. Between these two areas are seven regions of high hydrophobicity which electron microscopy and high resolution electron diffraction indicate as being transmembrane spanning domains arranged in a bundle perpendicular to the plane of the lipid bilayer [Henderson & Unwin, 1975; Engleman *et al.*, 1980; Henderson *et al.*, 1990]. G protein linked receptors are also thought to contain these distinctive seven transmembrane spanning regions (**figure 1. 4.**) [Dohlman *et al.*, 1992; Savarese & Fraser, 1992].

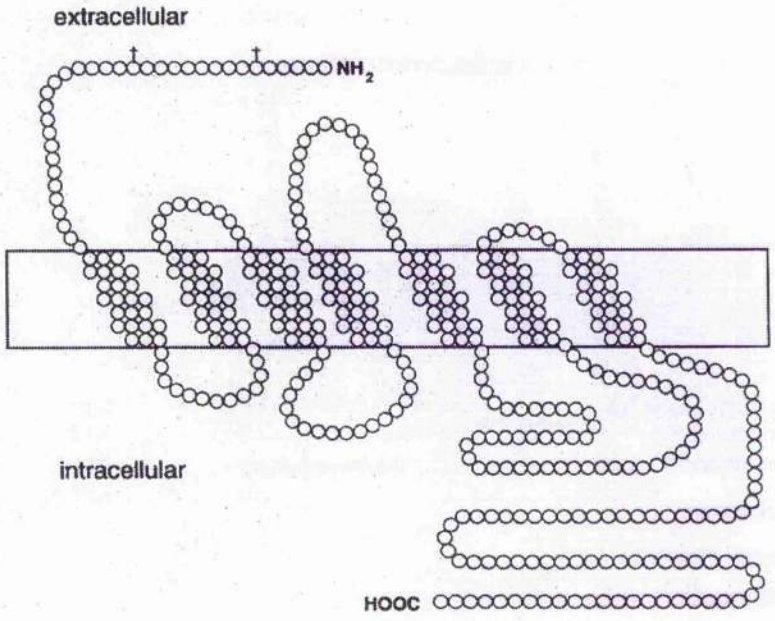
1. 3. 1. Structure-function relationships in serpentine receptors.

The following sections represent an outline of aspects of ligand binding to receptor and receptor activation. It is not intended to be an exhaustive review of the subject.

Figure 1. 4. Putative topology of serpentine receptors.

All G protein coupled receptors are thought to share the same basic membrane topology, namely the presence of seven transmembrane spanning helices. The model for these receptors places the N terminus extracellularly and the C terminus intracellularly. The receptor shown is the human β_2 adrenergic receptor, an archetypal serpentine receptor. Sites of glycosylation are shown as crosses on the N terminal tail. The sites of phosphorylation and palmitoylation of the C terminal tail are not shown. Other members of the family can contain very large extracellular and intracellular domains, but still share this basic structure. This figure was adapted from Lefkowitz *et al.*, [1993]

Figure 1.4



1. 3. 1. 1. Structural determinants for ligand binding.

Although serpentine receptors share the same basic structure, differences exist in the method of binding agonist. The receptors for small molecules such as light (using 11-*cis*-retinal as a chromophore ligand) [Khorana, 1992] catecholamines and acetylcholine [Dohlman *et al.*, 1992; Savarese & Fraser, 1992] bind these agonists in a cleft formed by the folding of the transmembrane helices of the receptor. Other serpentine receptors bind their agonists in different ways and these are illustrated in **figure 1. 5.**

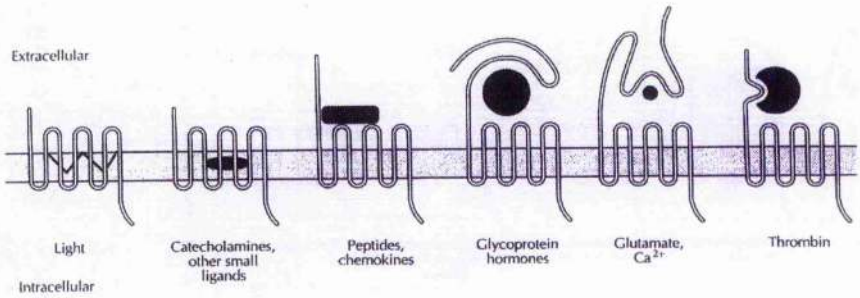
The β_2 adrenergic receptor has been used for many years as a model system for studying the function and regulation of serpentine receptors in transmembrane signalling. Large parts of the intracellular and extracellular domains can be deleted without appreciably altering the ligand binding properties of the receptor. Studies using alkylating agents [Dohlman *et al.*, 1988] and photoaffinity labels [Wong *et al.*, 1988] and a fluorescent antagonist [Tota & Strader 1990] have shown that the ligand binding pocket, formed from the seven transmembrane helices, is located approximately one third of the way down into the core of the protein.

Receptors for peptide agonists appear to recognise these agonists on their exofacial surface [Fong *et al.*, 1992a & Fong *et al.*, 1992b] while glycoprotein hormones such as thyrotropin bind to the N terminal tail [Moyle *et al.*, 1991; Nagayama *et al.*, 1991] of the receptor causing a conformational change in the receptor causing the tail to bind to the extracellular loops of the receptor.

Figure 1. 5. Graphical representation of the mechanism of ligand binding to various types of serpentine receptors.

It is thought that receptors for light in the eye, recognise the light photons via an 11-*cis*-retinal molecule bound in a cleft formed by the transmembrane helices (panel a) Receptors for small ligands such as catecholamines also bind agonists in a cleft formed by the seven transmembrane helices (panels b). Receptors for peptide agonists utilise a surface formed by the extracellular loops of the helices to bind agonists (panel c), while others utilise a large N terminal tail (panel d) to bind large glycoprotein agonists. The metabotropic glutamate receptor contains an extremely large N terminal tail which is thought to bind glutamate and cause a large pincer-like conformational change in the N terminus. This complex is then thought to act as a "tethered ligand" on the seven transmembrane helices (panel e). Thrombin, rather than being an ligand for its receptor is actually a specific protease which cleaves part of the N terminus off the thrombin receptor leaving the remaining portion of the N terminus to act on the transmembrane helices as a tethered ligand (panel f). This figure was adapted from Coughlin [1994].

Figure 1.5


Extracellular
Light
Intracellular
Catecholamines, other small ligands
Peptides, chemokines
Glycoprotein hormones
Glutamate, Ca^{2+}
Thrombin

The thrombin receptor detects agonist in a different way. The thrombin molecule is actually a protease which binds to the N terminal tail of the thrombin receptor and cleaves the first 25 amino acids of the receptor to create a new amino terminus which then acts as a "tethered ligand" for the receptor [Vu *et al.*, 1991a; Vu *et al.*, 1991b], at a site as yet undefined.

The metabotropic glutamate receptors and the parathyroid Ca^{2+} sensing receptor utilise yet another mechanism to detect their ligands [Tanabe *et al.*, 1992; Brown *et al.*, 1993]. These receptors contain extremely large N terminal domains which have stretches resembling bacterial transporters for small molecules such as amino acids [O'Hara *et al.*, 1993]. Studies on these bacterial periplasmic binding proteins (PBPs) [Oh *et al.*, 1993] suggest that glutamate and Ca^{2+} binding to these PBP domains induce a large conformational change in the metabotropic glutamate receptors and Ca^{2+} sensing receptor. This conformational change may then form yet another tethered ligand which binds to the exofacial surface of the receptor. More extensive reviews on the regions of serpentine receptors involved in ligand binding are available [Savarese & Fraser, 1992; Baldwin, 1994; Coughlin, 1994]

1. 3. 1. 2. Regions involved in receptor activation.

Following binding of a hormone to a receptor, the receptor is thought to undergo a conformational change causing an associated activation of the protein. This activation is responsible for the facilitation of the induction of the corresponding G protein activation. It is thought that regions of the

receptor involved in the activation of the polypeptide may be distinct from those involved in receptor-G protein coupling.

Based on the work done on the aspartate residues of bacteriorhodopsin and the human β_2 adrenergic receptor, a model for agonist activation of receptors has been proposed [Venter *et al.*, 1989]. It has been postulated that several acidic amino acids present in the transmembrane helices of the β adrenergic receptor (asp79 in helix II, asp113, glu122 and asp130 in helix III) bind water molecules or mono- or di- valent cations in the unliganded receptor. Upon agonist binding these residues may start an ion transfer across the membrane analogous to bacteriorhodopsin, a process which may in turn facilitate receptor-G protein interactions. Given the similarities between eukaryotic serpentine receptors and bacteriorhodopsin it may be that serpentine receptors may also act as proton pumps [Nerderkoorn *et al.*, 1995].

It is clear that the presence of a conserved aspartate in transmembrane helix II, near the centre of the transmembrane domain is obligatory for receptor activation. Virtually all receptors of this family possess an aspartate at this position. Mutagenesis of this residue in the human β_2 adrenergic receptor [Chung *et al.*, 1988], the human α_2 adrenergic receptor [Wang *et al.*, 1991], the rat D2 dopamine receptor [Neve *et al.*, 1991] or the rat M1 muscarinic receptor [Fraser *et al.*, 1989] decreased the ability of these receptors to interact with their effectors. This aspartate residue is thought to play a role in the allosteric actions of monovalent cations, such as Na^+. These ions are known to increase the affinity of α_2 adrenergic receptors for antagonists yet decrease the affinity for agonists [Limbird, 1984].

Several mutations in serpentine receptors, both naturally occurring and engineered, have indicated the importance of other residues in receptor activation. While some of these are thought to be involved in G protein binding and activation, others are found deep in the transmembrane helices of the receptor, and so are thought not to involve G proteins [Baldwin, 1994]. Mutations engineered in rhodopsin, in the third and seventh helices at positions glu113 and lys296 [Robinson *et al.*, 1992; Cohen *et al.*, 1993], and the naturally occurring mutation at ala292 [Dryja *et al.*, 1993] are thought to relieve a constraint on the receptor, which normally holds the receptor in an inactive state until activated [Robinson *et al.*, 1992; Cohen *et al.*, 1993]. This is also apparently true for glu92 of the melanocyte stimulating hormone receptor [Robbins *et al.*, 1993] and asp578 of the luteinising hormone receptor [Shenker *et al.*, 1993].

The α_{1b} and α_2 adrenergic receptors both contain single amino acids at the junction of the third intracellular loop and transmembrane helix VI which cause constitutive activation of the receptors and increase the affinity of the receptor for agonist. Kjelsberg *et al.* [1992] substituted all possible amino acids for ala293 of the α_{1b} adrenergic receptor while Ren *et al.* [1993] created five substitutions at the equivalent position of the α_2 adrenergic receptor. These results indicate that the receptor must first overcome a constraint in order to adopt an activated state to stimulate the G protein into guanine nucleotide exchange and trimer dissociation, and that agonist provides the necessary conformational change to faciliate this [Kjelsberg *et al.*, 1992; Ren *et al.*, 1993; Baldwin, 1994].

1. 3. 1. 3. Receptor regions involved in G protein coupling, activation and specificity

The intracellular loops and the C terminal tail formed by the folding of the receptor polypeptide are thought to be the main sites of receptor-G protein interaction. The precise nature of this interaction is still being investigated but it is already apparent that the nature of receptor selection of a G protein is located in this region and that even slight alterations in this area can alter the fidelity of interaction.

Most work in this area has focused on the third intracellular loop and the C-terminal tail of the receptor. It is within these regions that most diversity exists in the cytoplasmic areas of the protein. By contrast, the first and second intracellular loops are relatively conserved [Lefkowitz & Caron, 1988] and so have been the focus of less research. However, rhodopsin, which normally couples to G_t, possesses a relatively small third intracellular loop [Weiss *et al.*, 1988] yet G_t can also couple to α_2 adrenergic receptors and muscarinic M2 receptors, which have much larger third intracellular loops [Tsai, *et al.*, 1987]. The implication is that large areas of the loop have little to do in determining G protein-receptor coupling [Savarese & Fraser, 1992].

a) The second and third intracellular loops.

Adrenergic receptors have been model systems for studying serpentine receptors and their mechanism of interaction with G proteins. Studies employing deletion mutants of the β_2 adrenergic receptor indicate that a large portion of the third intracellular loop is important in activation of G_s and hence adenylyl cyclase. Deletion of residues 239-272 [Dixon *et al.*, 1987]

of the hamster β_2 adrenergic receptor, almost two thirds of the third intracellular loop, produced a receptor which could not activate adenylyl cyclase. Deletion of two relatively small segments of the receptor, residues 222-229 and 258-270 both greatly reduced the activation of adenylyl cyclase by the receptor [Strader *et al.*, 1987]. These residues are thought to be in the region proximal to the lipid bilayer The attenuation of the activation of adenylyl cyclase was shown to be due to an interruption of receptor G_s coupling [Strader *et al.*, 1987]. In addition, deletion of the residues at the C-terminal end of the third cytoplasmic loop of the human β_2 adrenergic receptor also resulted in stimulation of adenylyl cyclase to only a fraction of the level produced by the wild type receptor [Hausdorff *et al.*, 1990].

Kobilka *et al.* [1988] have attempted to address the role of the third cytoplasmic loop of the β_2 adrenergic receptor in mediating activation of G_s by constructing chimeric receptors where portions of the human β_2 adrenergic receptor were replaced with the corresponding regions of the human α_2 adrenergic receptor and assessing the chimeras' ability to stimulate adenylyl cyclase when expressed in *Xenopus* oocytes. If a portion of transmembrane helix V, all of helix VI and the intervening third cytoplasmic loop of the β_2 adrenergic receptor was introduced into the α_2 adrenergic receptor, the chimera could stimulate adenylyl cyclase to approximately one third the level of that produced by the wild type β_2 receptor [Kobilka *et al.*, 1988]. If only a portion of the third cytoplasmic loop of the receptor was introduced, however, then the ability to activate adenylyl cyclase was lost [Kobilka *et al.*, 1988]. These results indicate that the third cytoplasmic loop of the β_2 adrenergic receptor plays an important role in governing the interaction of the receptor and G_s and thus stimulation of adenylyl cyclase, but also that

this loop is insufficient on its own to fully activate the G protein. A highly conserved aspartate residue at the junction of transmembrane helix III and the second intracellular loop (asp130 in the human β_2 adrenergic receptor) may be one of these additional determinants for G protein binding. It is thought that the conformational changes which occur upon receptor activation may mask or unmask this residue, as it is located in or near the cytosol. Mutagenesis of this residue and corresponding residues in bovine rhodopsin, the rat M1 muscarinic receptor and the human α_2 adrenergic receptor either prevent receptor-G protein interaction or move the dose-effect curves for the receptors to the right [Fraser *et al.*, 1988; Fraser *et al.*, 1989; Franke *et al.*, 1990; Wang *et al.*, 1991].

Formation of chimeric α_1 adrenergic receptors has also been performed in order to delineate the domains of this G_q-linked receptor. When amino acids 252-259 of the third intracellular loop of the α_1 receptor, near the centre of the loop, were replaced by corresponding regions of the β_2 adrenergic receptor the α_1 receptor was only able to induce phosphoinositide hydrolysis to 10% of the wild type receptor [Cotecchia *et al.*, 1990]. Replacement of amino acids 288-294 caused a 100 fold increase in affinity for noradrenaline and a 300 fold increase in efficacy of phosphoinositide breakdown [Cotecchia *et al.*, 1990]. This replacement caused only 3 overall mutations and when each of these was examined in isolation, it was found that the K290H and A293L mutations were responsible for this increase in efficacy [Cotecchia *et al.*, 1990]. These mutant receptors also displayed an increase in basal inositol phosphate levels leading to the conclusion that these mutations lock the receptor in its activated state, suggesting a role for these residues in the agonist induced conformational change of the receptor which activates the G protein.

Franke *et al.* [1990] have found evidence to suggest that both the second and third intracellular loops of bovine rhodopsin function to bind G_t. Two mutants in which the sequence Cys-Lys-Pro-Met-Ser-Asn-Phe-Arg-Phe-Gly-Glu-Asn-His found in the central region of the second intracellular loop was replaced by the sequence Gly-Thr-Glu-Gly-Pro-Asn-Phe-Tyr-Val-Pro-Phe-Thr-Ser, and another in which residues 237-249 of the third intracellular loop were deleted, could bind G_t but could not cause guanine nucleotide exchange in the presence of GTP. It was postulated that these mutants could cause the release of GDP from G_t but not the formation of the GTP binding conformation of the G protein [Franke *et al.*, 1990]. The deletion mutant of G_t corroborated an earlier study in which lysine-248 was mutated to leucine. This mutant receptor was completely unable to activate the GTPase activity of G_t [Franke *et al.*, 1988].

b) The C-terminal tail.

The C-terminal tail of the α_1 adrenergic receptor is also thought to play a role in governing receptor-G_q interactions as substitution of a thirteen amino acid stretch near the cytoplasmic section of the tail (residues 353-365) with the corresponding section of the human β_2 adrenergic receptor produced a chimeric receptor whose capacity for activating phosphoinositidase C was impaired by 40% [Cotecchia *et al.*, 1990].

The C-terminal tail may be involved in governing the fidelity of interaction of serpentine receptors and G proteins. The splice variants of the EP3 prostanoid receptor display a marked infidelity in G protein coupling. These receptors are spliced so that they have different C terminal tails; the rest

of the protein is unaffected. These splice variants show remarkable promiscuity in coupling to G proteins. Namba *et al.* [1993] isolated 4 splice variants of the EP3 receptor and showed that they varied in their G protein specificity. EP_{3A}, with the longest tail, coupled to Gi family members, EP_{3B} and EP_{3C}, which had the shortest tails, both coupled to Gs while EP_{3D} coupled to Gi, Gs and Gq. In a subsequent series of experiments, it was shown that the EP_{3C} receptor coupled to Go as well as Gs, but while it stimulated the GTPase activity of Gs it inhibited the GTPase activity of Go by increasing the binding affinity of Go for GDP [Negishi *et al.*, 1993]. This may lead to the attenuation of signals generated through Go by the EP_{3C} receptor.

An approach similar to that used by Dalman & Neubig [1991] to identify the receptor-G protein interaction points on the α_{2A} adrenergic receptor was used originally by Takemoto *et al.* [1985] to investigate the role of cytoplasmic domains of bovine rhodopsin in rhodopsin-G_t interactions. A number of peptides corresponding to regions of the intracellular loops and cytoplasmic tail of the receptor were tested. Those peptides derived from the intracellular loops were incapable of inhibiting the GTPase activity of G_t in this system. Three peptides corresponding to amino acids 333-339, 324-331 and 317-321, all within the C-terminal tail, did however inhibit G_t activation; the 333-339 peptide was the most potent inhibitor. This region is, however, not conserved among other opsins and proteolysis of the C-terminal 12 amino acids had no effect on G_t binding to rhodopsin [Kuhn, 1984].

1. 4. Covalent modifications of signalling proteins by lipid moieties.

It has become apparent that lipid modification of proteins plays a major role in regulating their subcellular location and function [Deschenes *et al.*, 1990; Turner, 1992]. Many proteins involved in cellular signalling undergo one or more modifications by one or more lipid molecules [Resh, 1994; Casey, 1994; Wedegaertner *et al.*, 1995].

There are four known lipid modifications which occur on proteins, myristoylation, palmitoylation, polyisoprenylation and addition of glycosylphosphatidylinositol (GPI) [Turner, 1992].

1. 4. 1. Addition of glycosylphosphatidylinositol to proteins.

The addition of GPI to proteins was first documented following the observation that a soluble phosphoinositidase-C activity from certain bacterial species could release certain proteins from the plasma membrane [Ikezawa *et al.*, 1976; Low & Zilversmit, 1980]. This modification is thought only to occur on proteins expressed on the external surface of the cell. This then has very little to do with intracellular signalling, although a few cell surface proteins involved in receptor function have GPI anchors [Turner, 1992]. GPI anchors may be responsible for functions other than membrane anchorage, and may include cell-cell interaction and targeting of proteins.

The GPI anchor comprises a core phospholipid-glycan chain consisting of phosphatidylinositol-glucosamine-mannose-3-phosphoethanolamine attached to the C-terminal amino acid of the mature protein. This amino acid can be one of asp, gly, ala, cys or ser, suggesting that the donor amino acid must be small and aliphatic. The fatty acid associated with the core chain is apparently species specific. It would appear that the immature protein is transiently associated with the membrane via a predominantly hydrophobic region of 10-35 amino acids at the C-terminus. This region is then rapidly exchanged for the GPI anchor by an unidentified transamidase activity [Turner, 1992].

1. 4. 2. Polyisoprenylation of proteins.

Of far more importance in terms of transmembrane signalling is the addition of isoprene units to the C terminus of polypeptides. Many proteins involved in cellular signalling are modified by the addition of either farnesyl or geranylgeranyl groups [Deschenes *et al.*, 1990; Casey, 1994]. The prerequisite for the addition of isoprene units is the presence of a motif consisting of a cysteine-aliphatic-aliphatic-any amino acid (CAAX) or occasionally CC or CXC [Deschenes *et al.*, 1990; Casey, 1994]. This however is not the only requirement. The α subunits of G_{i1}, G_{i2}, G_{i3} and G_o have CAAX motifs at their C terminus yet are not substrates for isoprenylation, although the C terminal CAAX motif of $G_{i1}\alpha$ can support the isoprenylation of $p21^{ras}$, one of the small molecular weight G proteins, when fused to this protein. [Cox *et al.*, 1993]. This would indicate that the environment surrounding the CAAX motif is also important in determining specificity of prenoid transfer.

The farnesyl and geranylgeranyl units are derived from isopentenyl pyrophosphate. This molecule is the precursor to many important lipid molecules such as squalene (which goes on to form cholesterol), plant terpenes and carotenoids, vitamin K_2 and mitochondrial coenzyme Q_{10}. The condensation of two isopentenyl pyrophosphate groups forms the ten carbon geranyl pyrophosphate. Successive condensations of isopentenyl pyrophosphate gives rise to the fifteen carbon farnesyl pyrophosphate and the twenty carbon geranylgeranyl pyrophosphate. It is these two prenoid groups which are subsequently added to proteins [Turner, 1992].

A complex series of events accompanies the isoprenylation of proteins. In terms of the CAAX motif, addition of the isoprenoid group to the cysteine residue is followed by the removal of the AAX residues of the motif [Gutierrez *et al.*, 1989; Hancock *et al.*, 1989], leaving the prenylated cysteine residue at the extreme C terminus. This cysteine residue is then modified by carboxymethylation [Clarke *et al.*, 1988].

The process of isoprenylation is cytosolic and two separate enzymes have been identified specific for the addition of either farnesyl or geranylgeranyl groups to CAAX proteins, termed protein farnesyltransferase (FTase) and protein geranylgeranyltransferase-I (GGTase-I) respectively. These enzymes contain two polypeptides, a common α subunit of 48kDa and different β subunits of 46kDa (FTase) and 43kDa (GGTase-I) [Casey, 1994]. The proteolytic cleavage and subsequent carboxymethylation reactions are carried out in the microsomal membrane fraction by as yet uncharacterised enzymes [Casey, 1994]. The result of this complex series of modifications and

cleavages is believed to cause the targeting and/or anchoring of isoprenylated proteins to the plasma membrane. In some cases, most notably members of the Ras family, hydrophobicity of the protein is further increased by the addition of one or more palmitate groups to cysteine residues close to the C terminal isoprenylated cysteine residue [Hancock *et al.*, 1989], while others, notably $p21^{K\text{-}rasB}$ have a series of basic residues which are thought to interact with the lipid head groups of the phospholipids of the membrane.

A second geranylgeranyl transferase enzyme has also been identified which differs considerably from GGTase-I. This enzyme termed GGTase-II catalyses the transfer of the geranylgeranyl group members of the Rab family of small molecular weight G proteins, amongst others. These proteins, rather than containing the CAAX motif have the sequence CC or CXC at their C termini [Casey, 1994].

The addition of either farnesyl or geranylgeranyl groups to CAAX containing polypeptides is not a random process, but is instead governed by the exact composition of the CAAX motif. The G protein γ subunits γ_1 and γ_2 have the C terminal sequences CVIS and CAIL respectively, yet γ_1 is modified by the addition of a farnesyl residue while γ_2, like all other γ subunits, is modified by a geranylgeranyl group [Casey *et al.*, 1994]. It is believed that the extreme C terminal residue is responsible for the direction of the isoprenylation. If the residue is methionine, serine or glutamine then a farnesyl residue is added, while a leucine directs the addition of geranylgeranyl residues. All of the nonretinal γ subunits terminate in a leucine residue and are therefore modified by a geranylgeranyl isoprenoid while retinal γ subunit terminates in a serine and is thus farnesylated.

Transient co-expression studies using wild type and a cys–to-ser geranylgeranyl negative mutant of γ_2 and a β subunit indicate that isoprenylation is required for the membrane localisation of the $\beta\gamma$ complex [Simonds, 1994]. Studies to determine whether isoprenylation of γ_2 is required to form the $\beta\gamma$ complex have also been carried out. Some expressed β subunits localised in the cytosolic fraction along with unisoprenylated γ subunits indicating that the $\beta\gamma$ dimer can form in the absence of geranylgeranyl groups. Limited trypsinolysis of cellular extracts of these cells expressing β and γ_2 support this conclusion as the expected pattern of digestion was seen in the cytosolic fraction only of cells expressing the β and mutant γ_2 subunits but not in cells expressing β alone [Simonds *et al.*, 1991; Iñiguez-Lluhi *et al.*, 1992]. It was noted in these studies however that β subunits on their own could localise to the membrane in the absence of γ subunits. This was thought to be due to β subunits aggregating.

It is believed that differences exist between farnesylated and geranylgeranylated γ subunits. The farnesylated γ_1 subunit behaves somewhat differently to γ_2. It exists in two forms, a truncated form which does not contain farnesyl residues and a normal farnesylated form. Both of these forms associate with the membrane indicating the presence of determinants other than isoprenylation are important for the membrane localisation of transducin $\beta\gamma$ [Fukada *et al.*, 1990]. Transducin $\beta\gamma$ can be released from the membrane in the absence of detergent [Spiegel, 1990] indicating that farnesylation of γ_1 may have a roles other than, or in addition to, regulating membrane interaction. Functionally, farnesylation may have a role to play in activating the holomeric G protein. The farnesylated form of γ_1 is 30 times more efficient in promoting

binding of GTP to transducin [Fukada *et al.*, 1990]. Also carboxymethylation of farnesylated γ_1 enhances its binding to rhodopsin [Fukada *et al.*, 1994].

1. 4. 3. Addition of myristic acid to proteins.

Many cellular and viral proteins are now known to be acylated by the 14 carbon saturated fatty acid myristate [Towler *et al.*, 1988; Deschenes *et al.*, 1990]. The exact purpose of this modification is unclear at present although various hypotheses have been put forward. Being a hydrophobic molecule, the addition of myristic acid to proteins has been suggested to determine, or at least aid in, the regulation of the membrane association of the target protein. This simplistic view is at best only partially true as some myristoylated proteins are not membrane associated. In addition, myristic acid is only a relatively rare fatty acid in eukaryotic cells [Deschenes *et al.*, 1990], indicating perhaps another, more specialised, role for myristoylation. While myristoylation of many proteins, including viral proteins, MARCKS protein and some proteins involved in cellular signalling can cause their membrane association, others such as the catalytic subunit of protein kinase A, are not. Indeed, myristoylation of $pp60^{v\text{-}src}$ is not essential for membrane association [Resh, 1988; Kaplan *et al.*, 1990]. It has been proposed that specific receptors exist for myristoylated proteins [Schultz *et al.*, 1988] given that interaction of $p56^{lck}$ with CD4 is enhanced when $p56^{lck}$ is myristoylated. A putative binding protein for myristoylated $pp60^{v\text{-}src}$ has been identified [Resh & Ling, 1990].

The addition of myristic acid to proteins occurs co-translationally as addition of cycloheximide to cells prevents the incorporation of [^{3}H] myristate into cellular proteins but not lipids [Olson & Spizz, 1986;

Deichaite *et al.*, 1988]. The requirements for the addition of myristic acid to proteins appears very simple yet only a few proteins are actually modified therefore determinants other than those so far identified must be involved. A glycine at position 2 is absolutely essential. Once the initiating methionine residue has been removed, this becomes the N terminal residue and is then modified by myristic acid [Turner, 1992]. The enzyme responsible, myristoyl-Co-A:protein N-myristoyltransferase (NMT; EC 2.3.1.97), also prefers a serine at position 6 (position 5 in the mature protein) although this is not essential [Turner, 1992]. A lysine residue at position 8 has also been shown to be preferred by NMT to catalyse myristate transfer [Towler *et al.*, 1988]. The lipid substrate for NMT is more specific. The chain length of the lipid seems to be the governing factor as opposed to hydrophobicity as oxygen containing derivatives of myristic acid are specific inhibitors of the enzyme [Paige *et al.*, 1990]. Increases in the chain length of the lipid group are not tolerated, although shorter chain fatty acids such as lauric acid can serve as substrates [Towler *et al.*, 1987; Glover *et al.*, 1988]. This phenomenon allowed for the discovery of a degree of heterogeneity in acylation of $G_t\alpha$ as described below.

Some G protein α subunits have been demonstrated to incorporate [^{3}H] myristic acid. $G_s\alpha$, $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, $G_t\alpha$, $G_o\alpha$ and $G_z\alpha$ all have the required glycine residue, yet only $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, $G_o\alpha$ and $G_z\alpha$ incorporate myristate. $G_s\alpha$ contains a leucine residue at position 6 as opposed to the serine residue preferred by the transferase and so this is not myristoylated.

The myristoylation of G protein α subunits has been assessed by both site-directed mutagenesis of the target glycine residue and by

inhibition of NMT. These studies indicate a role for the addition of myristic acid in governing the association of the polypeptide with the plasma membrane and in the interaction of the α subunit with the βγ complex. Jones *et al.*, [1990] and Linder *et al.*, [1991] have demonstrated that myristoylation of $G_{i1}\alpha$ and $G_o\alpha$ causes a decrease in the affinity of these two α subunits for βγ. The myristoylation of $G_{i2}\alpha$ was also required for it to inhibit adenylyl cyclase and for the activating mutant of the polypeptide, the *gip2* oncoprotein, to cause plaque formation in Rat-1 fibroblasts [Gallego *et al.*, 1992]. The *gip2* protein is a mutant form of $G_{i2}\alpha$ in which a mutation inhibits the GTPase activity of the protein causing a constitutive activation of $G_{i2}\alpha$ [Pore *et al.*, 1991; Gupta *et al.*, 1992]. Thus, acylation of α subunits has an important function in determining the protein-protein interactions with other signalling molecules.

Although $G_s\alpha$ is not myristoylated, the glycine residue at position 2 is nevertheless important in the signalling properties of the molecule. Site-directed mutagenesis of the glycine, replacing it by an alanine residue, or deletion of the N terminal region of $G_s\alpha$, causes a reduction in the ability of $G_s\alpha$ to activate adenylyl cyclase and to interact with βγ [van der Neut *et al.*, 1993]. This may be due to an inhibition of palmitoylation as gly2 is required for proper palmitoylation of $G_{i1}\alpha$ [Galbiati *et al.*, 1994] and $G_z\alpha$ [Hallak *et al.*, 1994]. This region of $G_s\alpha$ may not, however, be required for membrane association of the molecule. A region near the C terminus of the polypeptide may be important in regulating the interaction with the membrane, although the N terminus is still necessary for βγ binding and has a role in effector activation [Journot *et al.*, 1991]. This C terminal region, termed the TENIR sequence, is thought to regulate membrane association of $G_s\alpha$ [Journot *et al*, 1991], although if this sequence is inserted into $G_{i1}\alpha$, it apparently does

not alter the membrane association of this α subunit [Degtyarev *et al*, 1994a]. Fusion of this sequence to an N terminally truncated $G_{i1}\alpha$, which is located in the cytoplasm, does however restore the membrane association of the fusion protein. $G_q\alpha$ and $G_{11}\alpha$ also contain this TENIR sequence, but the function of it in these polypeptides is not known.

Care must be exercised in interpreting the results obtained for these proteins in terms of mutational analyses of the role of myristoylation. Recent reports have demonstrated that the G2A mutation of $G_{i1}\alpha$ and $G_z\alpha$ actually abolishes the palmitoylation of these proteins as well as inhibiting myristoylation [Galbiati *et al.*, 1994; Hallak *et al.*, 1994]. This effect is not due to the lack of myristic acid on the protein as treatment of cells with the NMT inhibitor 2-hydroxymyristate does not prevent addition of palmitate to $G_{i1}\alpha$. Therefore the presence of the glycine residue at position 2 may be obligatory for the as yet uncharacterised palmitoyl transferase.

In retinal membranes, $G_t\alpha$ is not myristoylated, even though it has both the required gly2 and preferred ser6 residues. It is, however, myristoylated upon transient expression in COS-7 cells [Mumby *et al.*, 1990]. The solution to this apparent discrepancy lay in the observation of heterogeneity of acylation of $G_t\alpha$ occurred in retinal membranes. Kokame *et al.* [1992] and Neubert *et al.* [1992] demonstrated that $G_t\alpha$ in retinal membranes was present as 4 distinct forms differing in the lipid attached to the protein. These workers found that only around 5% of retinal $G_t\alpha$ contains covalently attached myristic acid; the remaining molecules contain, in decreasing amounts, lauric acid and two unsaturated 14 carbon fatty acids (C14:2 and C14:1). This heterogeneity of acylation probably reflects differing

acyl CoA species in the retina [Johnson *et al.*, 1994]. These lipids are less hydrophobic than myristic acid and so this could explain why $G_t\alpha$ can be washed from the membrane without detergent [Spiegel, 1990]. The presence of fatty acids on gly2 affects the $\beta\gamma$ interaction of G protein α subunits and Kokame *et al.* [1992] have also demonstrated that this heterogeneity of acylation may cause $G_t\alpha$ to dissociate more readily from $\beta\gamma$ as acylated peptides corresponding to the N terminus of $G_t\alpha$ were less able to bind to $\beta\gamma$ if lauric acid or the two unsaturated fatty acids were on the peptide.

1. 4. 4. Protein Palmitoylation.

The addition of palmitate to proteins is distinct from myristoylation in that it is post-translational as opposed to the co-translational myristate transfer. Also myristate is always amidically linked to glycine while palmitate is generally esterified to cysteine residues via thio-ester bonds [Casey, 1994; Casey, 1995]. This difference in the attachment of these acyl groups brings about another important functional difference. The amide bond attaching the myristic acid is very stable and once attached, the fatty acid is essentially permanently bound and is attached for the lifetime of the protein, although there has been a report of a demyristoylation of mature MARCKS protein [Manenti *et al.*, 1994]. The thio-ester bond linking the palmitic acid is, however, much more labile and can be enzymatically cleaved. This hints at the possibility of regulation of the process of palmitate transfer. Indeed a palmitoyl-esterase has recently been purified and has been demonstrated to cleave thio-ester linked palmitate from $p21^{H\text{-}Ras}$ and $G_o\alpha$ [Camp & Hofmann, 1993] although this enzyme has subsequently been shown to be secreted

[Camp *et al.*, 1994] and therefore could not be responsible for any intracellular regulation of palmitoylation.

The palmitoylation of some forms of $p21^{ras}$ is, as described earlier , part of the overall process of isoprenylation. Following the addition of farnesyl or geranylgeranyl groups, proteolysis and carboxymethylation, a palmitoyltransferase adds palmitate to specific cysteine residues near the site of isoprenylation [Hancock *et al.*, 1989]. This enzyme is poorly characterised and has yet to be purified. The enzyme does, however, require the prior isoprenylation of $p21^{ras}$. Given this specificity, this enzyme is unlikely to be responsible for addition of palmitate to heterotrimeric G protein α subunits and Src family members.

Many serpentine receptors have also been shown to be post-translationally and reversibly palmitoylated. This process occurs via cysteine residues located in the C terminal tail of the protein. At least for rhodopsin the palmitate attached to cys322 and cys323, is inserted into the lipid bilayer, forming a kind of 4th intracellular loop [Moench *et al.*, 1994]. The role of palmitoylation of receptors is at present unclear. Several groups have attempted to ascertain what effect the palmitic acid has on receptor mediated second messenger generation. Mutation of the palmitoylated cysteine residue in the β_2 adrenoceptor causes a marked decrease in efficacy of adenylyl cyclase stimulation [O'Dowd *et al.*, 1989] while similar studies with the α_{2A} adrenergic receptor indicates that it has no effect upon receptor-G protein coupling [Kennedy & Limbird, 1993] and studies on rhodopsin indicate an increase in the efficacy of second messenger generation [Morrison *et al.*, 1991; Weiss *et al.*, 1994]. Perturbation of palmitoylation of the β_2 adrenergic

receptor leads to a high degree of phosphorylation of the receptor in the basal state [Moffet *et al.*, 1993], a phenomenon associated with activation and subsequent desensitisation of the receptor [Milligan *et al.*, 1995b]. Given the proximity of the palmitoylated cysteine residues to the sites of phosphorylation, it may be that palmitoylation of the β_2 adrenergic receptor, which is an agonist regulatable process [Moffet *et al.*, 1993], serves as a signal for desensitisation of the receptor [Moffet *et al.*, 1993; Bouvier *et al.*, 1995].

The palmitoylation of G protein α subunits was simultaneously discovered by two groups when it was found that $G_s\alpha$ [Parenti *et al.*, 1993; Linder *et al.*, 1993], $G_{i2}\alpha$, $G_o\alpha$ and $G_q\alpha/G_{11}\alpha$ [Parenti *et al.*, 1993] could incorporate [^{3}H] palmitate via a thio-ester bond. The α subunits of G_{12} and G_{13} have since been shown to incorporate palmitate also [Viet *et al.*, 1994] as has gustducin α when expressed in insect Sf9 cells [Hoon *et al.*, 1995]. Mutagenesis studies by Parenti *et al.* [1993] and Degtyarev *et al.*, [1993a] have identified cys3 as the residue which is palmitoylated in $G_o\alpha$ and $G_s\alpha$ respectively. It has been demonstrated that $G_q\alpha$ is in fact dually palmitoylated on cys9 and cys10 [Wedegaertner *et al.*, 1993].

The role of palmitoylation of G protein α subunits has been the subject of intense speculation. Palmitate negative mutants of $G_o\alpha$ have been shown to have a decreased avidity of interaction with the plasma membrane [Grassie *et al.*, 1994]. The ability of this protein to still interact with the plasma membrane reflects the presence of other determinants for membrane attachment, e.g. the presence of myristic acid at the N terminus. The ability of palmitate negative mutants of $G_s\alpha$ to interact with the plasma membrane is less clear cut. Degtyarev *et al.* [1993a] have found no effect on membrane

attachment of C3S $G_s\alpha$ when expressed in COS-7 cells while Wedegaertner *et al.*, [1993] have found that palmitate is absolutely required for membrane association of $G_s\alpha$ when it is expressed in Human Embryonic Kidney 293 cells. The presence of the short sequence TENIR near the C terminus of the $G_s\alpha$ was thought to contribute to the membrane association of $G_s\alpha$ (see above) although this has been recently questioned. Work by Degtyarev *et al.*, [1994a] has thrown doubt on the role of the TENIR sequence in promoting membrane association in isolation. When mutagenically inserted into $G_{i1}\alpha$, the TENIR sequence was ineffective in promoting the membrane association of the polypeptide. Obviously there are other determinants which govern the membrane association of α subunits, especially $G_s\alpha$. Wedegaertner *et al.*, [1993] have also found that palmitoylation is mandatory for the interaction of $G_q\alpha$ with the plasma membrane. This α subunit was found to be doubly palmitoylated on cysteine 9 and cysteine 10. The mutagenesis of one of these cysteines to serine caused the majority of the immunoreactive $G_q\alpha$ protein to localise to the cytosol, but if both were mutagenised, all of the protein was located in the cytosol.

Like the palmitoylation of other proteins, the palmitoylation of G proteins is a dynamic process. The half life of palmitate on $G_s\alpha$ is approximately 50-90 mins [Degtyarev *et al.*, 1993b; Wedegaertner & Bourne, 1994] while the half life of the protein is approximately 11-22hrs [Levis & Bourne, 1992; Degtyarev *et al.*, 1993b]. Upon agonist activation of a receptor, the half life of the α subunit decreases to around 6hrs [Levis & Bourne, 1992] while the half life of the palmitate on the Gα subunit decreases to around 2mins [Wedegaertner & Bourne, 1994]. Clearly the regulation of the protein and the palmitate on it are under very different control processes. It may be

that reversible palmitoylation serves as a dampener of serpentine receptor mediate second messenger generation. In removing the palmitate, the G protein α subunit may no longer be able to interact with the target effector system [Wedegaertner *et al.*, 1993] and thus the second messenger generation would be curtailed [Wedegaertner *et al.*, 1995].

At the same time as G protein α subunits were found to be palmitoylated on cysteine residues, the src family members $p56^{lck}$ and $p59^{fyn}$ were also found to incorporate palmitic acid [Paige *et al.*, 1993; Shenoi-Scaria *et al.*, 1993]. Sequence analyses suggest that the other members of the family, with the exception of $pp60^{src}$ and blk, are also palmitoylated [Resh, 1994] although like G proteins the precise motif for palmitoylation varies; some contain more than one cysteine residue which may be a target for palmitoylation. Interestingly, these proteins are also targets for N-myristoylation. This dual acylation has been suggested to target G protein α subunits and src family members to caveolae. Caveolae are invaginiations of the plasma membrane which have been shown to contain high proportions of receptors, GPI linked proteins, G proteins and other signalling molecules. Many of the signalling polypeptides in caveolae have been demonstrated to by modified by lipids. These modifications are thought to stabilise the interactions of caveolar proteins. Lisanti *et al.*, [1994] have proposed that caveolin, a putative transmembrane polypeptide, forms a pH and cholesterol dependent co-clustering with GPI linked proteins. Lipid modified proteins such as G proteins and Src like tyrosine kinases may insert themselves along with lipid binding proteins at the cytoplasmic leaflet of the caveolae. Lipid modification of these proteins may act to anchor the proteins to the caveolar membrane and promote protein-protein interactions. In support of this finding, Shenoi-Scaria

et al. [1994] have demonstrated the requirement of palmitate for localisation to caveolae. What role the palmitoylation of G protein α subunits plays in their localisation to caveolae remains to be determined.

1. 5. The regulation of transmembrane signalling through G proteins.

The cascade of events regulated by G proteins is not a random occurrence and its regulation is complex. From the receptors to the G proteins themselves, regulation occurs to keep the system from "overheating".

When an agonist liganded receptor activates a G protein, the subsequent release of the activated G protein leaves the receptor unoccupied and the target for phosphorylation by various serine/threonine kinases. Consensus sequences for protein kinase A and G protein linked receptor kinases (GRKs) are present on many serpentine receptors [Savarese & Fraser, 1993]. The signal for phosphorylation by these kinases may be the removal of the palmitic acid on the C terminal tail of the receptor and the subsequent disappearance of the pseudo-4th intracellular loop [Bouvier *et al.*, 1995]. The presence of phosphorylated residues on the receptor attracts one of a family of proteins termed arrestins which bind to the phosphorylated receptor uncoupling the receptor from its associated G protein [Lohse, 1993] leading to short term homologous desensitisation of the agonist induced second messenger generation.

G protein α subunits by and large are not phosphorylated, the exceptions being $G_z\alpha$, $G_{i2}\alpha$ and $G_s\alpha$. The phosphorylation of $G_s\alpha$ has only

been demonstrated *in vitro* [Pyne *et al.*, 1992] so the physiological relevance of this is unclear at present. $G_z\alpha$ and $G_{i2}\alpha$ are both phosphorylated by protein kinase C [Katada *et al.*, 1985; Carlson *et al.*, 1989; Lounsbury *et al.*, 1991]. The sites for these phosphorylation events are thought to be located on ser247 ($G_{i2}\alpha$) [Kemp & Pearson, 1990] and ser27 ($G_z\alpha$) [Lounsbury *et al.*, 1993]. The effect of phosphorylation on $G_{i2}\alpha$ would appear to be negative; that is phosphorylated $G_{i2}\alpha$ is less able to cause inhibition of adenylyl cyclase [Katada *et al.*, 1985; Pyne *et al.*, 1989; Gordeladze *et al.*, 1989; Chen & Iyengar, 1993]. The lack of a known effector for $G_z\alpha$ makes it difficult to assign a function for phosphorylation of this α subunit. These results would indicate a level of cross-talk between the lipid signalling mechanisms regulated by the G_q family of G proteins and those systems regulated by G_s and G_i family members [Strassheim & Malbon, 1994]

Chronic agonist treatment of cells in culture produces a marked loss from the membrane of the specific G protein α subunit linked to the activated receptor. The precise understanding of the mechanisms governing this loss is incomplete. Treatment of NG108-15 cells with the prostanoid receptor agonist iloprost lead to a reduction in the levels of $G_s\alpha$ [McKenzie & Milligan, 1990a], while similar treatment of platelets with iloprost did not alter levels of the polypeptide [Molina *et al.*, 1989], a phenomoenon with no explanation at present [Milligan *et al.*, 1995a]. This change in NG108-15 cells is insensitive to cycloheximide and not influenced by regulation of mRNA levels [McKenzie & Milligan, 1990a; Donnelly *et al.*, 1992]. It is likely that the reduction in levels of $G_s\alpha$ and other α subunits following chronic agonist treatment reflects a change in the half life of the polypeptides. When CHO cells stably expressing the HM1 muscarinic acetylcholine receptor are

chronically challenged with agonist, a decrease in the half-lives of $G_q\alpha$ and $G_{11}\alpha$ is observed, from approximately 18 hours to approximately 2.5 hours. After around 8 hours of such a treatment the half-lives return to their normal value [Mitchell *et al.*, 1993]. No change in the levels of $G_q\alpha$ and $G_{11}\alpha$ mRNA were observed, as assessed by reverse transcription polymerase chain reaction [Mitchell et *al.*, 1993]. Similar results have been observed for $G_s\alpha$ [Levis & Bourne, 1992]. No change in the half life of $G_{i2}\alpha$ [Mitchell *et al.*, 1993], which is not activated by the HM1 receptor in these cells, was observed . It was also seen that the effect of agonist on the levels of $G_q\alpha$ could not be mimicked by phorbol myristate acetate, a direct activator of phosphoinositidase C, or be blocked by the phosphoinositidase C selective inhibitor chelerythrine [Shah & Milligan, 1994], indicating that the effect was not mediated by a protein kinase activated by the production of a second messenger. This data and results using sucrose gradient density centrifugation has lead Milligan *et al.*, [1995a] to propose the following mechanism to explain the agonist induced down regulation of G protein α subunits. Within 30 mins of receptor activation, there is a redistribution of the α subunit from the plasma membrane fraction to a light vesicular membrane fraction [Svoboda & Milligan, 1994] followed by a reduction in the levels of the polypeptide upon sustained challenge with receptor. While this mechanism is true in HM1 receptor expressing CHO cells and rat TRH receptor expressing HEK 293 cells, both of which are linked to members of the G_q family of α subunits, the universality or otherwise of this theory remains to be determined.

The mechanism of regulation of effector systems is less clear than for either receptors or G proteins. Upon chronic activation of β_2 adrenoceptor transfected NG108-15 cells with isoproterenol there is no

reduction in the amount of adenylyl cyclase [Kim & Milligan, 1995] present in the cell, as there is with both the β_2 adrenoceptor and $G_s\alpha$ [Adie & Milligan, 1994a; Adie & Milligan, 1994b] or with the IP prostanoid receptor and $G_s\alpha$ in wild type NG108-15 cells following challenge with the agonist iloprost [Adie *et al.*, 1992].

Modulation of effector systems is known to occur through phosphorylation of serine and/or threonine residues. For type II adenylyl cyclase, this causes an activation of the enzyme [Taussig & Gilman, 1995]; no data yet exists for the other isoforms of adenylyl cyclase.

It is unknown how chronic agonist activation affects the levels the β isoforms of phosphoinositidase C, however sustained activation of protein kinase C, either by agonist or by phorbol esters, leads to marked decreases in the levels of the protein, due to enhanced proteolysis of the polypeptide [Young *et al.*, 1987] giving rise to the down regulation of this particular arm of the lipid signalling system [Collins *et al.*, 1984; Rodriguez-Pena & Rozengurt, 1984]. The signal for proteolysis may be an autophosphorylation event [Mitchell *et al.*, 1989; Pears *et al.*, 1992; Hug & Sarre, 1993].The other result of phosphoinositdase C activation, release of Ca^{2+} from intracellular stores by IP_3 is also under control and the receptor for IP_3 on the endoplasmic reticulum undergoes rapid desensitisation and down regulation [Wojcikiewicz *et al.*, 1994a; Wojcikiewicz *et al.*, 1994b].

Certain ion channels are also subject to regulation by phosphorylation. Protein kinase A can selectively activate Ca^{2+} currents [Armstrong & Eckert, 1988; Artalejo *et al.*, 1992]. A delayed rectifier type K^+

channel, termed the RAK channel, can be negatively regulated by M1 muscarinic receptor activation via a tyrosine phosphorylation event [Huang *et al*, 1993] both in *Xenopus* oocytes and in native NG108-15 cells. At least one isoform of the plasma membrane ATP driven Ca^{2+} pump, which is a target for activation by $G_s\alpha$, is stimulated by phosphorylation by protein kinase A and protein kinase C, in addition to several other stimuli [Carafoli, 1992].

It is obvious that regulation of the various arms of signal transduction by G proteins is under many levels of control and much remains to be determined, such as the role of palmitoylation in G protein signalling. It is this question which this thesis aims to address.

Chapter 2.

Materials and Methods.

Chapter 2.

Materials and methods.

2. 1. Materials.

2. 1. 1. Chemicals.

Applied Biosystems, Warrington, Cheshire.

Amplitaq Dyedoexy terminator cycle sequencing kit (part no. 401150), phenol:water:chloroform reagent (68:18:14) (part no. 400765).

Appligene, Birtley, Co. Durham.

Aquaphenol.

Boehringer Mannheim UK, Lewes, East Sussex.

BSA, aprotinin, DNase free RNase.

Calbiochem-Novabiochem (UK) Ltd., Beeston, Nottinghamshire.

Pansorbin cells, DTT.

Difco, Detroit, Michigan, USA.

Yeast extract, Bacto agar, Bacto tryptone.

Du Pont NEN (UK) Ltd., Stevenage, Hertfordshire.

EN^{3}HANCE.

Gibco Life Technologies, Paisley, Strathclyde.

DMEM, inositol free DMEM, glutamine, sodium bicarbonate, newborn calf serum, penicillin/streptomycin solution, HAT, Lipofectin, ultrapure urea, agarose, 1kb DNA ladder, pSV Sport 1.

National Diagnostics, Aylesbury, Buckinghamshire.

Sequagel-6.

New England Biolabs, Hitchin, Hertfordshire.

Restriction endonuclease Bsa HI.

Porton Products, Porton Down, Wiltshire.

Pertussis toxin.

Promega Ltd., Southampton, Hampshire.

DNA minipreps, DNA maxipreps, PCR preps, Klenow DNA polymerase, CIAP, T4 polynucleotide kinase, all restriction endonucleases (except Bsa HI and Eag I), pGEM® 7Zf(+).

Scottish Antibody Production Unit, Lanarkshire, Scotland.

Horseradish peroxidase conjugated donkey anti-rabbit IgG.

Sigma Chemical Co. Ltd., Poole, Dorset.

Trypsin, ATP, arginine hydrochloride, NP-40, gelatine, *o*-dianisidine hydrochloride, 7-deoxycholic acid, bromophenol blue, TEMED, forskolin, Coomassie Blue R-250, hexamine cobalt chloride, hydroxylamine hydrochloride, GTP, thymidine, sodium azide, geneticin sulphate, soybean

trypsin inhibitor, PMSF, Triton X-100, thimerosal, ampicillin, CPSR-3, low melting point agarose.

Stratagene,

Pfu DNA polymerase.

All other reagents and chemicals were of analytical grade and obtained from **Fisons Scientific Equipment, Loughborough, Leicestershire.**

2. 1. 2. Radiochemicals.

Du Pont NEN (UK) Ltd., Stevenage, Hertfordshire.

Nicotinamide adenine dinucleotide di(triethanolammonium) salt [adenylate-^{32}P], ~29.6TBq (800Ci)/mmol (product no. NEG 023X), [9,10-^{3}H(N)] palmitic acid, 1.11-2.22TBq (30-60Ci)/mmol (product no. NET-043).

ICN Flow, Irvine, Strathclyde.

Tran^{35}S label, >37TBq (>1000Ci)/mmol (product no. 51006)

2. 1. 3. Antisera.

All antisera used in this study are detailed in **Table 2. 1**. The method of production of the antisera has been described previously [Goldsmith *et al.*, 1987].

Table 2. 1. Specificity of antisera employed in this study.

The antisera used during the course of this work are shown along with the peptide sequences to which they were raised, the corresponding areas of the G protein α subunits which correspond to these amino acids, the α subunits which the antisera identify and the references dealing with their production. All antisera were made in house apart from antiserum AS201, which was a kind gift of Dr G. Schultz, Institute of Pharmacology, Freie University, Berlin, Germany.

Table 2. 1.

Antiserum	Peptide Sequence	G protein Sequence	Antiserum identifies	Reference
IM1	NLKEDGISAAKDVK	$G_o\alpha$ 22-35	$G_o\alpha$	Mullaney & Milligan [1990]
OC1	ANNLRGCGLY	$G_o\alpha$ 345-354	$G_o\alpha$	Mullaney & Milligan [1990]
ON2	GCTLSAEERAALERSK	$G_o\alpha$ 1-16	$G_o\alpha$	Mullaney & Milligan [1990]
CQ2	QLNLKEYNLV	$G_q\alpha$ 349-359	$G_q\alpha$, $G_{11}\alpha$	Mitchell *et al.*, [1991]
SG1	KENLKDCGLF	$G_{t1}\alpha$ 341-350	$G_{t1}\alpha$, $G_{t2}\alpha$, $G_{i1}\alpha$, $G_{i2}\alpha$	McKenzie & Milligan, [1990]
CS3	RMHLRQYELL	$G_s\alpha$ 385-394	$G_s\alpha$	Milligan & Unson, [1989]
O1A	PGSNTYEDAAAYINTN	$G_{o1}\alpha$292-307	$G_{o1}\alpha$, $G_o{*}\alpha$	Mullaney & Milligan, unpublished observations.
AS201	GPSAFTEAVAHIQGQY	$G_{o2}\alpha$ 292-307	$G_{o2}\alpha$	Spicher *et al.*, [1991]
IQB	EKVTTFEHQYVNAIKT	$G_q\alpha$ 119-134	$G_q\alpha$	Milligan *et al.*, [1993]

2. 1. 4 Animals.

Adult Sprague-Dawley rats were killed by cervical dislocation, the brains removed and the cortex used in the preparation of crude plasma membranes as described in **section 2. 3. 1.**

2. 2. Cell Culture.

2. 2. 1. Growth conditions.

The cells were grown in continuous monolayer culture in $75cm^2$ sterile tissue culture flasks (Nunc, Roskilde, Denmark) in sodium pyruvate free Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) serum (as indicated for each cell line), 2mM L-glutamine, 100 I.U./ml penicillin, 100μg/ml streptomycin. Buffering of the medium was achieved by the addition of 0.375% (w/v) sodium bicarbonate and growing the cells in an atmosphere of O_2:CO_2 in the ratio of 95%:5%. Cells were incubated in 10ml of the above medium in a VSL incubator (Scotlab, Coatbridge, Strathclyde.) at 37°C and allowed to reach confluency. The medium was changed regularly, generally every 2 days.

2. 2. 2. Growth and maintenance of cells.

NG108-15 neuroblastoma x glioma hybrid cells were a kind gift from Dr. W. Klee (NIH, Bethesda, Maryland, U.S.A.). The cells were grown to confluency according to the method of Hamprecht *et al.* [1985], in DMEM medium supplemented with 10% (v/v) Controlled Process Serum

Replacement-3 (CPSR-3) and hypoxanthine, aminopterin, thymidine (HAT) at final concentrations of 100μM, 10μM and 16μM respectively.

Rat 1 fibroblasts and COS-1 cells were obtained from ECACC, Porton Down, Salisbury, Wiltshire. C5B and D3 cells were derived from Rat 1 fibroblasts by transfection with the cDNA encoding, respectively, wild type or C3S mutant forms of rat $G_{o1}\alpha$ in pcEXV-3 [Grassie *et al.*, 1994]. Parental Rat 1, C5B, D3 and COS-1 cells were grown to confluency in DMEM medium supplemented with 10% (v/v) newborn bovine serum. To minimise cell reversion, C5B and D3 cells were routinely passaged in medium containing 10% (v/v) newborn bovine serum and 700μg/ml geneticin sulphate.

2. 2. 3. Passaging of confluent cell cultures.

Upon reaching confluency, each flask of cells was usually split 1:5 into new flasks. Medium was removed and the monolayer trypsinised by addition of a solution containing 0.1% (w/v) trypsin, 10mM glucose, 0.67mM EDTA, pH 7.4. When all cells had detached from the flask, trypsinisation was terminated by addition of 10ml DMEM containing 10% CPSR-3 (NG108-15 cells) or NBS (Rat 1, transfectants and COS-1). The cell suspension was then decanted into a sterile 50ml polypropylene centrifuge tube (Nunc, Roskilde, Denmark) and centrifuged in a bench top centrifuge (MSE) at 1000rpm for 5mins. The supernatant was discarded and the pellet of cells resuspended in 5ml of serum containing DMEM and 1ml added to 5 new flasks containing 9ml of medium and placed in an incubator.

2. 2. 4. Freezing and storage of cells.

To provide a stock of cells for further use, cells were cryogenically stored in liquid N_2. After trypsinisation and centrifugation as above, cells were resuspended in 1ml per confluent flask of freezing medium. This was DMEM medium supplemented with 20% (v/v) of the appropriate serum and 8% (v/v) dimethyl sulphoxide. 1ml of the cell suspension was added to sterile freezing vials (Nunc, Roskilde, Denmark). To prevent formation of ice crystals in the cells, the freezing vials were placed in a polystyrene box packed with cotton wool and frozen slowly overnight at -80°C. The cells were then tranferred to liquid N_2.

2. 2. 5. Recovery of cells from liquid N_2.

Vials were removed from liquid N_2, thawed at 37°C and the contents placed in a 50ml centrifuge tube and diluted with 13ml of DMEM medium containing 10% serum and centrifuged at 1000rpm for 5mins as above. The supernatant was then discarded and the pellet resuspended in 10ml DMEM medium plus 10% serum and the whole added to a $75cm^2$ flask and the cells handled as normal.

2. 2. 6. Differentiation of NG108-15 cells.

Differentiation of NG108-15 cells was carried out using forskolin as previously described [Mullaney & Milligan, 1990]. Briefly, NG108-15 cells were trypsinised and centrifuged as described above and resuspended in 1ml per flask of serum reduced DMEM medium containing 1%

(v/v) CPSR-3 and HAT. A further 4ml per confluent flask of serum reduced DMEM medium was added and 1ml of the cell suspension was then added to 8ml of serum reduced DMEM medium. Forskolin was prepared as a 10x stock in serum reduced DMEM medium by taking an appropriate volume of 10mM forskolin stock in ethanol and filter sterilising through a 0.22μm syringe filter. 1ml of this stock was added to each flask to give a final concentration of 10μM. The cells were incubated as normal for 6 days without disturbing them or changing the medium. Cells were harvested as described in **section 2. 2. 10.**

2. 2. 7. Treatment of cells with pertussis toxin *in vivo*.

Cells which were to be pretreated with pertussis toxin were grown to approximately 70% confluency and the medium aspirated. 9ml of fresh DMEM medium was then added to each flask. Pertussis toxin was prepared fresh as a 10x stock in DMEM medium and filter sterilised as described above. 1ml was added to each flask to a final concentration of 25ng/ml. Cells were then grown for a further 16hrs before being harvested as described in **section 2. 2. 10.**

2. 2. 8. Labelling of cells with ^{3}H palmitic acid.

COS-1 cells were transfected for approximately 70hrs, as described in **section 2. 2. 12.**, before 200μCi/ml of [9, 10-^{3}H] palmitate was added to the flasks in DMEM medium containing 5% (v/v) dialysed newborn bovine serum, 5mM sodium pyruvate and incubated for 4hrs. Cells were then

harvested and total cell lysates immunoprecipitated as described in **section 2. 5. 3.**

2. 2. 9. Labelling of cells with Tran^{35}S label.

When COS-1 cells had been transfected for approximately 56hrs, cells were treated for 16hrs with 50μCi/ml of Tran^{35}S label. To maximise the incorporation of radioactivity into newly synthesised protein, DMEM, without methionine and cysteine, was supplemented with antibiotics as before, and 5% (v/v) dialysed newborn calf serum. This labelling was allowed to continue overnight before whole cell lysates were immunoprecipitated as described in **section 2. 5. 3.**

2. 2. 10. Harvesting of cells.

After cells reached confluency, or the particular treatment time had elapsed, they were harvested by scraping the monolayer into the medium and the cells collected in a 50ml centrifuge tube on ice. The tubes were centrifuged at 2000rpm for 5mins at 4°C in a Beckman TJ-6 benchtop centifuge. The supernatant was discarded and the cell pellet resuspended in 25ml of ice-cold PBS (137mM NaCl, 4mM Na_2HPO_4, 0.27mM KCl, 0.15mM KH_2PO_4, pH7.4) and centrifuged as before. Again the supernatant was discarded and the pellet resuspended in PBS and re-centrifuged. The supernatant was again discarded and the pellet stored at -80°C until required.

2. 2. 11. Transient transfection of COS-1 cells.

COS-1 cells were grown to approximately 50% confluency in $75cm^2$ flasks as described above. 80μg of DNA was added to a 13ml sterile polypropylene centrifuge tube containing 50μl of Lipofectin and sterile Milli Q H_2O, to a final volume of 300μl. DNA/liposome complexes were allowed to form for 15mins during which time the cells were washed twice with serum free DMEM medium. 10ml of serum free DMEM medium was then added to the DNA/liposome complexes and the whole added to one $75cm^2$ flask of COS-1 cells. Cells were then incubated for 8hrs to allow the DNA to enter the cell before the medium was replaced with DMEM containing 10% newborn bovine serum. Cells were then incubated for approximately 70hrs before being harvested as described in the preceding section.

2. 3. Preparation of membrane fractions.

2. 3. 1. Preparation of crude plasma membranes.

Membranes were prepared according to the method of (Koski & Klee, 1981). Frozen cell pastes were thawed on ice and resuspended in 2ml of ice-cold TE buffer (10mM Tris HCl, pH7.5, 0.1mM EDTA) and transferred to a pre-chilled glass homogeniser tube. The cells were then homogenised, on ice, with 20 strokes of a teflon homogeniser. Homogenates were transferred to polypropylene centrifuge tubes and the homogeniser tube washed with a further 1ml of ice-cold TE buffer and the contents added to the centrifuge tube.

Centrifuge tubes were placed in a type 50Ti rotor (Beckman) and centrifuged at 500xg_{av} for 10mins at 4°C in a Beckman L5-50B ultracentrifuge. The pellet, which contained nuclei and unbroken cells, was discarded and the supernatant transferred to fresh pre-chilled centrifuge tubes and centrifuged at 48,000xg_{av} for 10mins at 4°C. The supernatant was discarded and the pellet resuspended in 5ml of TE buffer and recentrifuged at 48,000xg_{av} for 10mins at 4°C. Again the supernatant was discarded and pellet resuspended in TE buffer to give an approximate protein concentration of 1-3mg/ml. The samples were then frozen at -80°C in 100μl fractions.

Crude plasma membranes from rat brain was prepared as above using cortex from brains of animals which had been sacrificed by cervical dislocation.

2. 3. 2. Preparation of membrane and cytoplasmic fractions.

Cell pastes were homogenised as described above, except that homogenisation was carried out in 1ml to prevent excessive dilution of the resulting cytoplasmic protein. Homogenates were centrifuged at 500xg_{av} as described above. The resulting supernatant was transferred to 11mm x 34mm centrifuge tubes and placed in a TL100a rotor and centrifuged at 200,000xg_{av} for 30mins at 4°C in a Beckman TL100 tabletop ultracentrifuge. The supernatant (designated "cytoplasmic fraction") was retained and stored in 200μl samples at -80°C. The pellet (designated "membrane fraction") was resuspended in TE buffer to give an approximate protein concentration of 3mg/ml and samples of 100μl were stored at -80°C.

2. 4. Determination of protein concentration.

Protein concentrations were determined by the method of [Lowry *et al.* [1951], using BSA as standard. The samples were read at 750nm on an LKB Biochrom Ultraspec II spectrophotometer.

2. 5. Preparation of samples for SDS - polyacrylamide gel electrophoresis (SDS -PAGE).

2. 5. 1. TCA/deoxycholate precipitation of samples.

The required amount of crude plasma membranes, or membrane and cytoplasmic fractions, as indicated for each experiment, were taken and placed on ice in a 1.5ml microcentrifuge tube and 6.3μl of 2% (w/v) 7-deoxycholic acid, sodium salt added. 700μl of dH_2O and 250μl of 24% (w/v) trichloroacetic acid were added sequentially and the tubes were vortexed briefly and incubated on ice for 15mins before being centrifuged on an MSE microcentrifuge at 13,000rpm for 5mins. The supernatant was discarded and the pellet dissolved by the addition of 20μl of 1M Tris base. 20μl of sample buffer (0.3g/ml urea, 0.06g/ml DTT, 0.05g/ml SDS, 50μl/ml 1M Tris HCl, pH8, with a few crystals of bromophenol blue) was then added. The sample was ready for loading onto the gel.

2. 5. 2. Hydroxylamine treatment.

Hydroxylamine cleaves thio-ester bonds attaching acyl chains to proteins. Using a modification of the method of Magee *et al.* [1984] and Magee & Courtneidge [1985], we used hydroxylamine to remove any cysteine linked acyl groups from G protein α subunits. The required amount of crude plasma membranes, as indicated for each experiment, were transferred to a 1.5ml microcentrifuge tube and the membranes pelleted in a microfuge at 4°C for 5 mins. The supernatant was discarded and the pellet resuspended in 100μl of 1M hydroxylamine, pH8 or 100μl of 1M Tris HCl, pH8 as control, and incubated for 4hrs at 25°C. The membranes were then transferred to TL100 centrifuge tubes and the microcentrifuge tubes washed with 100μl of PBS and the washings added to the contents of the TL100 tubes. The were placed in a TL100a rotor and centrifuged at 120,000xg_{av} for 30mins at 4°C in a Beckman TL100 tabletop ultracentrifuge .

The resulting supernatant was added to microcentrifuge tubes containing 10μg BSA to act as carrier protein. 100% (w/v) TCA was added, to a final concentration of 20%, and the tubes were left on ice for 15mins. The tubes were then centrifuged at 13,000prm for 5mins in an MSE microcentrifuge at 4°C. The pellet was then dissolved in 20μl of 1M Tris base and 20μl of sample buffer added.

20μl of sample buffer was added to the pellet from the ultracentrifugation step in the TL100 tubes. The pellet was dissolved in the sample buffer by incubating at 37°C for 1hr. The samples were then loaded onto the gel.

2. 5. 3. Immunoprecipitation of proteins.

The cell pellet from **sections 2. 2. 8.** and **2. 2. 9.** was resuspended in 200μl of 1% (w/v) SDS containing 0.2mM PMSF and 1μg/ml aprotinin. *In vitro* pertussis toxin catalysed ADP-ribosylation assays (see **section 2. 10.**) were diluted to 200μl with 1.33% (w/v) SDS containing PMSF and aprotinin. Both samples were then processed identically.

Tubes were boiled for 4mins and 800μl of ice cold solution I (1.25% (v/v) Triton X-100, 190mM NaCl, 6mM EDTA, 50mM Tris HCl, pH 7.5, containing PMSF and aprotinin) added to each. Samples were centrifuged in an MSE microfuge for 10mins at 4°C and the supernatant transferred to fresh tubes. 1μl of antiserum/10μg of protein (ribosylation experiments) or 15μl of antibody (palmitate and Tran^{35}S label experiments) was added to each tube and incubated with constant rotation overnight at 4°C. 50μl of Pansorbin Cells was then added to each tube and the tubes incubated again for 2-4hrs at 4°C with constant rotation. The tubes were centrifuged briefly for 20secs and the pellet resuspended 3 times, with centrifugation between washes, with solution I containing 0.2 volume of 1% (w/v) SDS. The Pansorbin was then washed once with 50mM Tris HCl, pH 6.8, as above and the final pellet resuspended in 50μl of Laemmli sample buffer (see **section 2. 5. 1.**) and boiled for 5mins. The suspension was then centrifuged at 13,000rpm for 2mins in an MSE microfuge and the supernatant subjected to SDS-PAGE as described in **section 2. 6. 1.**

2. 6. SDS-Polyacrylamide gel electrophoresis.

2. 6. 1. SDS-PAGE lower resolving gel - 10% (w/v) polyacrylamide gels.

SDS-PAGE was carried out as described by Laemmli, [1970] on 1.5mm wide slab gels. The gel plates measured 160mm x 180mm. The slabs were run on a Bio-Rad Protean I electrophoresis system (Bio-Rad Laboratories Ltd, Watford, Herts.)

Resolving gels were prepared by mixing stock acrylamide solution (30% (w/v) acrylamide, 0.8% (w/v) N, N' -methylene bisacrylamide), buffer 1 (1.5M Tris HCl (pH 8.8), 0.4% (w/v) SDS), 50% (v/v) glycerol and dH_2O to give final concentrations of 10% (w/v) acrylamide, 0.24% (w/v) N, N'-methylene bisacrylamide, 0.375M Tris HCl (pH8.8), 0.1% (w/v) SDS, 3% (v/v) glycerol. Polymerisation of the gel was achieved by the addition of 8μl of TEMED and 90μl of freshly prepared 10% (w/v) ammonium persulphate. Upper stacking gels were as described in **section 2. 6. 4.** and electrophoresis was carried out as described in **section 2. 6. 5.**

2. 6. 2. Lower resolving gel - urea gradient SDS-PAGE.

To achieve maximum separation of isoforms of $G_o\alpha$, urea gradient SDS-PAGE was carried out as a modification of the method of Scherer *et al.*, (1987) and Codina *et al.*, (1991). In order to maximise the separation, longer gels (160mm x 200mm plates, 1.5mm spacers) were used and ran as part of a Bio-Rad Protean II system. Resolving gels containing a

linear 4M-8M urea gradient were fashioned by preparing stock solutions of acrylamide (30% (w/v) acrylamide, 0.15% (w/v) N, N'-methylene bisacrylamide) and buffer 1 (as above) in both 4M and 8M urea and mixing them with 4M or 8M urea in separate containers, to a volume of 20ml. This gave final concentrations of 12.5% (w/v) acrylamide, 0.0625% (w/v) N,N'-methylene bisacrylamide, 0.375M Tris HCl (pH8.8), 0.1% (w/v) SDS. Polymerisation of the two mixtures was initiated by the addition of 3μl of TEMED and 15μl of freshly prepared 10% (w/v) ammonium persulphate and quickly added to the chambers of a gradient maker. The mix was then pumped into the gel plates and the gel allowed to polymerise, undisturbed, overnight. Ultrapure urea was used to avoid the usual Amberlite deionisation step involved. Upper stacking gels were as described in **section 2. 6. 4.** and electrophoresis was carried out as described in **section 2. 6. 5.**

2. 6. 3. Lower resolving gel - 6M urea SDS-PAGE.

In order to separate species variants of $G_{11}\alpha$, SDS-PAGE was carried out in the presence of 6M urea [Kim & Milligan, 1994]. Urea was added to the acrylamide mix described in **section 2. 6. 1.** to a final concentration of 6M and polymerised with 150μl of freshly prepared 10% (w/v) APS and 12μl TEMED. 37.5ml of this was loaded into the Bio-Rad Protean II gel plates described in **section 2. 6. 2.** and allowed to polymerise overnight. Upper stacking gels were as described in **section 2. 6. 4.** and electrophoresis was carried out as described in **section 2. 6. 5.**

2. 6. 4. Upper stacking gels.

The upper stacking gel was prepared by mixing stock acrylamide solution (30% (w/v) acrylamide, 0.8% (w/v) N, N'-methylene bisacrylamide), buffer 2 (0.5M Tris HCl (pH 6.8), 0.4% (w/v) SDS) and H_2O to give final concentrations of 3% (w/v) acrylamide, 0.08% (w/v) N, N'-methylene bisacrylamide, 0.125M Tris HCl (pH6.8), and 0.1% (w/v) SDS. This was polymerised by adding 8μl of TEMED and 150μl of freshly prepared 10% (w/v) ammonium persulphate. Samples were loaded onto the gels using a Hamilton microsyringe (Hamilton Co., Reno, Nevada, USA.).

2. 6. 5. Electrophoresis Running Buffers.

The running buffer contained 25mM Tris HCl (pH8.5), 0.192M glycine and 0.1% (w/v) SDS. Electrophoresis was toward the anode at 40V, 25mA per slab until the bromophenol blue dye front was 0.5cm from the bottom (standard 10% gels) or 160V, 60mA per slab until the prestained lactic dehydrogenase molecular weight marker was 5cm from the bottom (urea containing gels).

2. 7. Staining of SDS-PAGE gels.

2. 7. 1. Staining of SDS-PAGE gels with Coomassie Blue.

Following electrophoresis, gels were soaked, with gentle shaking on a rotary shaker, for 1hr in 45% (v/v) methanol, 10% (v/v) acetic

acid containing 0.25% (w/v) Coomassie Blue R-250. Gels were destained by washing with 45% (v/v) methanol, 10 % (v/v) acetic acid with frequent changes of the wash solution.

2. 7. 2. Staining of [^{3}H] containing gels.

Following electrophoretic separation of [^{3}H] palmitate labelled samples, gels were subjected to EN^{3}HANCE, according to the manufacturers instructions, before being dried and subjected to autoradiography as described in **section 2. 11.**

2. 8. Western blotting.

2. 8. 1. Transfer of proteins to nitrocellulose.

After SDS-PAGE, gels were transferred to nitrocellulose membranes essentially as described by Towbin *et al.* (1979). The sandwich was prepared by taking the plastic cage and submerging it in transfer buffer (25mM Tris, pH8.3, 192mM glycine, 25% (v/v) methanol). A sponge was laid on top and a piece of Whatman 3mm chromatography paper, cut slightly larger than the gel, laid on top of this. Next, the gel was placed on the chromatography paper followed by the nitrocellulose membrane (Costar, Cambridge, MA, USA). Another piece of chromatography paper was laid on top followed by a second sponge. The top half of the cage completed the sandwich. All manipulations were carried out with the sandwich completely submerged in the blotting buffer to minimise the formation of air bubbles which would interfere with transfer of the proteins. The sandwich was then

closed up and the proteins subjected to electrophoresis toward the anode at 2A for 1.5hrs.

2. 8. 2. Incubation of nitrocellulose membranes with antisera.

Following transfer of proteins, the nitrocellulose membranes were incubated in 5% (w/v) porcine gelatine in PBS, pH 7.4, containing a pinch of thimerosal, at 37°C for a minimum of 2hrs. The gelatine/PBS was removed and the membrane washed three times with 100ml dH_2O. The nitrocellulose was then incubated overnight at 37°C with the appropriately diluted anti-G protein antiserum in 1% (w/v) porcine gelatine in PBS, pH 7.4, containing 0.2% (v/v) NP-40 (gelatine/PBS/NP-40). The dilutions of primary antisera were removed and the blots washed three times with PBS containing 0.2% (v/v) NP-40 for 10mins and then three times with PBS for 10mins. Secondary antiserum (horseradish peroxidase conjugated donkey anti-rabbit IgG), at a dilution of 1:200 in gelatine/PBS/NP-40, was added and the nitrocellulose membranes incubated for 2hrs at 37°C. The secondary antiserum was removed and the nitrocellulose washed with PBS/NP-40 and PBS as before.

2. 8. 3. Development of immunoblots.

1ml of freshly prepared 1% (w/v)*o*-dianisidine hydrochloride was added to 40ml of PBS, pH7.4, and the whole added to the newly washed nitrocellulose membranes. Development of the immunoblot was initiated by addition of 20μl of stock H_2O_2 (30% v/v) and stopped by immediately

immersing the membranes in 1% (w/v) sodium azide. The developed immunoblot was rinsed in dH_2O and allowed to dry.

2. 9. Densitometric analysis of immunoblots.

Following development, immunoblots were densitometrically scanned on a Bio-Rad GS-360 imaging densitometer and analysed on an Apple Macintosh Quadra 800 microcomputer.

2. 10. Mono ADP-ribosylation of cell membranes with pertussis toxin.

Mono ADP-ribosylation of G-proteins was performed essentially as described by Hudson & Johnson, [1980]. Pertussis toxin was obtained as an 200μg/ml stock solution in 50mM phosphate buffer, pH 7.2, 500mM NaCl and 50% (v/v) glycerol. For *in vitro* ADP-ribosylations, pertussis toxin was first preactivated by taking equal volumes of stock solution and 100mM DTT and incubating at room temperature for 90 mins. 5μl (100ng) of this was added to each incubation tube.

The appropriate amount of membranes or cytoplasm, in a volume of 15-20μl, was added to each tube along with buffer containing 20mM thymidine, 0.1mM GTP, 250mM sodium phosphate buffer, pH7.0, 1mM ATP, 20mM arginine hydrochloride and spiked with 2μCi of [^{32}P] NAD^+ per assay tube. Reactions were started by the addition of preactivated pertussis toxin. The tubes were placed in a water bath at 37°C for 2hrs. The tubes were then placed on ice and either TCA precipitated as described in

section 2. 5. 1. or immunoprecipitated as described in **section 2. 5. 3.** The samples were then subjected to SDS-PAGE as described in **section 2. 6.**

2. 11. Autoradiography.

Destained gels from experiments containing [^{32}P] labelled proteins, or EN^{3}HANCE treated gels, were dried onto Whatman No.3 chromatography paper under a vacuum line attached to a Bio-Rad 583 gel drier. Autoradiography was at -80°C (^{32}P labelled proteins) or at room temperature (^{3}H labelled proteins), for an appropriate time on Fuji X-ray film in a Kodak X-o-matic cassette with intensifying screens. Films were developed in a Kodak X-o-mat developing machine.

2. 12. Phosphorimaging.

Some gels containing [^{32}P] labelled proteins and experiments from Tran^{35}S labelled proteins were dried as described in the preceding section and subjected to phosphor imaging for the indicated time before being analysed on a Fujix BAS1000 phosphorimager linked to an Apple Macintosh Quadra 650 microcomputer.

2. 13. Gel Filtration.

Separation of cytoplasmic proteins derived from D3 cells was achieved by gel filtration on a Superose 12 column (Pharmacia, Uppsala, Sweden) linked to a Pharmacia Fast Protein Liquid Chromatography (FPLC) system. The column was equilibrated overnight in buffer consisting of 10mM

Tris HCl, pH7.4, 1mM EDTA, 150mM NaCl. 200μl of crude cytoplasm, derived from the fractionation described in **section 2. 3. 2.**, was loaded onto the column and the column washed with the Tris/EDTA/NaCl buffer at a rate of 1ml/min. 500μl fractions were collected in microcentrifuge tubes containing 10μg of BSA, to prevent loss of protein, and TCA precipitated as described in **section 2. 5. 2.** The fractions were then run on SDS-PAGE gels as described and immunoblotted as described in **sections 2. 6. 1.** and **section 2. 8.**

2. 14. Growth of *Escherichia coli.* strain JM109.

E.coli were routinely grown in L broth. This consisted of 10g/litre Bacto-tryptone, 5g/litre yeast extract, 0.17M NaCl, pH 7.0. Depending on the strain and the plasmid, antibiotics were also included. The cultures were grown at 37°C with continuous shaking at 120-150 rpm.

When working with *E.coli*., all media and solutions, unless otherwise stated, were sterilised by autoclaving for 11mins at 126°C. Ampicillin was prepared as a 50mg/ml stock in dH_2O and sterilised by filtration through a 0.22μm syringe filter.

Stock JM109 *E.coli* cells were purchased from Promega Corporation and streaked onto an LB agar plate (15g/litre Bacto-agar in L broth) in the absence of any antibiotics. The agar plate was incubated overnight at 37°C. A single colony was picked for expansion and grown up overnight at 37°C in 10ml of L broth. 5ml of this was used to make competent cells and 5ml was used to make glycerol stocks.

Transformed JM109 cells were grown in the presence on antibiotics to prevent loss of the particular plasmid of interest. JM109 cells transformed with pCMV, pGEM® 7Zf(+) or pSV Sport 1 or recombinant plasmids derived from them were grown in L broth or on LB agar plates supplemented with 60μg/ml of ampicillin. The plasmid pCMV containing the cDNA for murine α11, cloned into the Cla I/Xho I sites, was a kind gift from Dr. Melvin I. Simon, California Institute of Technology, California, USA. Routinely, glycerol stocks of JM109 cells transformed with pCMV, pGEM 7Zf(+) or pSV Sport 1 or their recombinant derivatives were prepared.

2. 15. Preparation of chemically competent JM109 cells.

A stab from a glycerol stock of JM109 cells was grown up overnight in 5ml of L broth and 3ml of this used to inoculate 500ml of L broth. The OD_{600} was monitored until it reached approx. 0.25 (equivalent to approx. $1x10^8$ cells/ml). The cell suspension was then decanted into two 250ml centrifuge tubes, allowed to cool on ice for approximately 30mins and spun at 10,000rpm for 10mins at 4°C on a Beckman J2-21 centrifuge containing a JA14 rotor. The supernatant was discarded and the pellets resuspended in 20ml each of ice-cold sterile buffer I (100mM RbCl, 50mM $MnCl_2.4H_2O$, 30mM potassium acetate, 10mM $CaCl_2.2H_2O$, 15% (w/v) glycerol, pH 5.8). The suspension was left on ice for 15mins at 4°C and recentrifuged as before. The supernatants were again discarded and the pellets resuspended in a total volume of 3.5ml of ice-cold buffer II (10mM RbCl, 10mM MOPS, 75mM $CaCl_2.2H_2O$, 15% (w/v) glycerol, pH6.8) and pooled

into one tube. The suspension was left on ice for 15mins before 250μl aliquots were snap frozen in liquid N2 and stored at -80°C.

2. 16. Transformation of chemically competent *E.coli.* with plasmid DNA.

An aliquot of chemically competent JM109 cells were thawed on ice and an appropriate amount of DNA added. DNA and bacteria were mixed in a sterile 13ml polypropylene centrifuge tube. This was 10-100ng of supercoiled plasmid DNA or 5μl of a ligation reaction. The cells were incubated on ice for 15mins before being heat shocked at 42°C for exactly 90secs and returned to ice for 2mins. 800μl of SOC medium (20g/l Bacto-tryptone, 5g/l yeast extract, 20mM $MgCl_2$, 8.5mM NaCl, 2.5mM KCl, pH7.0) was added and the tubes incubated, with shaking at 150rpm, for 45-60mins at 37°C.

100μl and 200μl were spread onto LB agar plates containing 60μg/ml ampicillin. The plates were incubated overnight at 37°C and antibiotic resistant colonies picked and grown up overnight in 10ml L broth containing the required antibiotics as above. 3ml of this was used to prepare plasmid DNA and the remainder used to make glycerol stocks.

2. 17. Preparation of glycerol stocks.

Sterile 80% (v/v) glycerol was added to the overnight culture to 15% (v/v) and the suspension mixed gently to ensure thorough dispersion of the glycerol. 1ml aliquots of this were added to sterile microcentrifuge tubes

and snap frozen in liquid N_2. The tubes were stored at -80°C until required. The stocks were not allowed to thaw and scrapings from these frozen samples were used to inoculate fresh cultures.

2. 18. Preparation of plasmid DNA.

2. 18. 1. Preparation of double stranded plasmid DNA by alkaline lysis.

This method of plasmid DNA isolation was used to prepare plasmid DNA for diagnostic restriction digestion and not for subcloning or PCR reactions. The method used was as described in Sambrook *et al.*, (1988). 3ml of the overnight culture of interest was centrifuged in microcentrifuge tubes in an MSE microcentrifuge at 13,000rpm for 1min. The supernatant was discarded and the pelleted cells resuspended in 100μl of sterile, ice-cold solution I (50mM glucose, 25mm Tris HCl, pH 8.0, 10mM EDTA). 200μl of freshly prepared solution II (0.2M NaOH, 1% (w/v) SDS) was added and the tubes inverted several times to mix. Solution II was prepared by diluting stock solutions of 10M NaOH and 10% (w/v) SDS. 150μl of ice-cold solution III (60ml of 5M potassium acetate, 11.5ml of glacial acetic acid and 28.5ml dH_2O, giving final concentrations of 3M K^+ and 5M acetate) was added and the tube mixed by inversion again. The suspension was centrifuged at 13,000rpm for 5mins and the supernatant transferred to another microcentrifuge tube and ethanol precipitated as described in **section 2. 19**. Precipitates were resuspended in 50μl of 20μg/ml DNase-free RNase in sterile H_2O.

2. 18. 2. Preparation of double stranded plasmid DNA by Promega Minipreps.

This was carried out as described by the manufacturer. 3ml of overnight culture was centrifuged as described in **section 2. 18. 1** and the resulting pellet resuspended in 200μl buffer 1 (50mM Tris HCl, pH 7.5, 10mM EDTA, 100μg/ml RNase A). 200μl of buffer 2 added (0.2M NaOH, 1% (w/v) SDS) and the tube mixed by inversion. 200μl of buffer 3 (1.32M potassium acetate) was added to this and the tube mixed, again by inversion, and centrifuged at 13,000rpm in a MSE microcentrifuge. The supernatant was transferred to another tube and 1ml of Wizard purification resin added. The tubes were vortexed briefly and the contents transferred to a 2ml syringe barrel fitted with a Minicolumn. The resin was pushed through the column with the syringe plunger. The resin in the Minicolumn was washed with 2ml of wash buffer (0.09M NaCl, 9mM Tris HCl, pH 7.5, 2.2mM EDTA, 55% (v/v) analar ethanol) and the column attached to a microcentrifuge tube and centrifuged at 12,000rpm in a Sorvall MC12C microcentrifuge for 20secs. The Minicolumn was then transferred to a fresh microcentrifuge tube and 50μl of sterile Milli Q H_2O, preheated to 65°C, added. The column was allowed to sit at room temperature for at least 1min before being centrifuged as before. The resulting aqueous DNA solution was stored at -20°C until required.

2. 18. 3. Preparation of double stranded plasmid DNA for automated DNA sequencing.

This was done by a modification of the method in the preceding section. An entire 10ml overnight culture was centrifuged in a Beckman TJ6

benchtop centrifuge for 20mins at 3000rpm. The pellet was resuspended in 300μl of buffer 1 as above and the suspension transferred to a microcentrifuge tube. The lysis was carried out as described in **section 2. 18. 2.** using 300μl of each of buffers 2 and 3. Following centrifugation of the lysate, the supernatant was split into 2 microcentrifuge tubes (450μl each) and 500μl of resin added to each. The resins were then recombined into one syringe barrel and processed as described previously, except that 3ml of wash buffer was used and the column was centrifuged for 1min. The DNA was eluted with 100μl of preheated water followed by another 1min centrifugation step.

2. 18. 4. Large scale preparation of double stranded plasmid DNA by Promega maxiprep.

5ml of a 10ml overnight culture was used to inoculate 500ml of L broth containing 60μg/ml of ampicillin. Following incubation overnight at 37°C with shaking at 150rpm, the culture was decanted into 250ml centrifuge buckets and centrifuged at 10,00rpm in a Beckman JA14 rotor at 4°C for 10mins. The pellet was resuspended in 10ml of buffer 1 (50mM Tris HCl, pH 7.5, 10mM EDTA, 100μg/ml RNase A) and lysis carried out by the addition of 10ml of buffer 2 (0.2M NaOH, 1% (w/v) SDS). The lysate was neutralised by adding 10ml of buffer 3 (1.32M potassium acetate). The lysate was centrifuged at 10,000rpm for 20mins at 4°C in a Beckman JA14 rotor.

The supernatant was filtered through two layers of cheesecloth and the DNA precipitated by the addition of 0.6 volumes of iso-propanol. DNA was collected by centrifugation at 10,00rpm in a Beckman JA14 rotor at 4°C for 10mins and the pellet resuspended in 2ml of 10mM Tris HCl, pH 7.5,

1mM EDTA. 10ml of Wizard purification resin added and the whole added to a Wizard maxicolumn connected to a Vac-Man vacuum manifold (Promega). The resin was collected by drawing a vacuum through the column and washed by the addition of 25ml of wash buffer (0.09M NaCl, 9mM Tris HCl, pH 7.5, 2.2mM EDTA, 55% (v/v) analar ethanol) followed by 5ml of 80% (v/v) analar ethanol.

The resin was dried by centrifugation for 10mins at 2500rpm at 4°C in a Beckman TJ6 benchtop centrifuge. The DNA was eluted by addition of 1.5ml of sterile H_2O, preheated to 65-70°C, waiting 1min followed by centrifugation for 10mins at 2500rpm at 4°C in a Beckman TJ6 benchtop centrifuge. DNA was then ethanol precipitated as described in **section 2. 19.**

2. 19. Ethanol precipitation of DNA.

DNA was precipitated from solution by addition of 0.1 volumes of 3M sodium acetate pH 7.0 and 2.5 volumes of analar absolute ethanol at -20°C. The tubes were then placed at -80°C for 30mins before being centrifuged at 13,000 rpm in an MSE microfuge for 10mins. The supernatant was discarded and the pellet washed with 1ml of 70% (v/v) analar ethanol at -20°C. The tubes were recentrifuged for 5mins and the supernatant discarded. The resulting pellet was dried under vacuum for 15mins then resuspended in an appropriate volume of sterile Milli Q H_2O.

DNA from the preparation of plasmid DNA described in **section 2. 18. 1.** was precipitated at room temperature without the addition of 3M sodium acetate.

DNA obtained from PCR amplification which was to be used in automated sequencing was precipitated on ice in the presence of 0.1 volumes of 3M sodium acetate, pH 5.2.

2. 20. Quantitation of DNA.

The amount of DNA in a given sample was quantitated by measuring the absorbance at 260nm (A_{260}) of a 1:200 dilution of the sample in sterile Milli Q H_2O. An A_{260} of 1 was assumed to be equal to 50μg/ml of double stranded DNA or 20μg/ml of single stranded oligonucleotide. The purity of the DNA was assessed by measuring the A_{280} in parallel and calculating the A_{260}:A_{280} ratio. A ratio of approximately 1.8 was considered to be sufficiently pure for use otherwise a phenol:chloroform extraction was carried out as described in **section 2. 21.**

2. 21. Phenol:chloroform extraction of DNA.

DNA was brought to a volume of 300μl with sterile Milli Q H_2O and 0.5 volumes of both Aquaphenol, pH7.3, and chloroform:isoamyl alcohol (24:1) was added. The suspension was vortexed for 15secs and centrifuged for 2mins at 13,000rpm in an MSE microcentrifuge. The upper phase was transferred to a fresh tube and extracted again with 0.5 volumes each of aquaphenol and chloroform:isoamyl alcohol and centrifuged as before. The resulting upper phase was extracted once with an equal volume of chloroform:isoamyl alcohol and again centrifuged. The upper phase was

ethanol precipitated as described in **section 2. 20.** and the DNA stored at -20°C until required.

2. 22. Digestion of plasmid DNA with restriction endonucleases.

Plasmid DNA, generally 1μg, was digested in a volume of 10μl. The buffer used depended on the restriction enzyme used and was supplied with the enzyme as a 10x stock, thus 1μl was used per 10μl digestion. Digestion was carried out with 4-10 units of enzyme, depending on the concentration of enzyme supplied. One unit was defined as the amount of enzyme required to digest 1μg of supercoiled dsDNA in one hour at 37°C. The volume was made up to 10μl with sterile Milli Q H_2O and the digests incubated at 37°C for a minimum of 1hr, although digestion for longer was not detrimental and was considered prudent to ensure complete digestion. The composition of the buffers used is indicated in **table 2. 2. a.** and the enzymes used in these buffers are indicated in **table 2. 2. b.**

2. 23. Separation of digested plasmid DNA by electrophoresis.

Once digestion was completed, 2μl of loading buffer (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue) was added to each tube and the samples electrophoresed in an agarose gel. For fragments of >500bp and <6kbp, electrophoresis was on a 0.8% (w/v) agarose gel, while for fragments of <500bp, the percentage was increased to 1.2% (w/v) agarose. The gel was prepared by melting an appropriate weight of agarose in 25ml of 1xTAE buffer

Table 2. 2. Restriction enzyme buffer compositions and use by various restriction enzymes.

Restriction endonucleases vary in their buffer requirements and so a panel of buffers in supplied commercially to allow the most efficient digestion of DNA by various enzymes. Panel A gives the composition of the buffers required by the enzymes used during this study while panel B lists the buffers used by these enzymes

Table 2. 2.

A.

Buffer	pH (at 37°C)	Tris HCl (mM)	$MgCl_2$ (mM)	NaCl (mM)	MgOAc (mM)	DTT (mM)
A	7.5	6	6	6	-	1
B	7.5	6	6	50	-	1
C	7.9	10	10	50	-	1
D	7.9	6	6	150	-	1
J	7.5	6	7	50	-	1
M	7.8	25§	-	100*	10	1
NEB 4	7.9†	20§	-	50*	10	1

b.

Buffer	Enzymes	Buffer	Enzymes
A	Apa I	J	Sac I Sma I
B	Nhe I	M	Sac I Nhe I¶
C	Cla I Xho I‡	NEB 4	Bsa HI
D	Xho I		

§ Tris Acetate * Potassium Acetate † at 25°C

¶ Sac I and Nhe I both exhibit full activity in buffer M. This buffer was used only in double digests using both these enzymes.

‡ Xho I exhibits 75-100% of its full normal activity in buffer C as opposed to its recommended Buffer, buffer D. Buffer C was only used in double digests with Cla I.

(40mM Tris acetate, 1mM EDTA, pH 8.0) in a 700W microwave oven (Sanyo) for approximately 90secs. The solution was allowed to cool until it was hand hot and ethidium bromide was added to a final concentration of 2.5μg/ml. The solution was poured into the electrophoresis mould containing a comb with an appropriate number of teeth (5, 8 or 14) and any air bubbles removed with a yellow pipette tip. Once set, the gel was submerged in 1xTAE buffer and the samples loaded. To allow estimation of the fragment sizes, the samples were flanked by a 1kb DNA ladder.

Electrophoresis was carried out, towards the anode, at 75-100 mA at room temperature until the bromophenol blue dye front was 0.75 of the way along the gel. The fragments were visualised under UV light and photographed.

Occasionally, the fragments were to be purified from the agarose, in which case low melting point agarose of an appropriate final concentration was used. The samples were electrophoresed at 50mA at 4°C to prevent melting of the gel. Following photography, the fragment of interest was excised from the gel using a clean scalpel blade and placed in a sterile microcentrifuge tube. The gel was photographed again to unsure complete excision and that no carry over of unwanted DNA had taken place.

2. 24. Purification of DNA from agarose gels.

Following electrophoresis and excision of the desired fragment as described in **section 2. 22.**, the agarose containing the fragment was melted in a heating block (Techne) at 70°C for 5mins. Immediately, 1ml of Promega

PCR purification resin was added and the contents vortex mixed for 30secs. The resin was put into a Promega mini column attached to a 2ml syringe barrel and the slurry pushed through the column. The resin was washed with 80% (v/v) isopropanol and the mini column dried by centrifugation and the DNA eluted with preheated sterile Milli Q H_2O as described in **section 2. 18. 2.** The purified DNA was stored at -20°C until required.

2. 25. Phosphatase treatment of DNA fragments.

In some of the subcloning experiments (see **Chapter 5**), DNA fragments containing an antibiotic resistance marker could theoretically self ligate, thus causing false positive transformants later. To prevent this, such fragments were treated with CIAP to remove the 5' phosphate group required by DNA ligase. CIAP was supplied with buffer as a 10x stock, so 0.1 final vol of buffer was added to the linearised DNA fragment obtained in **sections 2. 22 . and 2. 23.** 0.5units of CIAP was added and the volume made up to that required with sterile Milli Q H_2O. The final 1x buffer concentration was 50mM Tris HCl, pH9.0, 1mM $MgCl_2$, 100μM $ZnCl_2$, 1mM spermidine. One unit of CIAP catalysed the hydrolysis of 1μmole of 4-nitrophenyl phosphate per minute at 37°C in diethanolamine buffer, pH9.8.

The reaction was carried out at 37°C for 30mins at which point SDS and EDTA were added to final concentrations of 0.5% (w/v) and 5mM respectively. The reactions were heated to 75°C for 10mins before being cooled to room temperature. As CIAP is an extremely hardy enzyme, not all the enzyme may have been inactivated by this treatment, so subsequently, a phenol:chloroform:isoamyl alcohol extraction was carried out as described in

section 2. 18. 1. followed by ethanol precipitation as described in **section 2. 19.**

2. 26. Filling in of 5' overhangs to create blunt ends.

The large fragment of mammalian DNA polymerase I (Klenow fragment) can be used to fill in a 5' overhang of digested DNA to create a blunt end. DNA obtained from **sections 2. 21., 2. 22** and **2. 23.** was treated as recommended by the supplier. Buffer, prepared as a 5x stock (250mM Tris HCl, pH 7.2, 50mM Mg SO_4, 500μM DTT) was added to the DNA to the required final concentration, 2.5units of Klenow polymerase, and sterile Milli Q H_2O were added to the required final volume. One unit of enzyme was defined as the amount required to catalyse the incorporation of 10nmoles of deoxyribonucleotides into TCA insoluble form at 37°C under the following conditions: 67mM potassium phosphate buffer, pH7.5, 6.7mM $MgCl_2$, 1mMβ-mercaptoethanol, 133μM activated calf thymus DNA, 33μM dATP, 33μM ^{3}H-dTTP and enzyme.

The reaction was carried out at 37°C for 30mins before being terminated by heating to 75°C for 10mins. A phenol:chloroform:isoamyl alcohol extraction was then carried out as described in **section 2. 18. 1.**

2. 27. Ligation of DNA fragments.

The desired fragments of plasmid vector and insert were ligated together to produce recombinant plasmids ready for further subcloning or

mutagenesis. T4 DNA ligase was used as this has the ability to catalyse the ligation of blunt ended DNA, something *E.coli* DNA ligase cannot. Three reactions were generally set up, varying the molar ratios of vector:insert DNA. Normally 1:1, 1:5 and 1: 10 ratios were considered. The required volumes of vector and insert DNA were added to sterile microcentrifuge tubes and the required volume of 10x ligase buffer added. Hexamine cobalt chloride was added at a final concentration of 1μM. 1.5 Weiss units (for sticky ended ligations) or 3 Weiss units (for blunt ended ligations) was added and the volume made up with sterile Milli Q H_2O. Ligation was carried out overnight at 4°C and transformation carried out as described in **section 2. 16. 1.**

Following transformation, the *E. coli* were grown up and plasmid DNA prepared as described in **section 2. 18. 1.**, the presence of the insert was assessed by restriction enzyme analyses as described in **section 2. 21.**

2. 28. Phosphorylation of oligonucleotides.

500μl of deprotected oligonucleotides, supplied in aqueous NH_4OH, were dried under vacuum in an RC 10.22 centrivap refrigerated solvent trap (Jouan) for 3hrs. The pellet was resuspended in 20μl of sterile Milli Q H_2O and dried under vacuum as described in **section 2. 20.** and resuspended again in 20μl of sterile Milli Q H_2O. 5μl of this was phosphorylated in the presence of 16 units of T4 polynucleotide kinase in a buffer consisting of 70mM Tris HCl, pH 7.6, 10mM $MgCl_2$, 5mM DTT supplemented with 1mM ATP. Phosphorylation was carried out at 37°C for 2hrs after which time the reaction was terminated by heating to 75°C for

10mins followed by phenol chloroform extraction of the phosphorylated oligonucleotide as described in **section 2. 22.**

2. 29. Site-directed mutagenesis by PCR.

25ng of DNA was added to a tube containing 250μM dNTP and 25pmoles each of sense and antisense oligonucleotides (see **Chapter 5**). The buffer consisted of a final concentration of 20mM Tris HCl, pH 8.2, 10mM KCl, 6mM $(NH_4)_2SO_4$, 2mM $MgCl_2$, 0.1% Triton X-100, 10μg/ml BSA. The volume was made up to 50μl with sterile H_2O and the samples overlayed with light mineral oil. The tubes were preheated to 94°C for 10mins before 5 units of *Pfu* DNA polymerase were added. The PCR reaction was carried out on a Hybaid Omnigene temperature cycler. The cycles consisted of 94°C for 42secs (denaturation), annealing for 60secs (see footnote§) , and 72°C for 10mins (extension), for 30cycles. 5μl of the amplified DNA was then analysed on an 0.8% agarose gel. The oligonucleotides used are indicated in **table 5.1.**

The desired fragment of DNA was excised from an 0.8% low melting point agarose gel, purified, ligated and transformed into JM109 *E.coli* as described in **sections 2. 25, 2. 28** and **2. 19. 2.** To prevent excessive sequencing, a small fragment spanning the mutated region was subcloned into wild type vector.

§ The annealing temperature was calculated to be 5°C lower than the melting temperature of the oligonucleotides.

2. 30. Sequencing of plasmid DNA.

2. 30. 1. PCR sequencing of double stranded DNA.

Sequencing was carried out by the dideoxy method using the Applied Biosystems Amplitaq Dyedoexy terminator cycle sequencing kit following the manufacturers instructions. 1μg of double stranded template DNA and 3.2pmol of primer were added to 500μl microcentrifuge tubes containing 9.5μl of reaction mix (4μl of 5x TACS buffer, 1μl of dNTP's, 1μl each of Dyedeoxy A, Dyedeoxy T, Dyedeoxy C and Dyedeoxy G terminators and 0.5μl of Amplitaq DNA polymerase). The volume was made up to 20μl with sterile Milli Q H_2O and one drop of light mineral oil was overlaid onto the reactions. The DNA was amplified on a Hybaid Omnigene temperature cycler, by cycling, for 30 cycles, at 94°C (denaturation) for 30secs, 50°C (annealing) for 50secs and 70°C (extension) for 4mins.

2. 30. 2. Extraction of amplified sequencing DNA.

Following cycling reactions, the amplified DNA was extracted by adding 90μl of sterile Milli Q H_2O to the lower aqueous phase and transferring 105μl of this to a fresh tube. The DNA was separated from the terminators by extracting twice with 100μl of Applied Biosystems phenol:water:chloroform reagent, first removing 105μl of the upper aqueous phase and second 93μl of the aqueous phase. The DNA was precipitated with analar absolute ethanol as described in **section 2. 19.** and the resulting dry

pellet either stored at -20°C until required or resuspended in formamide and run on the Applied Biosystems 373A DNA sequencer within a few hours.

2. 30. 3. Polyacrylamide gel electrophoresis of sequencing DNA.

Sequencing DNA was analysed on 0.5mm polyacrylamide slab gels using Sequagel 6. The Applied Biosystems gels plates were washed twice each with Alconox detergent, sterile Milli Q H_2O and analar ethanol and the spacers (0.5mm) placed between the two. 60ml of Sequagel 6 acrylamide solution was mixed with 15ml of buffer solution and the whole polymerised with 600μl of 10% (w/v) ammonium persulphate. This was quickly added to the plates and any bubbles removed with a Promega bubble getter. The gel was allowed to cure for at least 2hrs before being scanned and pre-run, in 1xTBE buffer (90mM Tris borate, 2mM EDTA, pH8.3) , in the Applied Biosystems 373A automated DNA sequencer according to the manufacturers instructions. Immediately prior to loading, the samples in formamide were heated to 90°C for 2mins and subsequently loaded in alternating fashion as described by the manufacturer. The gel was run overnight at 1200V and the data collected and analysed on an Apple Macintosh IIci microcomputer.

Chapter 3.

Separation of closely related G protein α subunits by SDS-PAGE.

Chapter 3.

Separation of closely related G protein α subunits by SDS-PAGE.

3. 1. Introduction.

The high degree of amino acid sequence homology between G protein α subunits has led to many difficulties in studying these proteins in isolation. Initial studies involving the use of an exotoxin from *Bordetella pertussis*, the causative agent of whooping cough, simplified the study of G proteins, as it could specifically modify members of the G_i family of α subunit proteins [Katada & Ui, 1982a; Katada & Ui, 1982b; Van Dop, *et al.*, 1984; Manning *et al.*, 1984; Sternweis & Robishaw, 1984; Neer *et al.*, 1984; Milligan & Klee, 1985], adding ADP-ribose from NAD^+ to a cysteine residue near their C terminus [West *et al.*, 1985]. These α subunits have very similar Mr values and the degree of separation achieved under standard 10% SDS-PAGE is small; indeed $G_{i1}\alpha$, $G_{i2}\alpha$ and $G_{i3}\alpha$ all run as an apparent single protein species under these conditions. SDS-PAGE conditions do exist to allow separation of these α subunits from each other and $G_o\alpha$. Here 12.5% acrylamide is employed in a matrix with a lower amount of N,N'-methylene bisacrylamide (0.0625%). Under these conditions $G_{i1}\alpha$ and $G_{i3}\alpha$ run as a single band, $G_{i2}\alpha$ migrates slightly more rapidly through the matrix, while $G_o\alpha$ migrates fastest of all.

This approach of using pertussis toxin as a label for α subunits has one drawback; only some α subunits are targets for its enzymatic action.

Only members of the G_i subfamily of α subunits, with the exception of $G_z\alpha$, are substrates. All other α subunits are insensitive to its actions.

The next advance in studying G protein α subunits was the advent of antisera as specific probes for the various components. Initial studies using antisera targeted against purified proteins were less successful than subsequent studies employing antisera raised against short peptides derived from the primary amino acid sequences of the subunits [Milligan, 1990]. These sequences were made available from cDNA clones isolated from a number of sources.

These antisera were, and still are, very effective in detecting specific subunits. However these antisera are not infallible. Cross reaction between antisera derived against a sequence from one α subunit which also recognises the α subunit of a closely related α subunit has sometimes been responsible for a certain ambiguity in interpreting results. For example the C-terminal decapeptide of $G_{i3}\alpha$ was employed as a target antigen but the resulting antiserum also recognised the α subunit of $G_o\alpha$, possibly due to the presence of a tyrosine residue at the extreme C terminus [Milligan, 1990]. This problem can be overcome by using the 12.5% acrylamide/0.0625% bisacrylamide gel conditions mentioned above. These conditions however cannot separate $G_q\alpha$ from $G_{11}\alpha$, two highly homologous α subunits of the G_q family which are both thought to couple to the phosphoinositidase C catalysed hydrolysis of phosphatidylinositol-4,5-bisphosphate. The C-terminal decapeptides of these α subunits are identical and the antisera raised against the C terminal decapeptide of $G_q\alpha$ recognises $G_{11}\alpha$ equally [Mitchell *et al.*, 1991]. Attempts to raise antisera to specific regions of these polypeptides in

our laboratory has met with mixed success, as have efforts to raise antisera to specific splice variants of $G_o\alpha$.

We have therefore attempted to overcome this problem by developing SDS-PAGE conditions to separate $G_q\alpha$ from $G_{11}\alpha$ and to separate splice variants of $G_o\alpha$ in order that we could study them using the antisera available. To do this we have modified the method of Scherer *et al.*, [1987] and Codina *et al.*, [1991]. These workers employed a linear 4M-8M urea gradient incorporated into the acrylamide matrix of the gel. We have modified this to use the 12.5% acrylamide/0.0625% bisacrylamide conditions mentioned above. In this chapter we set out to demonstrate the use of these SDS-PAGE conditions in the analysis of $G_q\alpha$, $G_{11}\alpha$ and $G_o\alpha$.

3. 2. Results.

3. 2. 1. Separation of pertussis toxin substrates on urea containing polyacrylamide gels.

35μg of rat brain cortex membranes, prepared as described in **section 2. 3. 1.** were electrophoresed on an polyacrylamide gel containing a linear 4M-8M urea gradient in the 12.5% acrylamide/0.0625% bisacrylamide gel matrix, as prepared in **section 2. 6. 2.** These gels were transferred to nitrocellulose and probed with antipeptide antisera designed to recognise $G_{i1}\alpha$ and $G_{i2}\alpha$ (SG1), $G_o\alpha$ (IM1), $G_{o1}\alpha$ (O1A) and $G_{o2}\alpha$ (AS201). Immunoblots were developed as described in **section 2. 8. 3.** and are displayed in **figure 3. 1. a.**

Antiserum SG1 recognised two immunoreactive bands. Since expression of $G_t\alpha$ is restricted to the retina, these polypeptides must represent $G_{i1}\alpha$ and $G_{i2}\alpha$. As $G_{i1}\alpha$ has a higher molecular weight, it is likely that the slower migrating band represents $G_{i1}\alpha$ and the more rapidly migrating band is $G_{i2}\alpha$. Indeed when rat brain cortex membranes were electrophoresed under the same conditions with plasma membranes of the NG108-15 neuroblastoma x glioma hybrid cell line, which are known to lack immunologically detectable $G_{i1}\alpha$ [McKenzie & Milligan, 1990a], the single SG1 reactive band in these membranes migrated with the more rapidly migrating band in rat brain cortex membranes (**figure 3. 1. b.**) indicating that this was indeed $G_{i2}\alpha$.

In **figure 3. 1. a. and c.** antiserum IM1 recognised three polypeptides. As the antiserum directed against the $G_{o2}\alpha$ splice variant

(antiserum AS201) recognised the most rapidly migrating band (**figure 3. 1. c.**), this must represent the $G_{o2}\alpha$ splice variant. The slowest migrating band was assumed to represent the $G_o*\alpha$ form of the protein described by Goldsmith *et al.* [1988] and Mullaney & Milligan [1990]. The broad centre band was thought to represent the $G_{o1}\alpha$ splice variant.

The antiserum raised to a peptide predicted from the cDNA sequence of $G_{o1}\alpha$ (antiserum O1A) to specifically recognise this splice variant [Hsu *et al.*, 1990; Strathmann, *et al.*, 1990] identified the broad $G_{o1}\alpha$ band and the slowly migrating $G_o*\alpha$ polypeptide [Goldsmith *et al*, 1988; Mullaney & Milligan, 1990], but not the most rapidly migrating polypeptide which represents $G_{o2}\alpha$. The O1A antiserum also produced a doublet from the $G_{o1}\alpha$ band. IM1, the other $G_o\alpha$ antiserum which was predicted not to distinguish between $G_o\alpha$ splice variants, could not resolve the $G_{o1}\alpha$ band into this doublet perhaps due to the intensity of the staining of the two bands causing an overlap. Indeed if only 15μg of rat brain membranes were electrophoresed under identical conditions and probed with antiserum IM1 (**figure 3. 1. d.**), a faint shadow could be seen under the $G_{o1}\alpha$ band. The nature of this doublet is unknown but proteolysis of the proteins can probably be excluded as there is no shadow under either of the bands corresponding to $G_o*\alpha$ or $G_{o2}\alpha$ and similar proteolysis of $G_{o1}\alpha$ and $G_{o2}\alpha$ would be expected as they are similar at both the extreme N and C termini.

Figure 3. 1. Separation of G_i family members on urea gradient SDS PAGE.

In panel A, 35μg of rat brain cortex membranes were subjected to urea gradient SDS-PAGE (lanes 2, 4 and 6, separated by prestained lactic dehydrogenase in lanes 1, 3 and 5) and probed with antisera IM1, O1A and SG1 (lanes 1-3, 3-5 and 5-6 respectively) at dilutions of 1:400, 1:200 and 1:1000 respectively.

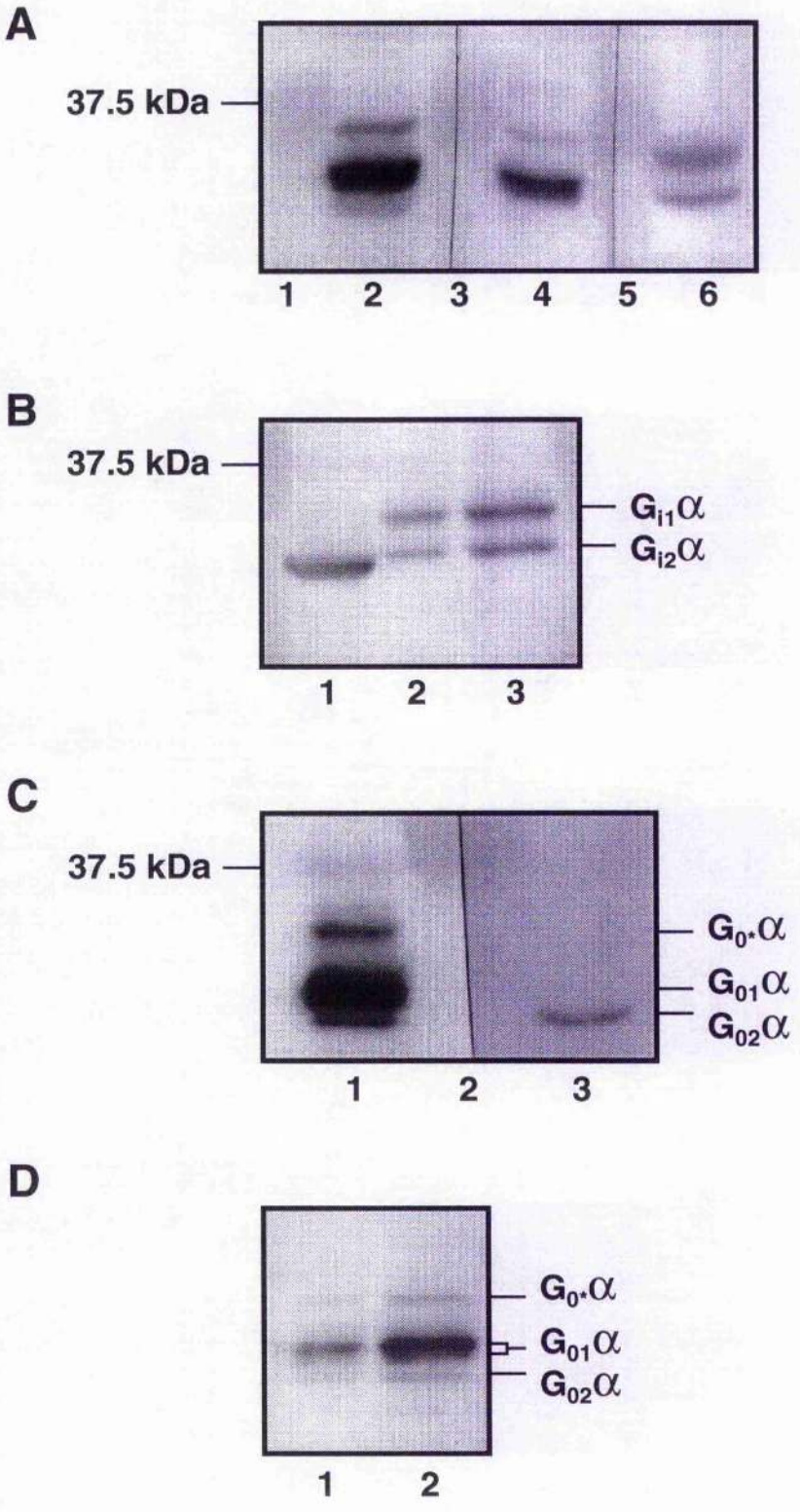
To determine which of the two SG1 reactive polypeptides represented $G_{i1}\alpha$ and which represented $G_{i2}\alpha$, 100μg of membranes from NG108-15 cells (panel B, lane 1) were electrophoresed under identical conditions with 15μg and 35μg of rat brain cortex (panel B., lanes 2 and 3 respectively). The resulting immunoblot was probed with SG1 at a dilution of 1:1000.

To determine which of the three IM1 reactive polypeptides represented $G_{o2}\alpha$, 35μg of rat brain cortex (panel C, lane 1) was electrophoresed with 200μg of rat brain cortex membranes (panel C, lane 3). The resulting immunoblot was probed with antiserum IM1 at a dilution of 1:400 and with antiserum AS201 at a dilution of 1:100 (lanes 1-2 and 2-3 respectively). Lane 2 contained prestained lactic dehydrogenase as a marker.

The presence of a $G_{o1}\alpha$ doublet was determined by electrophoresing 10μg (panel D, lane 1 and 30μg (panel D, lane 2) under urea gradient SDS-PAGE conditions and probing the resulting immunoblot with antiserum IM1 at a dilution of 1:400.

The mobility of the 37.5kDa marker is indicated and the identities of the polypeptides in panels B to D are shown. These immunoblots are representative examples of 3 separate experiments.

Figure 3.1


A
37.5 kDa
1 2 3 4 5 6
B
37.5 kDa
$G_{i1}\alpha$
$G_{i2}\alpha$
1 2 3
C
37.5 kDa
$G_{0*}\alpha$
$G_{01}\alpha$
$G_{02}\alpha$
1 2 3
D
$G_{0*}\alpha$
$G_{01}\alpha$
$G_{02}\alpha$
1 2

3. 2. 2. NG108-15 cell membranes contain only $G_{o1}\alpha$ and $G_{o2}\alpha$.

In **figure 3. 2.**, different amounts of rat brain cortex membranes and plasma membranes from NG108-15 cells were electrophoresed as described in **section 2. 6. 3.**, transferred to nitrocellulose and immunoblotted with antiserum IM1 as described in **section 2. 8.** It can be seen that while $G_o*\alpha$, $G_{o1}\alpha$ and $G_{o2}\alpha$ were present in rat brain cortex, as previously seen in **figure 3. 1.**, only $G_{o1}\alpha$ and $G_{o2}\alpha$ were detected in membranes of NG108-15 cells. This observation has previously been noted by Mullaney & Milligan [1990].

3. 2. 3. Differential expression of $G_o\alpha$ splice variants in rat brain regions.

Membranes of a series of rat brain regions were prepared and kindly donated by Dr. Christine M. Brown (Syntex Research Centre, Riccarton, Edinburgh, UK) and electrophoresed in urea gradient containing SDS-PAGE. As shown in **figure 3. 3. a.**, differential expression of $G_o\alpha$ splice variants was detectable in these rat brain regions. All regions contained detectable levels of $G_{o1}\alpha$ and $G_{o2}\alpha$ but the relative expression of these polypeptides varied between the regions. Some areas contained no detectable $G_o*\alpha$ immunoreactivity. Quantitation of a series of such immunoblots is displayed in **figures 3. 3. b., c.** and **d.** Expression of the $G_o*\alpha$ splice variant was undetectable in membranes prepared from olfactory bulb and the frontal cortex, indeed expression of total $G_o\alpha$ was reduced in these two regions compared to that observed in other regions. The expression of $G_o*\alpha$ was very

Figure 3. 2. NG108-15 cell membranes contain only $G_{o1}\alpha$ and $G_{o2}\alpha$.

30μg of rat brain cortex membranes (lane 1) were electrophoresed under urea gradient SDS-PAGE conditions with 100μg of NG108-15 cell membranes and the resulting immunoblot probed with antiserum IM1 at a dilution of 1:400. The identities of the immunoreactive polypeptides are displayed. This experiment is a representative example of 3 separate experiments.

Figure 3.2

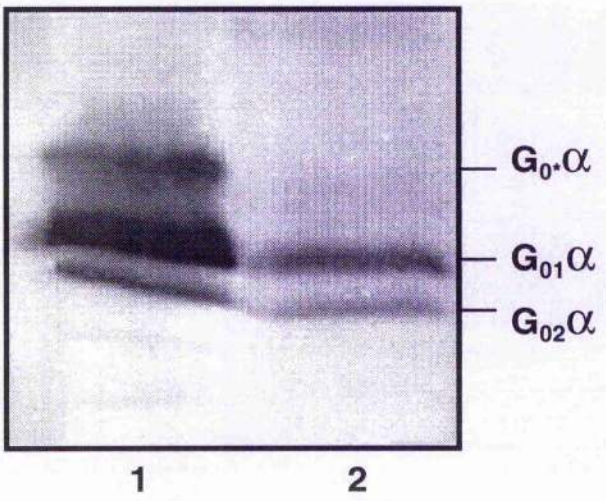


Figure 3. 3. Differential patterns of $G_0\alpha$ isoform expression in rat brain regions.

Panel A depicts a representative immunoblot of urea gradient SDS-PAGE analysis of 5μg of rat brain cortex membranes (lane 1) together with 5μg each of membranes from olfactory bulb (lane 2), frontal cortex (lane 3), parietal occipital cortex (lane 4), caudate putamen (lane 5), globus pallidus (lane 6), hippocampus (lane 7), thalamus (lane 8), hypothalamus (lane 9), pituitary (lane 10), optic chiasma (lane 11) and cerebellum (lane 12). Immunoblots were probed with antiserum 1M1 at a dilution of 1:400. The identities of the immunreactive polypeptides and the mobility of the 37.5 kDa marker are shown.

Panels B C and D show the quantitative analyses of 3 such experiments for G_o*α, $G_{o\ 1}\alpha$ and $G_{o\ 2}\alpha$ respectively The optical density of each immunoreactive polypeptide, corrected for amount of protein, was plotted against brain region. Results are shown as the mean ± SEM of these 3 experiments.

Figure 3.3

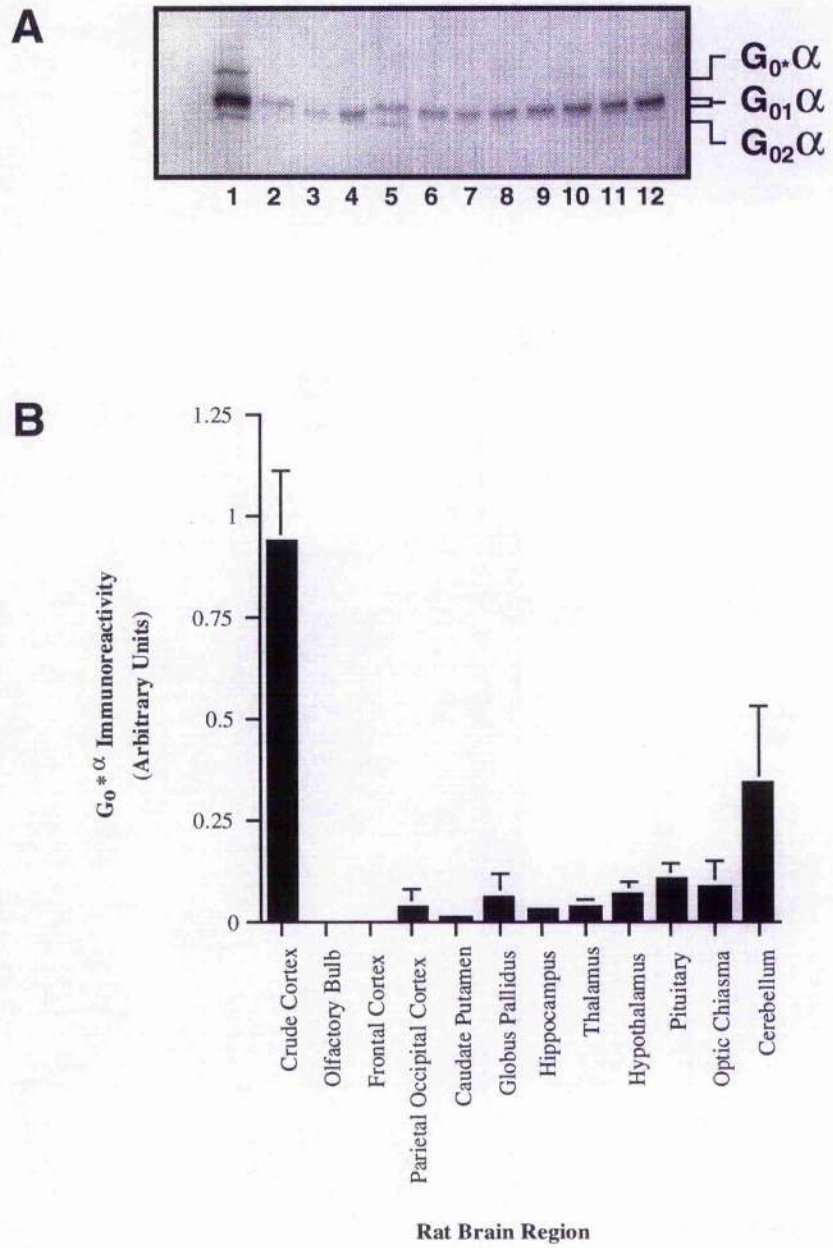
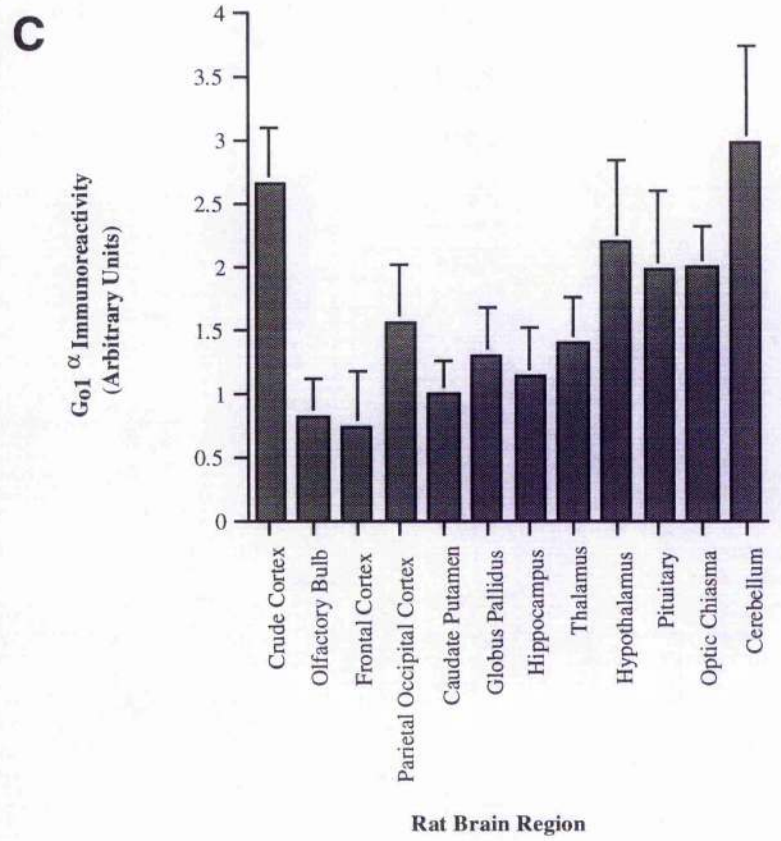
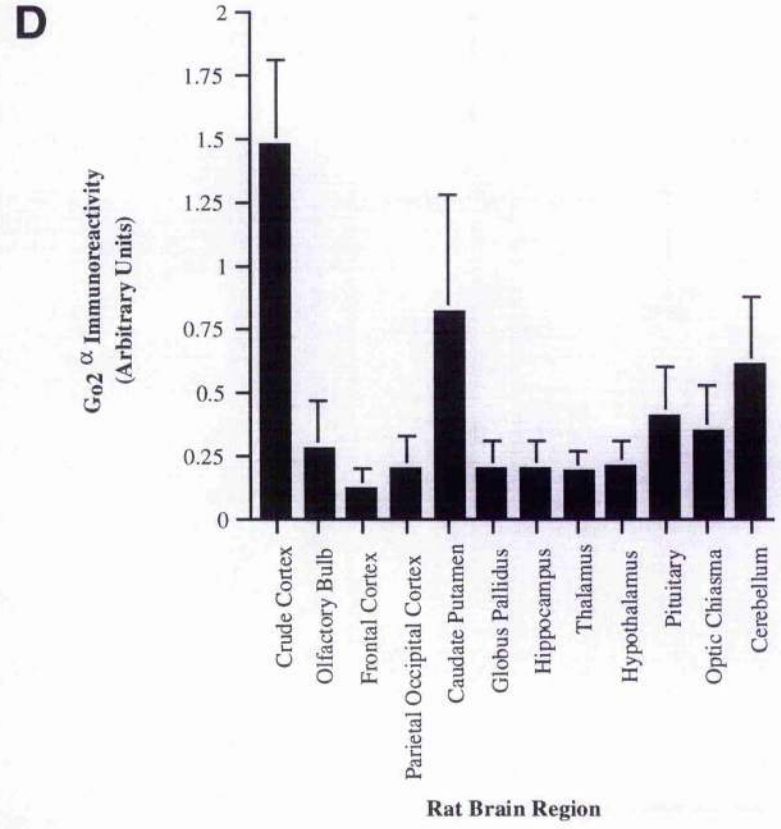

A
$G_{0*}\alpha$
$G_{01}\alpha$
$G_{02}\alpha$
1 2 3 4 5 6 7 8 9 10 11 12
B
Go * α Immunoreactivity
(Arbitrary Units)
1.25
1
0.75
0.5
0.25
0
Crude Cortex
Olfactory Bulb
Frontal Cortex
Parietal Occipital Cortex
Caudate Putamen
Globus Pallidus
Hippocampus
Thalamus
Hypothalamus
Pituitary
Optic Chiasma
Cerebellum
Rat Brain Region

Figure 3.3


C
Go1 α Immunoreactivity (Arbitrary Units)
4
3.5
3
2.5
2
1.5
1
0.5
0
Crude Cortex
Olfactory Bulb
Frontal Cortex
Parietal Occipital Cortex
Caudate Putamen
Globus Pallidus
Hippocampus
Thalamus
Hypothalamus
Pituitary
Optic Chiasma
Cerebellum
Rat Brain Region


D
Go2 α Immunoreactivity (Arbitrary Units)
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
Crude Cortex
Olfactory Bulb
Frontal Cortex
Parietal Occipital Cortex
Caudate Putamen
Globus Pallidus
Hippocampus
Thalamus
Hypothalamus
Pituitary
Optic Chiasma
Cerebellum
Rat Brain Region

low in most areas of the brain with the exception of crude cortex membranes and membranes from the cerebellum. $G_{o1}\alpha$ expression was relatively high in crude cortex, cerebellum, hypothalamus and parietal occipital cortex. The expression of $G_{o2}\alpha$ in olfactory bulb was higher than in some other regions, and $G_{o2}\alpha$ expression was increased relative to other variants in the caudate putamen. Overall, the relative expression of $G_o\alpha$ splice variants was $G_{o1}\alpha > G_{o2}\alpha > G_o{*}\alpha$.

3. 2. 5. Regulation of $G_o\alpha$ splice variant expression in NG108-15 cells.

Treatment of NG108-15 cells with agents which increase intracellular levels of cAMP such as nonhydrolysable cAMP analogues, for example dibutyryl cAMP, forskolin or agonists of adenylyl cyclase coupled receptors such as prostaglandin E_1 (PGE_1) acting at an IP prostanoid receptor, cause these cells to differentiate into neurone-like cells [Mullaney & Milligan, 1989]. This differentiation produces a characteristic morphological change in these cells [Mullaney & Milligan, 1989]. The cells lose their rounded shape and flatten out. Neurone-like processes appear which form synaptic connections with each other.

NG108-15 cells were differentiated with 10μM forskolin as described in **section 2. 2. 6.**, membrane fractions prepared and analysed on urea gradient containing SDS-PAGE and probed with antipeptide antisera against $G_{o2}\alpha$ and all $G_o\alpha$ splice variants. **Figure 3. 4. a.** showed how the expression of $G_o\alpha$ splice variants of changes following differentiation. An increase in $G_{o1}\alpha$ expression and a lesser or negligible increase in $G_{o2}\alpha$

expression was seen following forskolin treatment. These changes were quantified on a Bio Rad imaging densitometer and the results displayed in **figure 3. 4. b.** These indicated an approximately 200% increase in $G_{o1}\alpha$ expression and an approximately 150% increase in $G_{o2}\alpha$ expression.

3. 2. 6. Urea containing SDS-PAGE can separate $G_q\alpha$ from $G_{11}\alpha$.

The phosphoinositidase C linked G protein α subunits $G_q\alpha$ and $G_{11}\alpha$ share 88% homology at the amino acid level. They have 359 amino acids, as deduced from their cDNA sequences, and are both 42kDa in size as assessed by SDS-PAGE [Mitchell *et al.*, 1991]. This presents problems in designing antipeptide antisera with significant selectivity in discerning these two polypeptides. While an antiserum against their C termini has a high titre, it cannot distinguish between the two α subunits [Mitchell *et al.*, 1991]. Analysis of the primary amino acid sequence indicates few regions of the polypeptides which are significantly divergent to create more specific antisera. An attempt was therefore made to separate these two polypeptides by SDS-PAGE such that the antipeptide antiserum raised to their common C terminus would be a sufficient means of immunological detection.

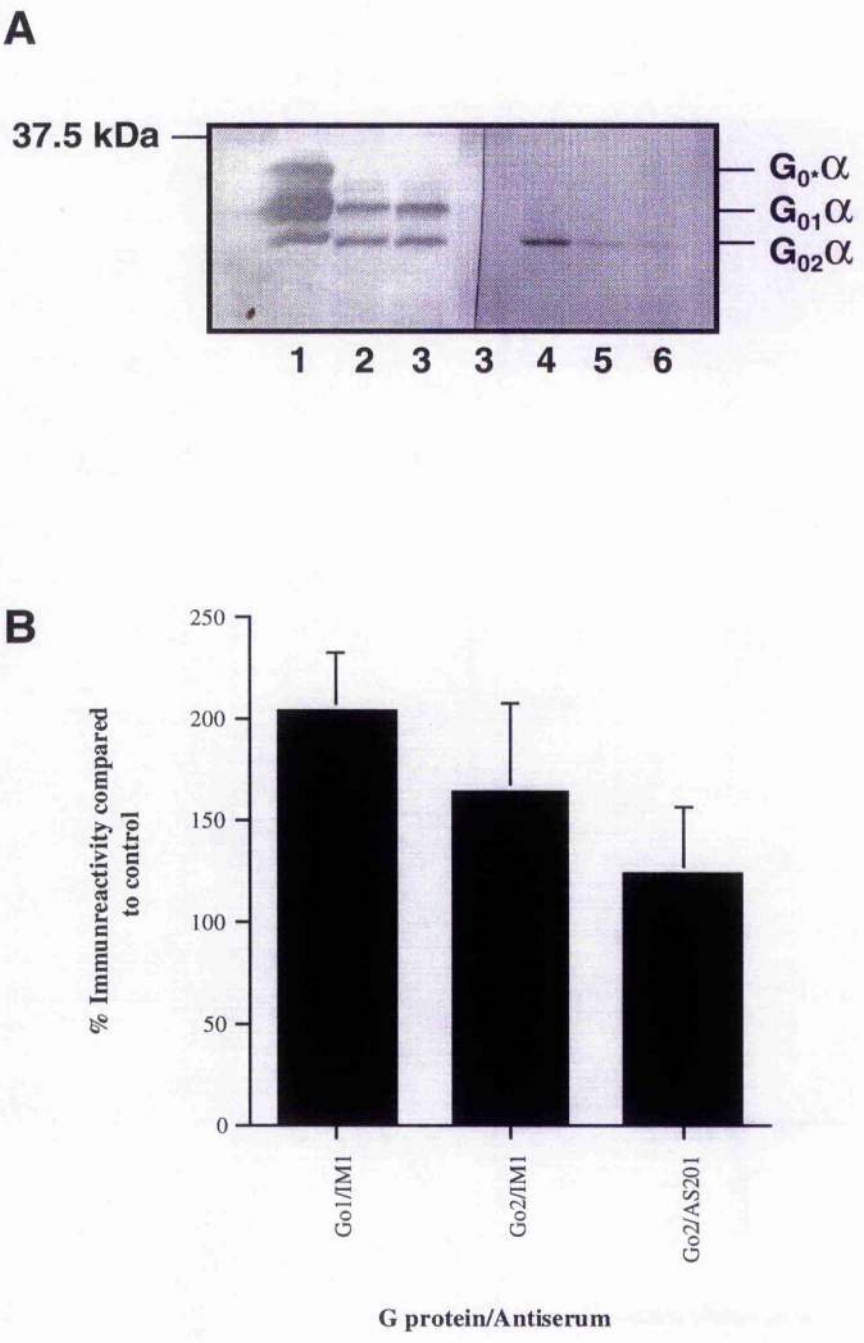
When membranes of rat brain cortex were electrophoresed under urea gradient SDS-PAGE conditions as before and resultant immunoblots probed with the antipeptide antiserum CQ2, raised against the common C terminus shared between $G_q\alpha$ and $G_{11}\alpha$, three immunoreactive bands were seen (**figure 3. 5. a.**).

Figure 3. 4. Regulation of $G_o\alpha$ splice variant expression in NG108-15 cells.

Panel A shows a representative immunoblot from rat brain cortex membranes (25μg in lane 1 and 150μg in lane 5), control NG108-15 cell membranes (50μg in lane 2 and 150μg in lane 6) and forskolin differentiated NG108-15 cell membranes (50μg in lane 3 and 150μg in lane 7) which had been analysed on a 4M-8M urea gradient polyacrylamide gel as before. Lane 4 contained prestained molecular weight markers. The resulting immunoblot was probed with antiserum IM1 at a dilution of 1:400 (lanes 1-4) or antiserum AS201 at a dilution of 1:100 (lanes 4-7).The position of the 37.5kDa marker is shown, as is the identities of the immunoreactive polypeptides.

Quantitation of this experiment is shown in panel B The percent increase in the optical density of differentiated NG108-15 cell membranes, compared to control cell membranes, for each immunoreactive band was plotted against the identity of the band and the antiserum used to detect it.

Figure 3.4


A
37.5 kDa
$G_{o}\alpha$
$G_{o1}\alpha$
$G_{o2}\alpha$
1 2 3 3 4 5 6
B
% Immunreactivity compared to control
250
200
150
100
50
0
Go1/IM1
Go2/IM1
Go2/AS201
G protein/Antiserum

If samples were instead immunoblotted with the $G_q\alpha$ selective antiserum IQB only one band is observed (**figure 3. 5. b.**), the fastest migrating polypeptide not being observed. This indicated that this fastest migrating polypeptide probably represented $G_{11}\alpha$ while the slowest migrating band represented $G_q\alpha$. The nature of the polypeptide with intermediate mobility is unknown. It does not represent proteolysis of the proteins as $G_{11}\alpha$ does not have a similar immunoreactive band beneath it and preparation of the samples in the presence of protease inhibitors does not prevent its detection [Milligan *et al.*, 1993].

3. 2. 7. Urea containing SDS-PAGE can discern between species variants of $G_{11}\alpha$.

Recently it has been demonstrated that a modified procedure for urea containing SDS-PAGE can not only separate $G_q\alpha$ from $G_{11}\alpha$ but can also discern between species variants of $G_{11}\alpha$ [Kim & Milligan, 1994].

This system was used to separate murine $G_{11}\alpha$ from primate $G_{11}\alpha$. **Figure 3. 6.** demonstrates that if mouse brain cortex membranes and plasma membranes from simian COS-1 cells were electrophoresed on a polyacrylamide gel consisting of 10% acrylamide/0.24% bisacrylamide gel matrix containing 6M urea as described in **section 2. 6. 3.**, murine $G_{11}\alpha$ migrated more rapidly through the matrix than its simian equivalent.

Figure 3. 5. Separation of $G_q\alpha$ and $G_{11}\alpha$ using urea gradient SDS-PAGE.

In panel A 40μg (lane 1) and 20μg (lane 2) of CHO cell membranes, kindly prepared by Dr. I Mullaney, Division of Biochemistry and Molecular Biology, IBLS, University of Glasgow, were electrophoresed on a 4M-8M urea gradient SDS-PAGE gel as before and the resulting immunoblot probed with antiserum CQ2 at a dilution of 1:1000.

In panel B 40 μg of CHO cell membranes were electrophoresed on a urea gradient SDS-PAGE gel as before and probed with either antiserum 1QB at a dilution of 1:200 (lane 1) or CQ2 at a dilution of 1:1000 (lane 2).

The identities of the immunoreactive bands are shown.

Figure 3.5

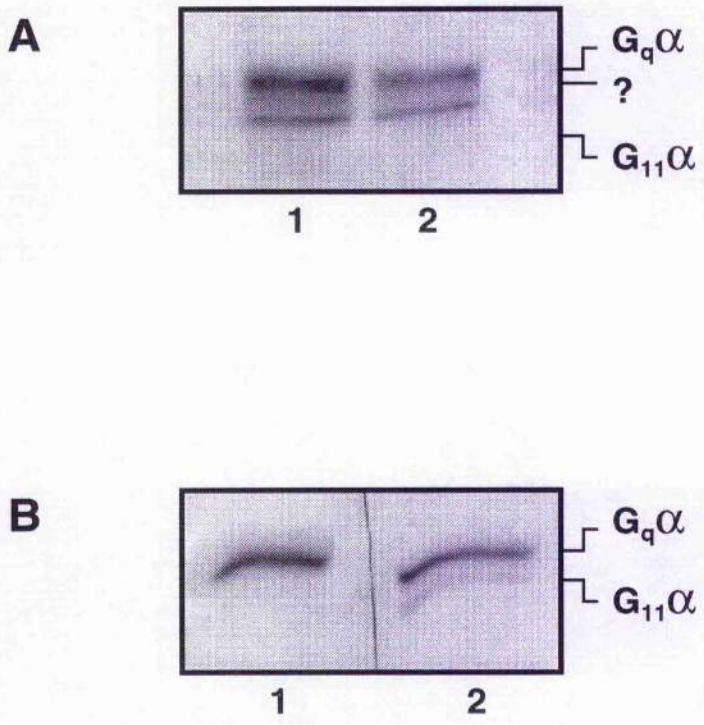
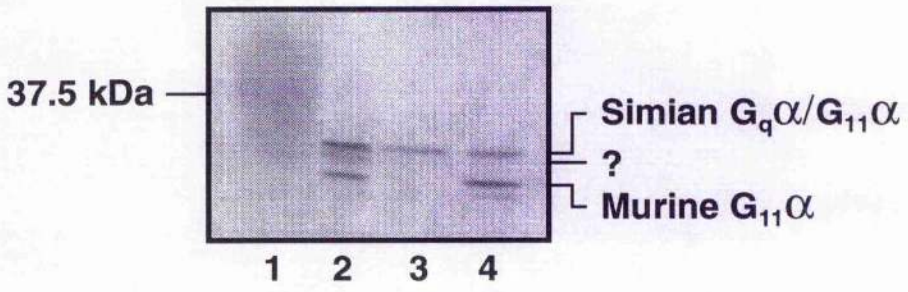


Figure 3. 6. Separation of species variants of $G_{11}\alpha$ on urea containing SDS-PAGE.

40μg of mouse brain membranes (lane 2), kindly prepared by Mr. G.-D. Kim, Division of Biochemistry and Molecular Biology, IBLS, University of Glasgow, were electrophoresed with membranes of untransfected COS-1 cell membranes (lane 3) or with membranes of COS-1 cells which had been transiently transfected with mouse $G_{11}\alpha$ in the mammalian expression vector pCMV (lane 4). Lane one contained prestained lactic dehydrogenase marker. The subsequent immonoblot was probed with antiserum CQ2 at a dilution of 1:1000. The mobility of the lactic dehydrogenase marker and the identity of the immunreactive polypeptides are indicated. The question mark indicates a polypeptide of unkown identity.

The band visible beneath murine G11a in lane 4 is thought to represent a truncated "read through" translation product.

Figure 3.6



3. 3. Discussion.

The high degree of similarity between G proteins has led to some difficulty in studying sections of the superfamily, especially if members are closely related as is the case for $G_q\alpha$ and $G_{11}\alpha$ or the gene responsible for their production undergoes alternate splicing to create more diversity such as $G_s\alpha$ and $G_o\alpha$.

Variants which arise from the splicing of the $G_s\alpha$ mRNA are relatively easily discerned as they vary considerably in size due to the addition of an entire exon (exon 3) in the long form of the protein. This addition represents 15 amino acids and approximately 1.5kDa [Kaziro, *et al.*, 1990]. This difference has yet to have a functional significance assigned to it, even though there is a different expression pattern of the splice variants in various cell types and tissues.

As demonstrated in this Chapter, urea gradient containing SDS-PAGE conditions can be employed to give considerable separation of the only other G protein α subunits known to be derived from a single gene and to undergo alternative splicing, $G_o\alpha$. In rat brain cortex membranes 3 forms of the protein can be discerned under these conditions and, similarly to $G_s\alpha$, the expression of these splice variants is under some form of tissue specific control as some variants are more highly expressed in some regions of the rat brain than others (**figure 3. 4. a.**).

This tissue specific control of expression can be modelled in the neuroblastoma x glioma hybrid cell line NG108-15. These cells express no

detectable G_o*α but do express considerable levels of the other two splice variants detectable under these SDS-PAGE conditions. These cells can be induced to differentiate into neurone-like cells upon chronic treatment with cAMP elevating agents such as the diterpene forskolin [Mullaney *et al.*, 1988; Mullaney & Milligan, 1989]. If one follows the expression of G_oα splice variants upon differentiation, we can see a concomitant increase in the expression of the G_{o1}α splice variant with a lesser change in the expression of the G_{o2}α splice variant. Similar results have been noted by Mullaney & Milligan [1990].

Complementary DNA molecules representing 2 splice variants of G_oα were first identified by Hsu *et al.*, [1990] and Strathmann *et al.*, [1990]. Previously, heterogeneity of G_oα had been demonstrated by SDS-PAGE and chromatographic analyses [Scherer *et al.*, 1987; Goldsmith *et al.*, 1988]. Four forms of G_oα have been purified from bovine brain by Mono Q column chromatography [Kobayashi *et al.*, 1989]. Analysis of the kinetic parameters of the GTPase activities of these four forms indicated differences in their K_m and V_{max} values [Inanobe *et al.*, 1990]. It is unknown which of these forms corresponds with the splice variants reported here and by others. Inanobe *et al.*, 1990 speculated that these different forms may represent a covalent modification of both of the G_{o1}α and G_{o2}α polypeptides, thus creating four proteins with distinct properties [Inanobe *et al.*, 1990]. Exactly where G_o*α fits into this scheme is unclear. A third isoform of G_oα, which reacts with a G_{o1}α directed antiserum has been identified in rat brain membranes analysed on urea containing SDS-PAGE [Spicher *et al.*, 1992]. This form of G_oα is not G_o*α however and has been termed G_{o3}α [Nurnberg *et al.*, 1994]. Spicher *et al.* [1992] also postulated that post translational

modification of the three forms could give rise to six forms detected electrophoretically.

The functional significance of the alternate splicing of $G_o\alpha$ mRNA is a little clearer than that for the splice variants of $G_s\alpha$. Kleuss *et al.* [1991] employed antisense oligonucleotides specific for $G_{o1}\alpha$ and $G_{o2}\alpha$ in an attempt to assign specific functions to one or other of the splice variants expressed in the rat pituitary tumour cell line GH_3. Using this technique they determined that the $G_{o1}\alpha$ splice variant could couple to a muscarinic acetylcholine receptor but not to the somatostatin receptor while the converse was true of the $G_{o2}\alpha$ splice variant. As the $G_o\alpha$ mRNA is spliced near what will become the C terminal region of the proteins [Hsu *et al.*, 1990 and Strathmann *et al.*, 1990], this result is not surprising as the C terminal region is thought to be the receptor contact site.

The splice site is also close to the putative effector interaction site and so differences may exist in the coupling of the $G_o\alpha$ splice variants to effector systems. The classical effector to which G_o couples is the inhibition of opening of the N-type Ca^{2+} channel [Hescheler *et al.*, 1987]. It has been demonstrated, by microinjection of purified, activated $G_{o1}\alpha$ and $G_{o2}\alpha$ into neuronal somata of the pond snail *Helisoma trivolvis*, that only the $G_{o2}\alpha$ polypeptide is capable of inhibiting a pertussis toxin sensitive, voltage sensitive Ca^{2+} current. The activated $G_{o1}\alpha$ protein could not inhibit this Ca^{2+} current [Man-Son-Hing *et al.*, 1992]. This again may reflect the different amino acid composition at the splice site such that this area is not recognised by the N-type channel, giving an insight into the effector activating regions of the $G_o\alpha$ protein. However, it has been recently demonstrated that $G_{o1}\alpha$ is

indeed capable of inhibiting Ca^{2+} conductance in transfected cells. This may reflect a more physiological environment for the α subunit [Charpentier *et al.*, 1993].

Developmental regulation of the expression of $G_{o1}\alpha$ and $G_{o2}\alpha$ splice variants in neonatal rat brain, similarly to those reported for NG108-15 cells [this study and Mullaney & Milligan, 1990], has been demonstrated [Rouot *et al.*, 1991]. Over the time course studied (from the 15th day of gestation to 30th day *post partum*), the expression of the $G_{o1}\alpha$ variant increased, while the expression of the $G_{o2}\alpha$ variant decreased. Taken together these pieces of information indicate another level of regulation of the signalling pathways involved in neuronal Ca^{2+} signalling. This differential regulation of expression of $G_o\alpha$ splice variants may stem from a distinct metabolism of the two protein species. It has been previously noted that the rates of degradation of two $G_o\alpha$ splice variants changes upon differentiation of neuroblastoma cells [Brabet *et al.*, 1991].

Overall, there seems to be different levels of the $G_o\alpha$ splice variants in different tissues and cell types. As described here, areas of the rat brain have varying levels of the splice variants. In addition, various cultured cells vary in expression of $G_o\alpha$ isoforms [Asano *et al.*, 1992].

It would be interesting to see how the changes in expression of the $G_o\alpha$ splice variants relate to the development of the brain in general. Does expression of other parts of the G_o-linked signalling cascade change in response to developmental stimuli?

The α subunits of the phosphoinositidase C linked G proteins G_q and G_{11} share 88% identity at the amino acid level and possess few linear regions useful for the production of selective antisera. A $G_q\alpha$ selective antipeptide antisera, IQB, which does not identify $G_{11}\alpha$ has been generated, but one which can selectively identify $G_{11}\alpha$ has not been generated in this laboratory, even if the peptide sequence used was identical to that successfully employed by other groups [G. Milligan, personal communication]. We therefore attempted to separate $G_q\alpha$ from $G_{11}\alpha$ such that we could use the non selective antiserum CQ2, which was raised against the predicted C terminal decapeptide of $G_q\alpha$, to detect $G_q\alpha$ and $G_{11}\alpha$ independently.

When rat brain cortex membranes were electrophoresed on urea gradient containing SDS-PAGE, three immunoreactive polypeptides were observed. The fastest migrating polypeptide is $G_{11}\alpha$, as the polypeptide is not recognised by antiserum IQB, which was raised against an internal sequence of $G_q\alpha$ specific for that α subunit. The slowest migrating polypeptide is therefore $G_q\alpha$. Also purified $G_q\alpha$ migrated with the slower moving species while purified $G_{11}\alpha$ migrated with the fastest migrating species [Milligan *et al.*, 1993]. The nature of the polypeptide with the intermediate mobility is unclear. It does not represent a proteolytic clip of $G_q\alpha$ as preparation of membranes in the presence protease inhibitors did not eliminate its presence [Milligan *et al.*, 1993]. It may represent $G_{14}\alpha$, another member of the G_q family of α subunits, as the antisera employed in this study could theoretically cross react with $G_{14}\alpha$.

Another group has also recently reported a method for the separation of $G_q\alpha$ and $G_{11}\alpha$ [Blank *et al.*, 1991]. They employed an imidazole

buffer system in a 13% acrylamide gel to achieve this separation. In the hands of our group, this method achieves a lesser degree of separation and is not as reproducible as the urea gradient method employed here [Milligan *et al.*, 1993].

Recent work from our laboratory has demonstrated that $G_{11}\alpha$ from different species can be separated from each other and from $G_q\alpha$ [Kim & Milligan, 1994]. This method employed a 10% acrylamide matrix into which 6M urea had been incorporated. It is technically less demanding than the urea gradient method previously employed and allows examination of exogenous $G_{11}\alpha$ which has been transfected into a suitable cell type. This is an very powerful technique given that the species of $G_{11}\alpha$ shown in **figure 3. 6.**, namely mouse (rodent) and simian (primate) have so few amino acid differences that production of selective antisera is impossible. Indeed murine and human (also primate) $G_{11}\alpha$ differ in only 9 amino acids over their entire lengths.

Other groups have employed another technique to examine members of the G_q family of α subunits. Bourne and co-workers [Wedegaertner, *et al.*, 1993], and Johnson and co-workers [Qian *et al.*, 1993] have created $G_q\alpha$ cDNAs into which a peptide tag sequence has been inserted. This sequence encodes a stretch of amino acids recognised by a commercially available monoclonal antibody to the haemagglutinin protein (HA) of the human influenza virus. As this sequence is not present in the wild type polypeptide, detection of transfected mutated $G_q\alpha$ with the anti-HA antibody will exclude any wild type $G_q\alpha$ or $G_{11}\alpha$. Although this technique has apparently no effect on the function of a protein bearing this tag [Levis &

Bourne, 1992; Wedegaertner, *et al.*, 1993; Qian *et al.*, 1993], the possibility still exists that insertion of the HA-tag sequence may affect some, as yet undefined, capacity of the polypeptide. The use of urea gels to separate different species variants of $G_{11}\alpha$ allows us to compare members of the G_q family without the need to mutate the wild type protein.

Upon examination of **figure 3. 1. a.**, it can be seen that the pertussis toxin substrates do not migrate according to the sizes reported for them by conventional SDS-PAGE or by analyses of their reported amino acid sequences. While $G_{i1}\alpha$ and $G_{i2}\alpha$, which are both approximately 40kDa in size, migrate according to their relative sizes, i.e. $G_{i2}\alpha$ migrates faster than the larger $G_{i1}\alpha$, $G_{o1}\alpha$ and $G_{o2}\alpha$ co-migrate with them despite being only approximately 39kDa in size. Indeed the prestained lactic dehydrogenase used to follow their mobility through the gel also migrates with G protein α subunits despite being only approximately 37kDa. Urea containing gels therefore do not separate protein purely on the basis of their sizes. The function of urea in such systems is to abolish hydrophobic interactions of polypeptides. Thus although there are so few amino acid differences between $G_o\alpha$ splice variants and between $G_q\alpha$ and $G_{11}\alpha$, these differences alter the level of hydrophobicity of the polypeptides sufficiently to allow their separation on such urea containing gels.

The use of urea gels to separate G proteins with identical electrophoretic characteristics is a very useful tool in addressing several questions. It allows the specific identification of $G_o\alpha$ isoforms and the concurrent measurement of $G_q\alpha$ and $G_{11}\alpha$. More importantly for this study, the ability to simultaneously detect two species variants of $G_{11}\alpha$ allows us to

transfect the cDNA encoding murine $G_{11}\alpha$ 1 into simian COS-1 cells. We can therefore go on to examine mutants of the protein in isolation.

Chapter 4.

The effect of chemical and mutational depalmitoylation on $G_o1\alpha$ function.

Chapter 4

The effect of chemical and mutational depalmitoylation on $G_{o1}\alpha$ function.

4. 1. Introduction.

The role of lipidation of intracellular proteins has been a contested point over the past few years. Many cellular and viral proteins have been shown to be modified by the addition of the 14 carbon fatty acid myristic acid (tetradecanoic acid). The role of this modification is controversial. Some myristoylated proteins are targeted to cell membranes, such as endothelial nitric oxide synthase, while myristate minus mutants are not [Busconi & Michel, 1993]. Some myristoylated proteins, such as the catalytic subunit of cAMP dependent protein kinase, are cytosolic [Carr *et al.*, 1982]. The free energy of myristic acid is barely sufficient to cause membrane attachment of a peptide [Peitzsch & McLaughlin, 1993]. Therefore the addition of myristic acid cannot *per se* lead to membrane association but the nature and environment of the modified polypeptide can also be important in governing what effect the modification has on membrane association.

Prenylation of proteins does have a definite effect on membrane association of the polypeptide. Mutational removal of the prenoid group precludes membrane association of members of the Ras and Rab families of small molecular weight G proteins and of the γ subunit of heterotrimeric G proteins [Casey *et al*, 1989; Hancock *et al.*, 1989; Kinsella & Maltese, 1991; Fukada *et al.*, 1990; Finegold *et al.*, 1990]. Indeed, if H-Ras is not prenylated,

it loses its transforming ability, so prenylation is a necessary modification for function of target proteins. However, prenylation by itself may not be sufficient to anchor the protein to the membrane. Another factor is also required. This may be the addition of the 16 carbon unsaturated fatty acid, palmitic acid (hexadecanoic acid) to a position near the site of prenylation as is the case for, H-Ras, N-ras and K-Ras(A) or the presence of a long stretch of basic residues near the prenylated cysteine residue, as in K-Ras(B) [Hancock *et al.*, 1990]. These together may then facilitate full membrane association.

Recent evidence has pointed to the importance of another type of acylation in the function of proteins involved in intracellular signalling such as heterotrimeric G proteins. These, and other proteins, can be modified at their N-terminus by the addition of palmitic acid, in a process distinct from that which adds this fatty acid to the C-terminal region of Ras proteins. This modification is more widespread than the addition of myristic acid to G proteins, which is restricted to members of the G_i family of G proteins. Parenti *et al.*, [1993], Linder *et al.*, [1993], Degtyarev *et al.*, [1993a], Wedegaertner *et al.*, [1993] and Veit *et al.*, [1994] have demonstrated the incorporation of [^{3}H] palmitic acid into $G_s\alpha$, $G_o\alpha$, $G_{i1/i2}\alpha$, $G_{q/11}\alpha$ and $G_{12/13}\alpha$. This modification is thought to occur via labile thio-ester bonds to cysteine residue(s) near the N terminus of the proteins. This modification was also simultaneously reported for $p56^{lck}$ and $p59^{fyn}$, members of the Src family of non-receptor tyrosine kinases [Paige *et al.*, 1993; Shenoy-Scaria *et al.*, 1993]. Other members of this non-receptor tyrosine kinase family also contain possible target cysteine residue(s) [Resh, 1994]. The growth cone associated protein GAP-43 of neuronal cells has also been shown to be dually palmitoylated on cysteine residues close to its N terminus [Skene & Virág, 1989] and this contributes to

its membrane association [Skene & Virág, 1989; Zuber *et al.*, 1989]. Palmitoylation of GAP-43 also affects the function of the protein [Sudo *et al.*, 1992].

The effect of this modification on both G proteins and Src family members has since remained a hotly contested point. Parenti *et al.* [1993] reported that palmitoylation of $G_{o1}\alpha$ contributed to the membrane association of the polypeptide as mutation of the cys3 not only abolished detectable palmitoylation but reduced the ability of the polypeptide to interact with the plasma membrane. Degtyarev *et al.* [1993a] have also identified cys3 of $G_s\alpha$ as the palmitoylated residue in that protein. However substitution of this residue by serine causes no change in the ability of $G_s\alpha$ to interact with the plasma membrane. Mumby *et al.* [1994] have similarly shown no effect on the membrane interaction of $G_s\alpha$ when cys3 was mutated to alanine, whereas Wedegaertner *et al.*, [1993] have demonstrated an absolute requirement for palmitoylation for the membrane association of $G_s\alpha$. Wedegaertner *et al.*, [1993] have also identified cysteines 9 and 10 of $G_q\alpha$ as the palmitoylated residues in that α subunit. If these residues are mutated to serines, $G_q\alpha$ loses all its ability to interact with the plasma membrane.

Functionally, mutants of $G_s\alpha$ and $G_q\alpha$ which lack detectable palmitic acid have little or no ability to activate their effector systems [Wedegaertner *et al.*, 1993]. Conversely palmitoylation of GAP-43 abolishes its ability to stimulate the guanine nucleotide exchange of $G_o\alpha$ [Sudo *et al.*, 1992].

Thus, the role of palmitoylation in regulating protein-membrane and protein-protein interaction is still unclear. This chapter assesses how palmitoylation affects membrane association, by chemically depalmitoylating G protein α subunits, and assessing how mutationally depalmitoylated α subunits interact with the $\beta\gamma$ complex of heterotrimeric G proteins. Treatment of membranes from NG108-15 cells and Rat-1 cells with hydroxylamine, a substance known to remove thio-ester linked fatty acids from proteins, can induce the release of $G_{i1/i2}\alpha$, $G_o\alpha$, and $G_{q/11}\alpha$ from cell membranes to equivalent degrees while causing a lesser release of $G_s\alpha$. Also, when a C3S mutant of $G_{o1}\alpha$ was stably expressed in Rat-1 fibroblasts, this caused a significant release of immunodetectable $G_{o1}\alpha$ to the cytoplasmic fraction. Both wild type and C3S $G_{o1}\alpha$ could bind $\beta\gamma$ in these Rat-1 transfectants.

4. 2. Results.

4. 2. 1. Treatment of cell membranes with hydroxylamine causes release of G protein α subunits from the membrane.

4. 2. 1. 1. Analysis of NG108-15 membranes.

It is well established that hydroxylamine (NH_3OH) at neutral pH can cleave thio-esterified acyl groups from the cysteine residues of proteins by nucleophilic attack. To assess the effect of any thio-esterified acyl groups present on G protein membrane association, cell membranes from NG108-15 cells were treated with 1M hydroxylamine at pH 8 as described in **section 2. 5. 2.** and separated into particulate and soluble fractions by ultracentrifugation at 120,000xg_{av}. These fractions were then subjected to SDS-PAGE and immunoblotted as described in **section 2. 8.** These immunoblots were probed with antisera selective for $G_s\alpha$, $G_o\alpha$, $G_{i1/i2}\alpha$ and $G_{q/11}\alpha$. Representative examples of these immunoblots are shown in **figure 4. 1.**

As NG108-15 cells are known to express no $G_{i1}\alpha$ [McKenzie & Milligan, 1990a], the immunoblot probed with antiserum SG1 represents only the protein levels of $G_{i2}\alpha$. It can be seen that hydroxylamine causes a relocation of a fraction of the immunoreactive protein. Quantitiation of this, as described in **section 2. 9.** and shown in **figure 4. 2.**, indicated that approximately 30% of immunoreactive $G_o\alpha$, $G_{i2}\alpha$ and $G_{q/11}\alpha$. was released into the soluble fraction, once the amount of immunoreactivity present in the

Figure 4. 1. Hydroxylamine causes the release of G protein α subunits from the membranes of NG108-15 cells.

100μg of NG108-15 cell membranes were treated with either 1M hydroxylamine, pH8, or 1M Tris HCl, pH8, as described in **Chapter 2.** Following centrifugation at 200,000xg_{av}, the resulting pellet and supernatant fractions from Tris treated samples (lanes 2 and 3 respectively) and hydroxylamine treated samples (lanes 4 and 5 respectively) were electrophoresed on a standard 10% SDS-PAGE gel along with 100μg of membranes which had not undergone any treatment (lane 6) and prestained molecular weight markers (lane 1). The resulting immunoblots were probed with antisera CS3 (dilution 1:250), IM1 (dilution 1:400), SG1 (dilution 1:1000) and CQ2 (dilution 1:1000) in panelsA B C and D respectively. The identities of the immunoreactive polypeptides and the mobility of the 43kDa molecular weight marker are shown.

Figure 4.1

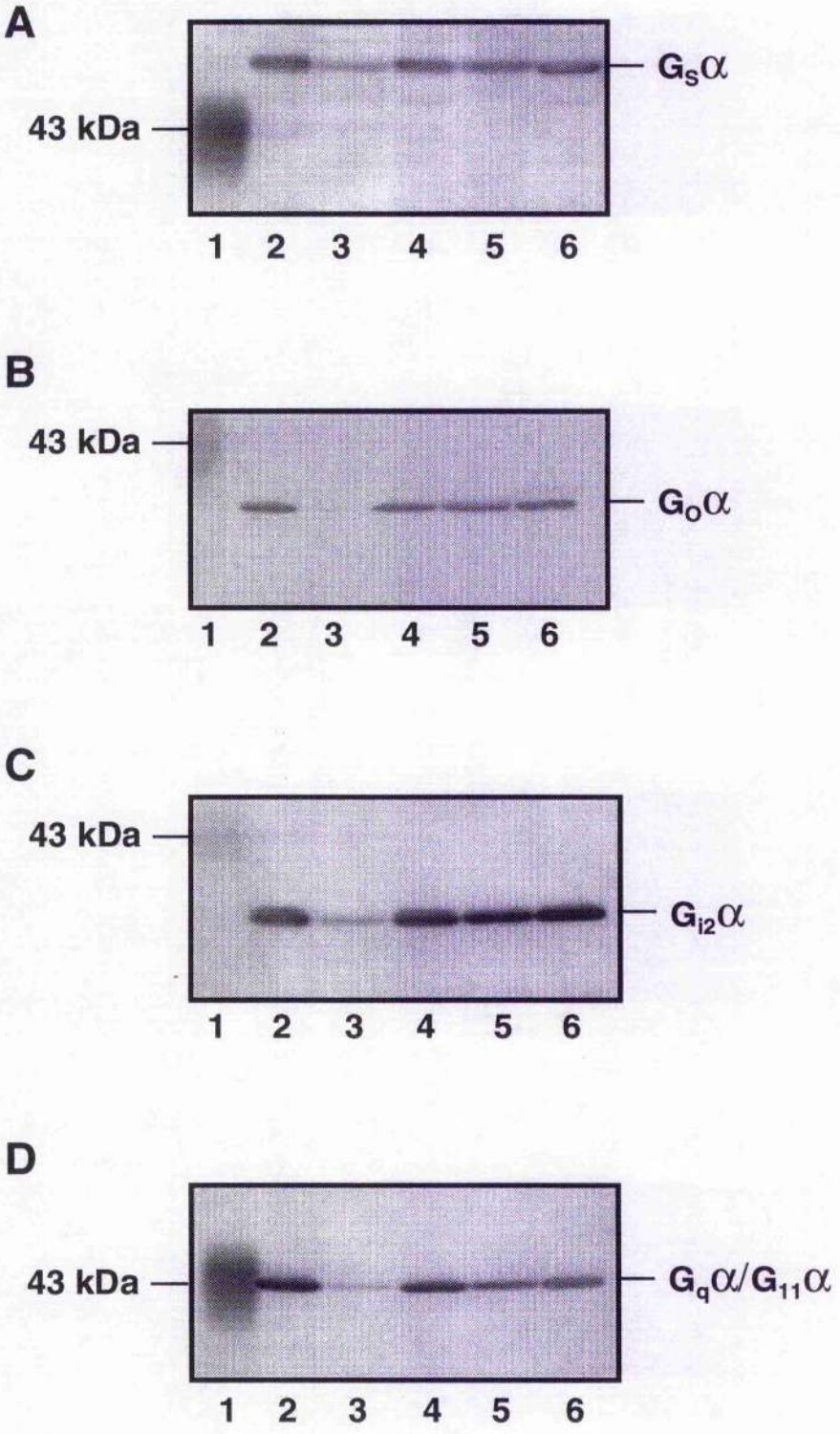
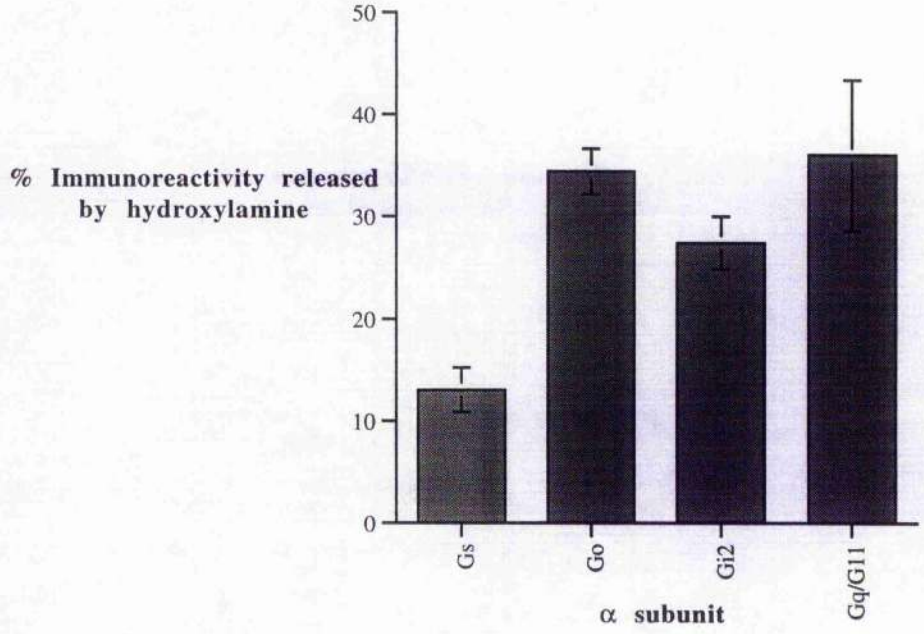

A
43 kDa
$G_s\alpha$
1 2 3 4 5 6
B
43 kDa
$G_o\alpha$
1 2 3 4 5 6
C
43 kDa
$G_{i2}\alpha$
1 2 3 4 5 6
D
43 kDa
$G_q\alpha/G_{11}\alpha$
1 2 3 4 5 6

Figure 4. 2. Quantitation of the amount of immunoreactive $G_s\alpha$, $G_o\alpha$, $G_{i2}\alpha$ and $G_q\alpha/G_{11}\alpha$ released from NG108-15 cell membranes by hydroxylamine.

Immunoblots from the experiment shown in **figure 4. 1.** were densitometrically analysed on a Bio-Rad GS670 imaging densitometer. After correction for the amount of immunoreactive polypeptide released by Tris HCl, the amount of polypeptide released by hydroxylamine was expressed as a percentage of the total amount immunoreactive polypeptide (the amounts in the pellet fractions + the amounts in the supernatant fractions) and graphed against the G protein α subunit. Results are shown as the mean±SEM of 4 independent experiments.

Figure 4. 2.


% Immunoreactivity released by hydroxylamine
50
40
30
20
10
0
Gs
Go
Gi2
Gq/G11
α subunit

soluble fraction of Tris-HCl treated control samples had been accounted for. A smaller amount, approximately 10%, of $G_s\alpha$ was released by hydroxylamine.

Antisera CQ2, used to detect $G_q\alpha$ and $G_{11}\alpha$, and IM1, used to detect $G_o\alpha$, cannot distinguish between $G_q\alpha$ or $G_{11}\alpha$, or between the splice variants of $G_o\alpha$. Therefore, following treatment with hydroxylamine as before, particulate and soluble fractions were electrophoresed by urea gradient SDS-PAGE as described previously, in an attempt to determine whether both $G_q\alpha$ and $G_{11}\alpha$, and $G_o\alpha$ variants, are affected by chemical depalmitoylation, and to what extent. A representative experiment is shown in **figure 4. 3. a.** and the quantitation of this experiment is displayed in **figure 4. 3. b.** These demonstrated that hydroxylamine caused the release of $G_q\alpha$ and $G_{11}\alpha$, and the $G_{o1}\alpha$ and $G_{o2}\alpha$ splice variants to an equal degree, that is 30%.

4. 2. 1. 2. Analysis of Rat-1 transfectant membranes.

Complementary DNAs encoding either wild type $G_{o1}\alpha$ or $G_{o1}\alpha$ in which the codon for cysteine-3 has been mutated to encode serine have been stably transfected into Rat-1 fibroblasts [Grassie *et al.*, 1993; Grassie *et al.*, 1994]. This mutation has previously been shown to abolish detectable palmitoylation of the $G_{o1}\alpha$ protein upon transient expression in COS-7 cells [Parenti *et al.*, 1993].

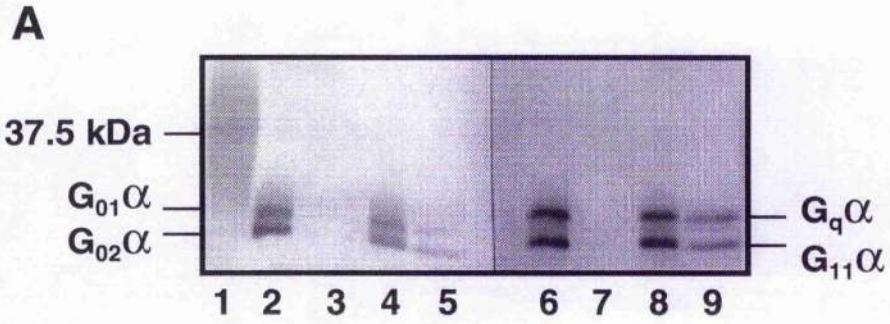
In order to determine whether these transiently transfected proteins were equivalent to the $G_{o1}\alpha$ detected in rat brain as described in **Chapter 3**, membranes of these transfectants, termed C5B and D3 for the wild

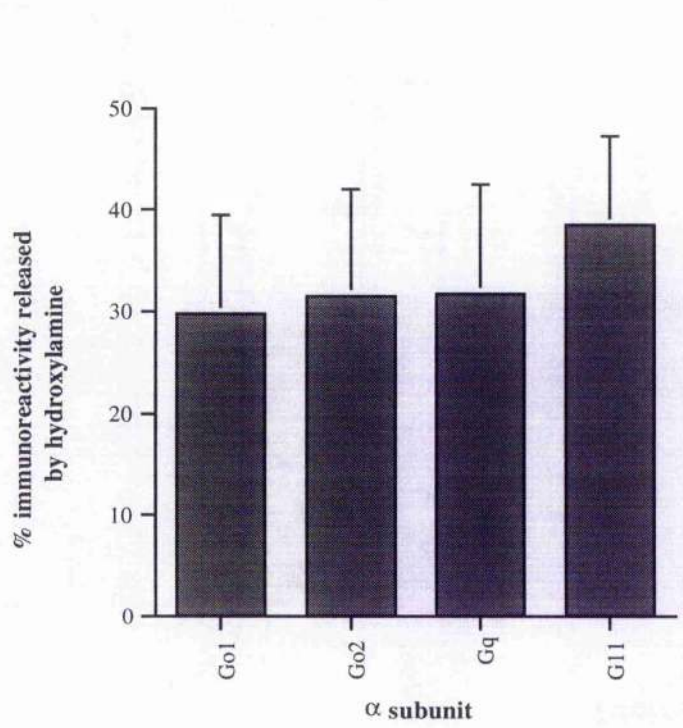
Figure 4. 3. Analysis of the ability of hydroxylamine to cause release of $G_{o1}\alpha/G_{o2}\alpha$ and $G_q\alpha/G_{11}\alpha$ from NG108-15 cell membranes.

Following treatment of 100μg of NG108-15 cell membranes with either 1M Tris HCl, pH8, or 1M hydroxylamine, pH8, pellet and supernatant fractions from the 200,000xg_{av} centrifugation step were analysed on a 4M-8M urea gradient SDS-PAGE gel. Panel A shows a representative immunoblot from such an experiment. Lane 1 contained prestained lactic dehydrogenase marker, lanes 2 and 3 and lanes 6 and 7 contained the pellet and supernatant fractions (respectively) of Tris HCl treated samples and lanes 4 and 5 and lane 8 and 9 contained the pellet and supernatant fractions (respectively) of hydroxylamine treated samples. The resulting immunoblot was probed with antisera IM1 (dilution 1:400; lanes 1-5) and CQ2 (dilution 1:1000; lanes 6-9. The mobility of the lactic dehydrogenase marker and the identities of the immunoreactive polypeptides are shown.

Panel B shows the quantitative analysis of several such experiments. Following densitometric scanning as before, the amount of immunoreactive $G_{o1}\alpha$, $G_{o2}\alpha$, $G_q\alpha$ and $G_{11}\alpha$ released by hydroxylamine was corrected for that released by Tris and expressed as a percentage of the total immunoreactive protein from both pellet and supernatant fractions. These results were graphed against the G protein α subunits of interest and expressed as the mean±SEM of 3 independent experiments

Figure 4.3


A
37.5 kDa
$G_{01}\alpha$
$G_{02}\alpha$
$G_q\alpha$
$G_{11}\alpha$
1 2 3 4 5 6 7 8 9


B
% immunoreactivity released by hydroxylamine
50
40
30
20
10
0
Go1
Go2
Gq
G11
α subunit

type and mutant cells respectively, were assayed by urea gradient SDS-PAGE as before. **Figure 4. 4.** indicated that both C5B and D3 cells express the same IM1 immunoreactive polypeptide, and this co-migrated with the $G_{o1}\alpha$ splice variant of rat brain cortex membranes. It can also be seen that the absence of palmitic acid on the $G_{o1}\alpha$ protein expressed in D3 cells did not alter its mobility under urea gradient gel conditions as has previously been suggested [Nurnberg *et al.*, 1994].

Membranes of these cells were subjected to hydroxylamine treatment as described for NG108-15 cell membranes above. The immunoblots were probed for the same range of α subunits as for NG108-15 membranes. **Figure 4. 5.** shows representative examples of these immunoblots and quantification of several experiments is displayed in **figure 4. 6.** It can be seen that the amount of any given α subunit is the same for each of the C5B and D3 membranes, with the exception of $G_{o1}\alpha$, indicating that the transfection has not altered the level of expression of the endogenous α subunits.

While the amount of immunoreactive $G_{o1}\alpha$ released by hydroxylamine in C5B cell membranes was equivalent to that for $G_{i1/i2}\alpha$ and $G_{q/11}\alpha$ (approximately 25%), only around 5% of immunoreactive $G_{o1}\alpha$ was released by hydroxylamine in D3 cell membranes. This would indicate that the C3S $G_{o1}\alpha$ in D3 cells is not palmitoylated. Also the amount of $G_s\alpha$ released from the membranes was less than that for the other α subunits as previously seen for $G_s\alpha$ in NG108-15 cell membranes.

Figure 4. 4. C5B and D3 cells both express an immunoreactive $G_{o}\alpha$ species which co-migrates with rat brain $G_{o1}\alpha$ on urea gradient SDS-PAGE.

25μg of rat brain cortex membranes (lane 1) were electrophoresed on a 4M-8M linear urea gradient gel with 50μg of D3 cell membranes (lane 2) and 50μg of C5B cell membranes (lane 3). The resulting immunoblot was probed with antiserum IM1 at a dilution of 1:400. The identities of the immunoreactive polypeptides are shown. This gel is a representative example of 3 independent experiments.

Figure 4.4

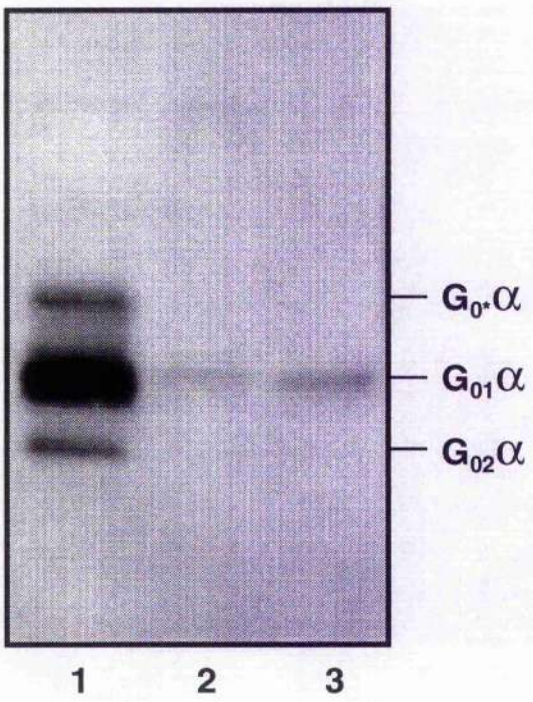


Figure 4. 5. Analysis of the effect of hydroxylamine on the G protein a subunits expressed in C5B and D3 cells.

100μg of cell membranes from either C5B cells (lanes 2-5) or D3 cells (lanes 6-9) were either treated with 1M Tris HCl, pH8, or with 1M hydroxylamine, pH8, and separated into pellet and supernatant fractions by centrifugation at 200,000xgav. The pellet and supernatant fractions from Tris treated samples (lanes 2 and 3 and lanes 6 and 7, respectively) were electrophoresed on a standard 10% SDS-PAGE gel along with the pellet and supernatant fractions from hydroxylamine treated samples (lanes 4 and 5 and lanes 8 and 9, respectively). The resulting immunoblots were probed with antisera CS3 (dilution 1:250), IM1 (dilution 1:400), SG1 (dilution 1:1000) and CQ2 (1:1000) as shown in panels A, B, C and respectively. The mobility of the prestained lactic dehydrogenase marker (lane 1) and the identities of the immunoreactive polypeptides are shown. These immunoblots are representative examples of 4 independent experiments.

Figure 4.5

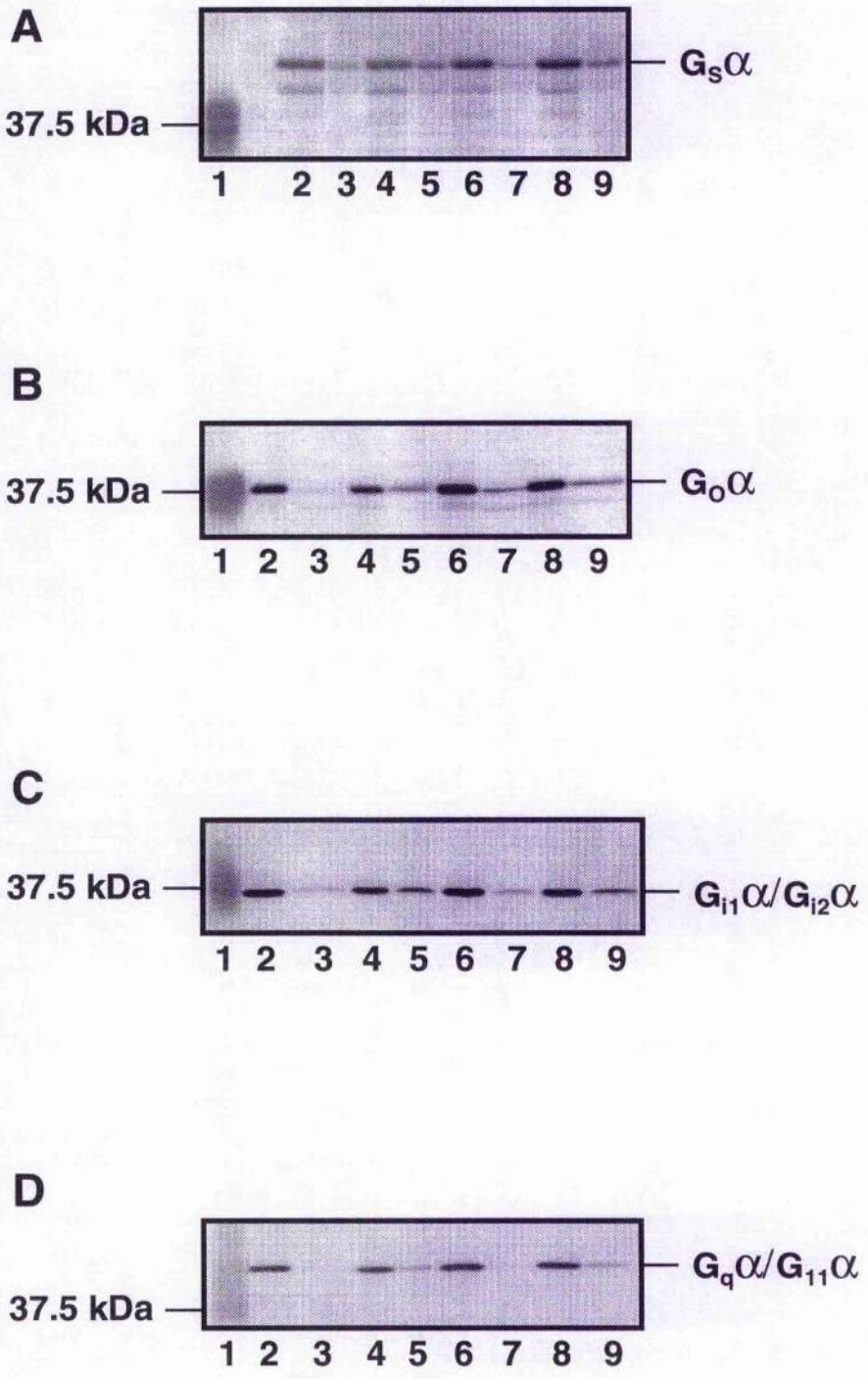
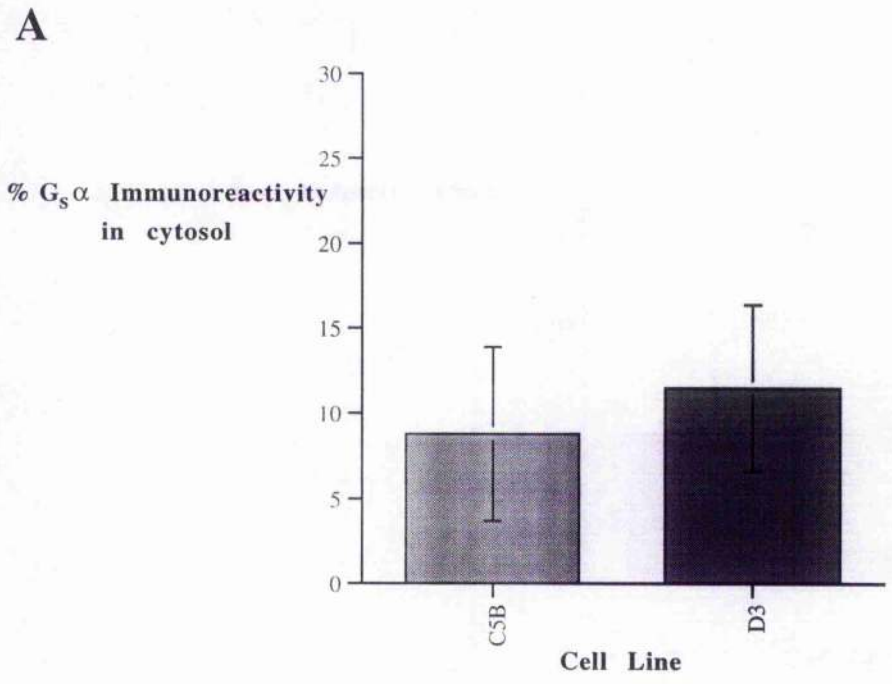

A
37.5 kDa
$G_s\alpha$
1 2 3 4 5 6 7 8 9
B
37.5 kDa
$G_o\alpha$
1 2 3 4 5 6 7 8 9
C
37.5 kDa
$G_{i1}\alpha/G_{i2}\alpha$
1 2 3 4 5 6 7 8 9
D
37.5 kDa
$G_q\alpha/G_{11}\alpha$
1 2 3 4 5 6 7 8 9

Figure 4. 6. Quantitative analysis of the effect of hydroxylamine on the G protein α subunits expressed in C5B and D3 cells.

Following correction for the amount of immunoreactive polypeptides released by Tris, densitometrically scanned analyses of samples from hydroxylamine treated samples were expressed as a percentage of total immunoreactive polypeptide and graphed against the G protein α subunits examined; that is $G_s\alpha$, $G_o\alpha$, $G_{i1}\alpha/G_{i2}\alpha$ and $G_q\alpha/G_{11}\alpha$ in panels A B C and D respectively. The analyses are given as the mean±SEM of 4 independent experiments.

Figure 4. 6.


A
% $G_s\alpha$ Immunoreactivity in cytosol
30
25
20
15
10
5
0
C5B
D3
Cell Line

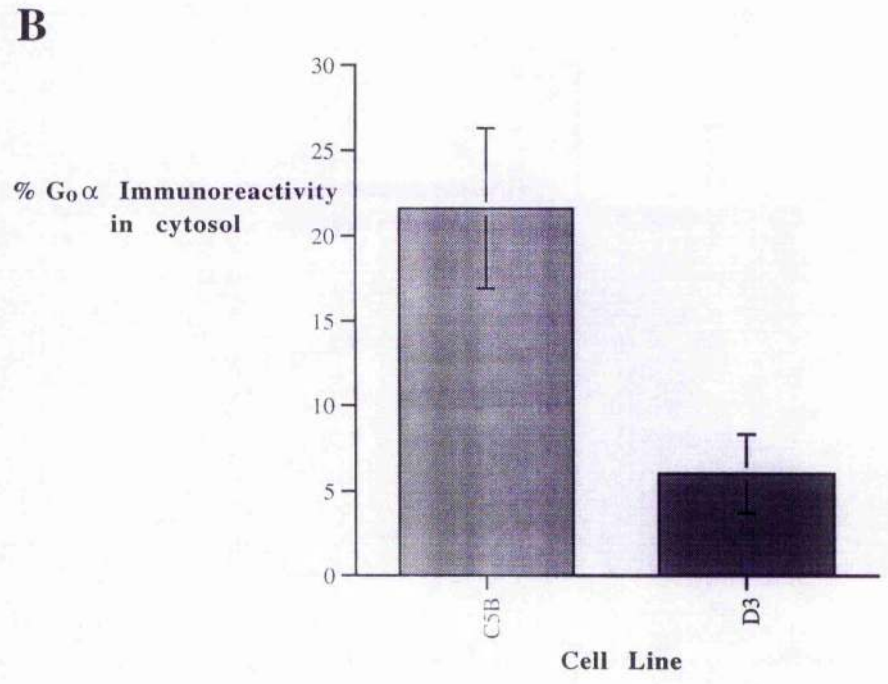
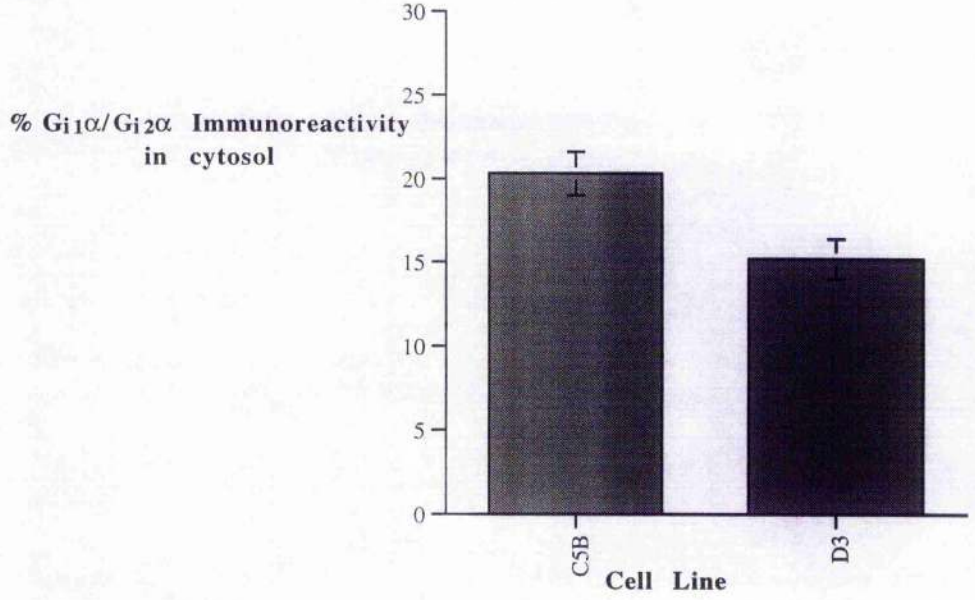
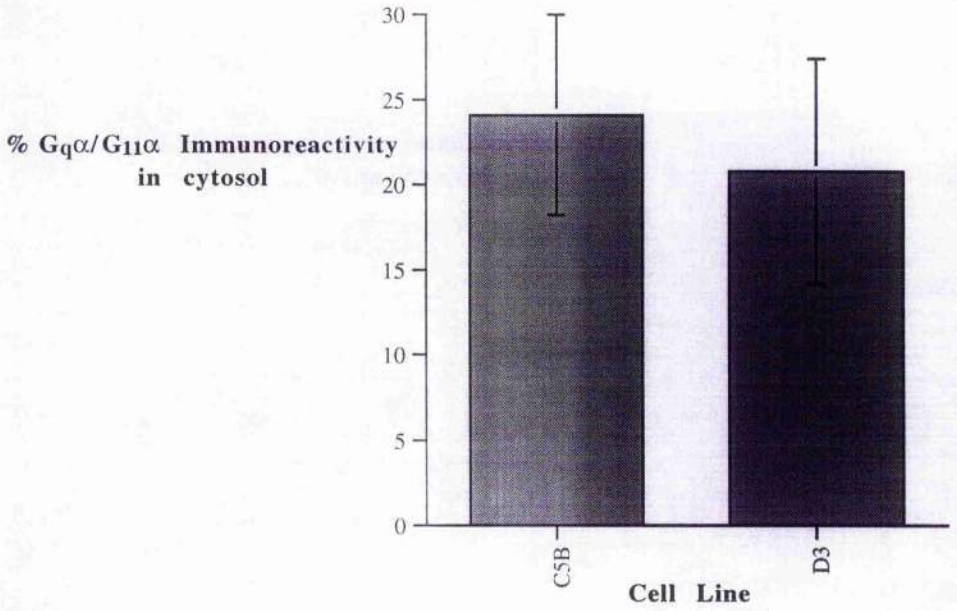

B
% $G_o\alpha$ Immunoreactivity in cytosol
30
25
20
15
10
5
0
C5B
D3
Cell Line

Figure 4. 6.

C


% Gi1α/Gi2α Immunoreactivity in cytosol
30
25
20
15
10
5
0
C5B
D3
Cell Line

D


% Gqα/G11α Immunoreactivity in cytosol
30
25
20
15
10
5
0
C5B
D3
Cell Line

4. 2. 2. Mutation of cysteine 3 to serine alters the membrane association of $G_{o1}\alpha$ when expressed in Rat-1 cells.

Mutation of target cysteine residues to serine has been shown to abolish detectable palmitoylation of several proteins including $p21^{ras}$ and GAP-43 [Hancock *et al.*, 1989; Zuber *et al.*, 1989]. Recently this approach has been used to identify palmitoylated cysteine residues of $G_s\alpha$, $G_{o1}\alpha$ and $G_q\alpha$ when transiently expressed in either COS-7 cells ($G_{o1}\alpha$ and $G_s\alpha$) or HEK 293 cells ($G_s\alpha$ and $G_q\alpha$) [Parenti *et al.*, 1993; Degtyarev *et al.*, 1993a; Wedegaertner, *et al.*, 1993]. These experiments have demonstrated that both $G_s\alpha$ and $G_{o1}\alpha$ are palmitoylated on cys-3 while, similarly to GAP-43, $G_q\alpha$ is dually palmitoylated, on cys9 and cys10.

The effect on membrane association of the addition of palmitic acid to these G protein α subunits is still unclear. $G_q\alpha$ is apparently wholly dependent on the addition of palmitic acid for its membrane interaction [Wedegaertner *et al.*, 1993], while the membrane association of $G_{o1}\alpha$ is less dependent on acylation [Parenti *et al.*, 1993] and the membrane association of $G_s\alpha$ is not dependent on its palmitoylation state at all [Degtyarev *et al.*, 1993a; Mumby *et al.*, 1994]. These studies were carried out under transient transfection conditions. To prevent the necessity of repeated transfections the membrane association of $G_{o1}\alpha$ was assessed in stably transfected Rat-1 fibroblasts expressing either wild type or C3S forms of the α subunit. Particulate membrane and soluble cytosolic fractions were prepared and immunoblotted for the presence of immunoreactive $G_o\alpha$ using the selective antiserum IM1 (**figure 4. 7. a.**). Quantification of several such experiments

indicated that 27.2±5.5% of total immunoreactive $G_{o1}\alpha$ was present in the cytosol of the C3S mutant expressing D3 cells while in wild type $G_{o1}\alpha$ expressing cells, only 4.6±4.6% of immunoreactive $G_{o1}\alpha$ was detectable in the cytosolic fraction (**figure 4. 7. b.**).

4. 2. 3. Both wild type and mutant $G_{o1}\alpha$ are substrates for pertussis toxin *in vivo*.

The binding domain on α subunits for the $\beta\gamma$ dimer is thought to be located at the N terminus of the polypeptide, as enzymatic cleavage of the first 21 amino acids of $G_o\alpha$ eliminates $\beta\gamma$ binding [Winslow *et al.*, 1986]. The presence of palmitic acid on cys3 may therefore affect α–$\beta\gamma$ interactions, as this modification has been shown to have a negative effect on the association of GAP-43 with $G_o\alpha$. [Sudo *et al.*, 1992]. Also as the C3S mutation decreases the ability of $G_o\alpha$ to interact with the plasma membrane, it may not be in the correct environment to associate with $\beta\gamma$ at all.

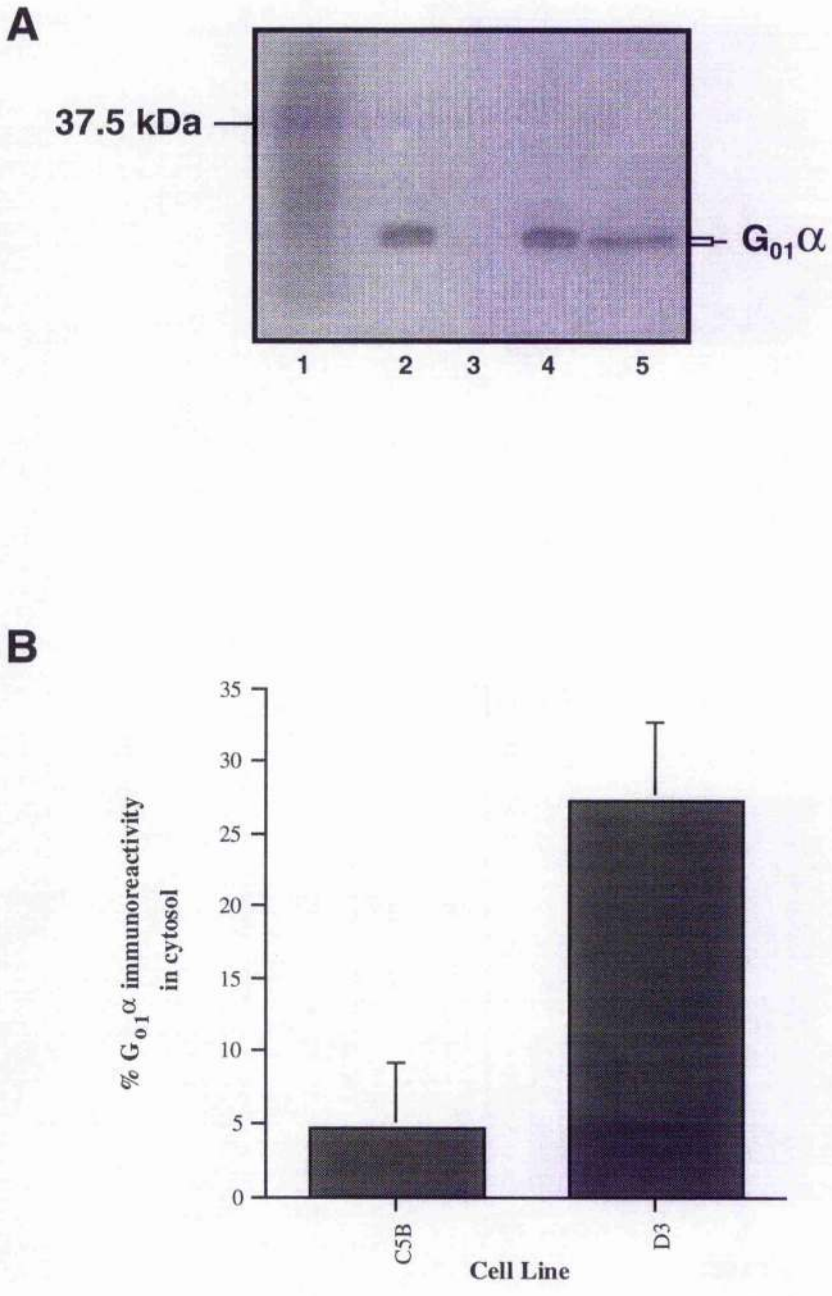
To address this point use was made of the *Bordetella pertussis* exotoxin, pertussis toxin, to assess the ability of the wild type and mutant $G_{o1}\alpha$ to interact with $\beta\gamma$. The toxin requires the α subunit to be complexed to $\beta\gamma$ in order to efficiently transfer the ADP-ribose group of NAD^+ to the target C terminal cysteine residue. Therefore if the $G_o\alpha$ from either C5B or D3 cells (or both) is complexed to $\beta\gamma$, pertussis toxin will be able to ADP-ribosylate the α subunits.

The addition of ADP-ribose to the α subunits of the G_i family of G protein α subunits causes a slight reduction in mobility of the subunit

Figure 4. 7. A $G_{o1}\alpha$ immunoreactive polypeptide is present in the cytosolic fraction of D3 cells while all the immunoreactive $G_{o1}\alpha$ in C5B cells is present at the membrane.

C5B (lanes 2-3) and D3 cells (4-5) were homogenised and separated into membrane (particulate) and cytosolic (supernatant) fractions by centrifugation at 200,000xgav. 100μg of the membrane fraction and an equivalent amount of cytosolic fraction (such that the amount of cell protein from membrane and cytosolic fractions was the same) from each cell type was subjected to SDS-PAGE on a standard 10% polyacrylamide gel and the resulting immunoblot probed with antiserum IM1 at a dilution of 1:400 (panel A). This immunoblot is a representative example of 3 experiments. Following densitometric analysis of these immunoblots, the immunoreactive $G_{o1}\alpha$ was expressed as a percentage of the total immunoreactive $G_{o1}\alpha$ present in each cell type and graphed against the cell type involved (panel B). The results are expressed as the mean±SEM of 3 independent experiments.

Figure 4.7


A
37.5 kDa
$G_{01}\alpha$
1
2
3
4
5
B
% $G_{01}\alpha$ immunoreactivity in cytosol
35
30
25
20
15
10
5
0
C5B
D3
Cell Line

through acrylamide gels. This mobility shift can be exaggerated if the proteins are analysed by urea gradient SDS-PAGE. Therefore C5B and D3 cells were treated with 25ng/ml of pertussis toxin *in vivo* as described in **section 2. 3. 6.** before membrane and cytosolic fractions were prepared by ultracentrifugation at 200,000x $g_{av.}$ Analysis of these toxin treated fractions on urea gradient SDS-PAGE is displayed in **figure 4. 8.** It can be seen that all of the wild type $G_{o1}\alpha$ was a substrate for pertussis toxin. Also, as expected from previous experiments, none of the immunoreactive wild type polypeptide was present in the cytosolic fraction of either pertussis toxin treated or untreated cells. In D3 cells a fraction of the immunoreactivity was present in the cytosol of both treated and untreated cells, as was seen previously. All the immunoreactive $G_{o1}\alpha$ in these cells was, however, a substrate for pertussis toxin, as the $G_{o1}\alpha$ present in both the membrane and cytosolic fractions derived from pertussis toxin treated D3 cells underwent a mobility shift under urea gradient SDS-PAGE. This indicated that either the $G_{o1}\alpha$ in these cells was at the membrane at some time and perhaps a cycling process ensured that all the $G_{o1}\alpha$ was complexed to a $\beta\gamma$ dimer at some point, or that $\beta\gamma$ was present in the cytosol of these cells and that $G_{o1}\alpha$ was complexed to it here. The $\beta\gamma$ complex has never been observed in the cytosol due to the prenylated C terminus of the γ subunit, but the possibility does exist.

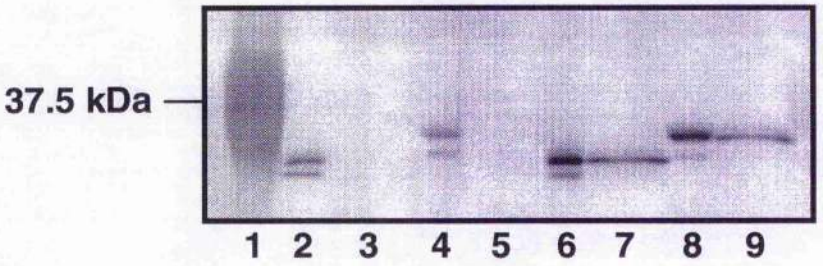
4. 2. 4. Only membrane associated $G_{o1}\alpha$ is a substrate for pertussis toxin.

In order to assess whether the cytosolic fraction of D3 cells contained heterotrimers of $G_{o1}\alpha$ which could support pertussis toxin catalysed ADP-ribosylation, membrane and cytosolic fractions of C5B and D3 cells

Figure 4. 8. All the immunreactive $G_{o}1\alpha$ present in both C5B and D3 cells is a substrate for pertussis toxin catalysed mono ADP-ribosylation *in vivo*.

C5B and D3 cells were treated for 16hrs with 25ng/ml of pertussis toxin before membrane and cytosolic fractions were prepared. 100μg of membranes, and an equivalent amount of cytosol, from untreated C5B cells (lanes 2 and 3 respectively) were electrophoresed on a 4M-8M linear urea gradient with 100μg of membranes and the equivalent amount of cytosol from each of treated C5B cells (lanes 4 and 5, respectively), untreated D3 cells (lanes 6 and 7, respectively) and pertussis toxin treated D3 cells (lanes 8 and 9, respectively). The resulting immunoblot was probed with antiserum ON1 (dilution 1:1000). Lane 1 contained prestained lactic dehydrogenase marker, the mobility of which is shown. The experiment shown is a representative example of 3 independent experiments.

Figure 4.8



were subjected to *in vitro* ADP-ribosylation in the presence of [^{32}P]NAD$^+$. $G_{o1}\alpha$ was subsequently immunoprecipitated using antiserum OC1 and subjected to SDS-PAGE.

A sample autoradiogram from these experiments is displayed in **figure 4. 9.** While the $G_{o1}\alpha$ in the membrane fractions of both C5B and D3 cells was subject to ADP-ribosylation neither cytosolic fraction provided a substrate for pertussis toxin catalysed ADP-ribosylation indicating that the cytosolic fraction of $G_{o1}\alpha$ in D3 cells was not complexed to $\beta\gamma$ dimers.

4. 2. 5. The cytosolic $G_{o1}\alpha$ of D3 cells exists as a free α subunit.

In order to assess whether the $G_{o1}\alpha$ present in the cytosolic fraction of D3 cells was bound to another protein or found free in the soluble fraction, this fraction was analysed on a Superose 12 gel filtration column as described in **section 2. 10.** The resultant fractions from the column were precipitated using TCA and subjected to SDS-PAGE and immunoblotting. Immunblots were then probed with antiserum OC1. The elution volume of the fraction with the highest level of $G_o\alpha$ immunoreactivity (**figure 4. 10. a.**) was compared to the elution volume of several standard proteins with known molecular mass (**figure 4. 10. b.**). This gave a value for the molecular mass of $G_o\alpha$ of approximately 40kDa, which was close to the reported value for the molecular mass of the α subunit.

To determine whether the free α subunit of D3 cell cytosol was capable of binding the $\beta\gamma$ complex, and thus forming a substrate for pertussis toxin. Exogenous $\beta\gamma$, purified from bovine brain, kindly donated by Dr M.

Figure 4. 9. Only membrane associated $G_{o1}\alpha$ is a substrate for pertussis toxin *in vitro*.

50μg of membranes, or an equivalent amount of cytosol, from C5B and D3 cells were mono ADP-ribosylated with DTT activated pertussis toxin in the presence or absence of 1mM Gpp(NH)p. Following this 150μl of 1.33% (w/v) SDS was added to each sample, to give a final concentration of SDS of 1% (w/v) and $G_{o1}\alpha$ was immunoprecipitated from these samples using antiserum OC1. Immunoprecipitates were subjected to SDS-PAGE on a 10% (w/v) gel. This was then dried down and exposed to a phosphorimaging screen for 1hr. The lanes contained C5B membranes in the absence (1) or presence (2) of Gpp(NH)p, C5B cytosol in the absence (3) or presence (4) of Gpp(NH)p, D3 membranes in the absence (5) or presence (6) of Gpp(NH)p and D3 cytosol in the absence (7) or presence (8) of Gpp(NH)p. The identity of $G_{o1}\alpha$ is shown. Similar results were obtained in 3 independent experiments.

Figure 4.9

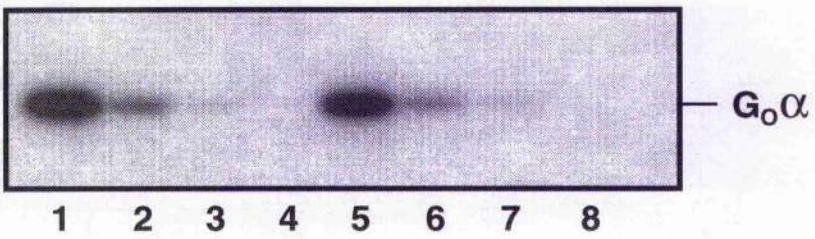
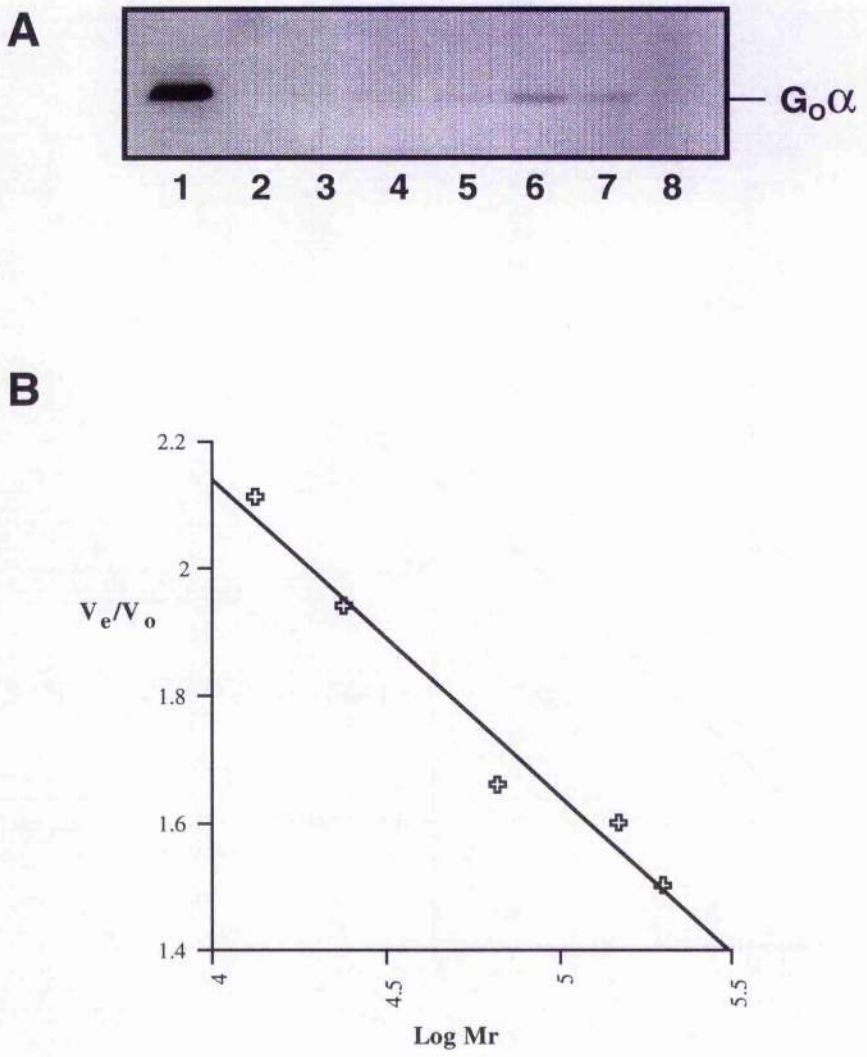


Figure 4. 10. The $G_{o1}\alpha$ in the cytosol of D3 cells exists as a free α subunit.

To determine if the $G_{o1}\alpha$ of D3 cell cytosol was complexed to another protein, 200μg of crude ctyosol from these cells was chromatographed on a Superose 12 gel filtration column against standards of known molecular mass. 500μl fractions from this column were TCA precipitated in the presence of 10μg of BSA to prevent protein loss and subjected to electrophoresis on a 10% (w/v) SDS-PAGE gel. The resulting immunoblot (panel A) showed the presence of an immunoreactive polypeptide which eluted from the column and co-migrated with $G_o\alpha$ from rat brain membranes (lane 1). The eluted fraction (lanes 2-6) were compared to the elution volumes of known proteins (panel B). This gave an Mr for $G_o\alpha$ from D3 cell cytosol of approximately 40kDa, close to that reported for $G_o\alpha$ in the literature.

Figure 4.10



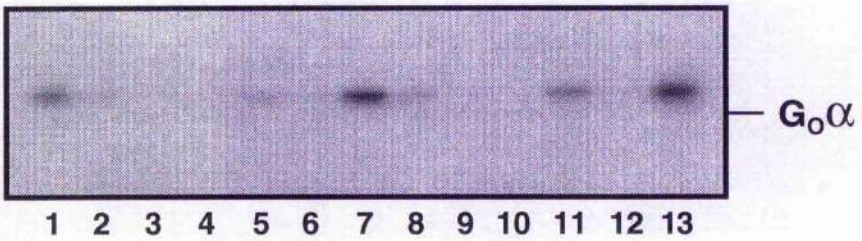
Freissmuth, Department of Pharmacology, University of Vienna, Vienna, Austria, was therefore added to samples of cytosol from C5B and D3 cells and *in vitro* pertussis toxin catalysed ADP-ribosylations performed in the presence of [^{32}P]NAD^{+}.

A sample autoradiogram from this experiment is shown in **figure 4. 11.** Unfortunately it would appear that a considerable amount of $G_o\alpha$ was present in the purified brain $\beta\gamma$. This obscured any [^{32}P]ADP-ribose labelled $G_{o1}\alpha$ in the cytosolic samples. We were unable to obtain a sufficiently pure sample of $\beta\gamma$ from another source so were unable to resolve whether the $G_{o1}\alpha$ found in the cytosolic fraction of D3 cells was capable of binding $\beta\gamma$.

Figure 4. 11. Addition of purified bovine brain βγ to the membrane and cytosolic fractions of C5B and D3 cells.

50μg of membranes, or an equivalent amount of cytosol, from C5B and D3 cells were mono ADP-ribosylated with DTT activated pertussis toxin in the presence or absence of 1mM Gpp(NH)p following addition of 1ng/ml final concentration of purified bovine brain βγ. Samples were then immunoprecipitated using antiserum OC1 and subjected to SDS-PAGE on a 10% (w/v) gel. This was then dried down and exposed to a phosphorimaging screen for 1hr. The identity of $G_o\alpha$ is shown. Lanes 1 and 2 contained C5B membranes, lanes 3-6 contained C5B cytosol either with (lanes 5 and 6) or without (lanes 3 and 4) added bovine brain βγ. Lanes 7 and 8 contained D3 membranes, lanes 8-12 contained D3 cytosol either with (lanes 11 and 12) or without (lanes 8 and 9) βγ. Lane 13 contained only purified bovine brain βγ. Similar results were obtained in 3 other experiments.

Figure 4.11



4. 3. Discussion.

Protein lipidation is thought to lead to a variety of effects on target proteins and their interaction with other proteins and with cellular membranes. Some extracellular receptors, for example folate binding protein, have no significant hydrophobic domains capable of forming any transmembrane α-helices, yet are located on the exofacial leaflet of the plasma membrane. These proteins are modified by a complex lipid moiety derived from phosphatidylinositol. This glycerophosphatidylinositol (GPI) link achieves two things. It increases the hydrophobicity of the protein sufficiently to promote its membrane association alone. It also gives the protein the ability to transduce its activation state, via the GPI link, to proteins associated with the membrane and from them to the inside of the cell. This modification thus changes the proteins' ability to interact with the plasma membrane and facilitates protein-protein interaction.

This dual effect has also been identified for other proteins. Members of the small molecular G protein superfamily undergo modification by a complex series of lipid modifications. The initial addition of prenoids and the subsequent proteolysis and carboxymethylation are insufficient to promote full membrane association [Hancock, *et al.*, 1989; Hancock *et al.*, 1990]. Another factor is required. In some members, particularly some of the Ras family, another lipidation is required to promote membrane interaction. Palmitoylation of the protein, occurring on cysteine residues near the prenylation site, provides the additional hydrophobicity required to promote full membrane interaction [Hancock *et al.*, 1990]. Other members, for example Rab and $p21^{K\text{-}ras(B)}$, have no cysteine residues capable of supporting acylation

by palmitic acid near the isoprenylated C terminus [Hancock *et al.*, 1990]. Instead, they have a long stretch of basic amino acids close to the prenylation site. This stretch is thought to contribute to membrane association by binding to the phosphate groups on lipid molecules [Hancock *et al.*, 1990].

Ras proteins have been demonstrated to be transforming in a variety of cell lines and types. This ability to transform can be overcome by the elimination of prenylation, although it is unclear whether this is a reflection of a change in protein-membrane or protein-protein interaction. Mutagenesis of the prenylated cysteine residue obliterates the modifications of the proteins and prevents membrane association. Mutagenesis of either the site of palmitoylation or the polybasic domain did not prevent Ras' transformation ability indicating that these domains were not required for the proteins' function, whereas the addition of the prenoid group was required for transformation implying a role of prenylation in protein-protein interactions [Hancock *et al.*, 1989; Hancock *et al.*, 1990]. Thus although prenylation *per se* does not lead to total membrane association, this modification has a significant role in promoting it [Hancock *et al.*, 1990]. These proteins are important in several areas of intracellular trafficking and cellular signalling. The Ras proteins are important in activating Raf, the initial enzyme in the MAP kinase cascade [Van Aelst *et al.*, 1993; Vojtek *et al.*, 1993]. It is unclear whether prenylation and acylation of the proteins plays any direct role in coupling of these proteins to their targets.

The γ subunits of heterotrimeric G proteins are also subject to prenylation. The lipid is required for the proper function of the βγ complex [Fukada *et al.*, 1990; Wedegaertner *et al.*, 1995]. Mutation of the prenylated

cysteine to serine does not preclude formation of the βγ dimer. Once formed, however, the dimer cannot insert in the membrane [Simonds, 1994] or interact with the α subunit [Wedegaertner *et al.*, 1995]. Thus it would appear that the presence of prenoid groups on proteins can affect protein-membrane as well as protein-protein interactions.

G protein α subunits are also subject to lipidation. Members of the G_i subfamily have been shown to be acylated by the addition of myristic acid at gly2 [Jones *et al.*, 1990; Mumby *et al.*, 1990] Following removal of the initiating methionine, this residue becomes the N-terminal residue and is thus in the correct environment for myristoylation by N-myristoyl transferase [Sefton & Buss, 1987; Towler *et al.*, 1988]. The exception to this is $G_t\alpha$, which is predominantly laurylated. This is due not to a separate enzyme but to a difference in the lipid environment of the retina making lauryl-CoA more prevalent than myristoyl-CoA [Kokame *et al.*, 1992].

It appears that myristoylation also affects both protein-membrane and protein-protein interactions. Mutagenic alteration of the target glycine residue and inhibition of the N-myristoyl transferase enzyme using 2-hydroxymyristate [Paige *et al.*, 1990], a substituted analogue of myristic acid, both lead to a reduction of the membrane association of $G_{i1}\alpha$ and $G_o\alpha$ [Jones *et al.*, 1990; Mumby *et al.*, 1990]. It also appears that myristoylation affects the function of the polypeptide. Myristoylation increases the affinity of $G_o\alpha$ and $G_{i1}\alpha$ for the βγ dimer [Linder *et al.*, 1991] and seems to interfere with the ability of $G_i\alpha$ to inhibit adenylyl cyclase [Taussig *et al.*, 1993].

Mutations of the $G_{i2}\alpha$ protein, which occur naturally in certain cancers, are known to function by inhibiting the GTPase activity of the polypeptide causing constitutive activation of the α subunit [Graziano & Gilman, 1989; Masters *et al.*, 1989]. One of these mutations, the Q205L mutation, causes transformation and constitutively activates the MAP kinase cascade when stably expressed in Rat-1 fibroblasts [Pore *et al.*, 1991; Gupta *et al.*, 1992]. Transfection of a double mutant encoding both the Q205L and the G2A mutations does not lead to cellular transformation or MAP kinase activation [Gallego *et al.*, 1992], indicating a role for myristoylation in the ability of $G_{i2}\alpha$ to interact with target proteins.

Not all myristoylated proteins are membrane bound. For example the catalytic subunit of the cAMP dependent protein kinase and calcineurin (protein phosphatase 2B) are also modified by myristoylation yet are located in the cytosol [Carr *et al*, 1982; Aitken *et al.*, 1982}. Therefore an additional signal may be required for a myristoylated protein to bind to the membrane. This may reflect a lesser ability of myristate to anchor acylated peptides to lipid vesicles. Myristoylated peptides have barely enough energy to anchor the peptides to membranes and the target protein would probably require other factors, such as the presence of positive charges or a protein-protein interaction to interact with the membrane [Peitzsch & McLaughlin, 1993]. Myristoylation of calcineurin and A-kinase catalytic subunit is presumed to affect the protein-protein interactions, perhaps by promoting the interactions of catalytic and regulatory subunits of the proteins [Turner, 1992].

Members of the Src family of non-receptor tyrosine kinases are also myristoylated on gly2. These proteins have recently been shown to be

post-translationally palmitoylated as well [Paige *et al.*, 1993; Shenoy-Scaria *et al.*, 1993], a lipid modification capable of anchoring acylated peptides alone [Peitzsch & McLaughlin, 1993] thus providing an additional factor to provide membrane association. The purpose of these two independent acylations is unclear as yet. Members of the Src family function to couple extracellular and membrane bound receptors to transduce their signals. Src family members, like G protein α subunits contain no hydrophobic domains capable of supporting membrane association alone. These twin acylations may play the key role in localising these proteins to the correct membrane compartment.

GAP-43, a protein associated with the neurite growth cone [Basi *et al.*, 1987], is also known to be palmitoylated at two adjacent cysteine residues at positions 3 and 4 [Skene & Virág, 1989]. This palmitoylation has two effects on GAP-43 function. It determines the membrane association of GAP-43 [Skene & Virág, 1989; Zuber *et al.*, 1989] and also affects the ability of GAP-43 to interact with and regulate the guanine nucleotide exchange activity of $G_o\alpha$ [Sudo *et al.*, 1992]. The acylation apparently regulates the relative amounts of inactive, membrane associated, palmitoylated protein and active, cytosolic, unmodified protein [Sudo *et al.*, 1992]. Palmitoylation appears therefore to affect both protein-membrane and protein-protein interactions.

Serpentine receptors are also subject to post-translational modification by palmitic acid in their intracellular C-terminal tail. The effect this has on the function of the receptor is unclear. While some believe the modification has a positive effect on the receptor's function [Morrison *et al.*, 1991] others think that it has no effect [Karnik *et al.*, 1988; Karnik *et al.*, 1993;

Kennedy & Limbird, 1993] or even inhibit the receptors' ability to transduce signals [O'Dowd *et al.*, 1989]. The palmitic acid on rhodopsin has been shown to be in a lipid environment [Moench *et al.*, 1994], therefore the palmitoylation of these receptors may induce the formation of a fourth intracellular loop [Karnik *et al.*, 1988; Ovchinikov *et al.*, 1988; König *et al.*, 1989]. The palmitoylation of the β_2 adrenergic receptor has also been demonstrated to be reversible and regulated [Mouillac *et al.*, 1992]. The presence of this fourth loop is thus transient and regulatable. Bouvier and co-workers believe that the depalmitoylated receptor, thus possessing only 3 intracellular loops, is a target for desensitisation [Bouvier *et al.*, 1995]. Consequently palmitoylation of serpentine receptors may have a role in the regulation of transmembrane signalling by receptors.

Recently, the presence of palmitic acid has been found on G protein α subunits. This modification differs significantly from myristoylation in several key areas. The presence of myristate is restricted to some members of the G_i family, while palmitoylation is prevalent amongst α subunits of the other families. Of the α subunits studied to date, only $G_t\alpha$ lacks the fatty acid [Wedegaertner *et al.*, 1995]. The post-translational addition of the fatty acid is via a labile thio-ester bond, making regulation of palmitoylation a possibility. This regulation of palmitoylation has been demonstrated for G protein α subunits [Degtyarev *et al.*, 1993b; Mumby *et al.*, 1994; Wedegaertner *et al.*, 1994], receptors [Mouillac *et al.*, 1992] and other proteins [Huang, 1989; Paige *et al.*, 1993]. Regulation of the co-translational addition of myristic acid is not possible as it is via a stable amide bond.

How palmitoylation of G protein α subunits affects their membrane association is a contentious subject at present. In this study it has been shown that chemical removal of palmitic acid releases approximately 30% of immunoreactive $G_{i2}\alpha$, $G_{o1}\alpha$, $G_{o2}\alpha$, $G_q\alpha$, $G_{11}\alpha$ and a lesser amount of $G_s\alpha$ from cell membranes. This would indicate that palmitoylation contributes to, but does not entirely govern, the membrane association of these α subunits. Clearly the presence of myristic acid may increase the hydrophobicity of the $G_i\alpha$ and $G_o\alpha$ proteins to cause a slight increase in corresponding membrane association, but as the $G_q\alpha$ and $G_{11}\alpha$ proteins are not subject to myristoylation, another mechanism must exist to promote the full membrane interactions of these proteins.

It is unclear why $G_s\alpha$ was not as susceptible to release from the membrane following hydroxylamine treatment as other α subunits under these conditions. Audigier *et al.* [1990] have hypothesised that a sequence, TENIR, near the C terminus of $G_s\alpha$ targeted the protein to the membrane. This theory has recently been challenged [Degtyarev *et al.*, 1994a], as mutagenesis of the analogous site in $G_{i1}\alpha$ to encode TENIR produces no change in the membrane association of that polypeptide. Therefore, $G_s\alpha$ may contain another factor which contributes to its membrane association.

Stable expression of wild type and C3S mutant forms of the $G_{o1}\alpha$ protein in Rat-1 cells produced an immunoreactive $G_o\alpha$ band in the cytosolic fraction of the mutant expressing cells. Here, following breakage of the cells, approximately 30% of immunoreactive, palmitate negative, C3S $G_{o1}\alpha$ was found in the cytosolic fraction of these cells. Three possible interpretations exist to explain this. First, 30% of C3S $G_{o1}\alpha$ is present in the

cytosol at all times in these cells *in vivo*. Secondly, all the C3S $G_{o1}\alpha$ is present at the membrane in the whole cell and the 30% found in the cytosolic fraction only redistributes there upon lysis of the cells in a hypotonic buffer. Finally, the C3S $G_{o1}\alpha$ may be cycling between membrane associated and cytosolic compartments and that these fractions are trapped when the cell is broken.

The experiments involving pertussis toxin (**Figures 4. 6.** and **4. 7.**) exclude the first possibility. Fractionation of wild type and C3S mutant $G_{o1}\alpha$ expressing cells following *in vivo* pertussis toxin treatment indicate that all of the C3S $G_{o1}\alpha$ in the cell, whether subsequently located in the membrane or cytosolic fraction following disruption, is a substrate for ADP-ribosylation by the toxin. Both the *in vitro* pertussis toxin catalysed ADP-ribosylation of cytosolic fractions from these cells and the gel filtration data (**Figures 4. 7.** and **4. 8.**), indicated that the cytosolic fraction of C3S $G_{o1}\alpha$ was present as a free α subunit and that there were no $\beta\gamma$ dimers present in the cytosol. Thus all the C3S $G_{o1}\alpha$ must have been located at the membrane at some point. Whether it was all bound to the membrane *in vivo* until the cell was lysed, or was cycling on and off the membrane cannot be addressed by these experiments. What is clear from these experiments is that palmitoylation of $G_{o1}\alpha$ increases the avidity of interaction of this α subunit with the plasma membrane. Recent evidence points to the cycling hypothesis. Scanning laser confocal microscopic studies indicate that, while the $G_{o1}\alpha$ of wild type transfected cells is located only at the membrane, C3S $G_{o1}\alpha$ is present in the cytosol in the whole cell [M. A. Grassie & G. Milligan unpublished observations]

Other groups have come to different conclusions. Experiments by Bourne and co-workers, using mutants of $G_s\alpha$ and $G_q\alpha$, in which the target cysteine residues were replaced by serines, demonstrated that these mutant α subunits were entirely cytosolic in cellular location, indicating that $G_s\alpha$ and $G_q\alpha$ were wholly dependent on palmitoylation for their membrane interaction [Wedegaertner *et al.*, 1993]. Other groups have found that palmitoylation of $G_s\alpha$ had no detectable part to play in the membrane association of the α subunit [Degtyarev *et al*, 1993a; Linder *et al*, 1993; Mumby *et al*, 1994]. These two groups employed a C3A mutant of $G_s\alpha$ to answer this question, whilst Wedegaertner *et al.*, [1993], like this report, employed a C3S mutation. Whether this discrepancy is responsible for the differences observed in terms of membrane interaction is unclear.

Wedegaertner *et al.*, [1993] have postulated that palmitoylation affects the function of G protein α subunits by also affecting protein-protein interactions. Neither the palmitate negative $G_s\alpha$ nor the palmitate negative $G_q\alpha$ were able to interact with and stimulate their effector systems, adenylyl cyclase and phosphoinositidase C respectively. Thus it would appear that like the other lipidations described here, palmitoylation can influence both the protein-membrane and protein-protein interactions of G proteins

Recent work has shown that the palmitoylation status of G proteins, and indeed Src family members, can be regulated. The half life of palmitate on $G_s\alpha$ is approximately 50-90mins [Degtyarev *et al.*, 1993b; Wedegaertner & Bourne, 1994] while the half life of the protein is approximately 11-22hrs [Levis & Bourne, 1992; Degtyarev *et al.*, 1993b]. The rate of turnover of palmitate on $G_s\alpha$ can be increased to around 2mins, beyond

that of the polypeptide itself (approximately 6hrs), upon stimulation of the β_2 adrenergic receptor of S49 *cyc*$^-$ cells stably expressing epitope tagged $G_s\alpha$ with isoprenaline [Levis & Bourne, 1992; Wedegaertner & Bourne, 1994]. Constitutive activation of $G_s\alpha$, either mutationally by the introduction of the R201C constitutively activating mutation or by treatment with cholera toxin, also led to an increase in palmitate turnover [Degtyarev *et al*, 1993b; Mumby *et al*, 1994; Wedegaertner & Bourne, 1994]. Whether this means a net increase, or decrease, in the total pool of palmitoylated $G_s\alpha$ over non-palmitoylated protein is unclear as yet. The kinetics of stoichiometry indicated that there was a faster depalmitoylation of $G_s\alpha$ [Wedegaertner & Bourne, 1994].

Treatment of intact S49 *cyc*$^-$ cells with β_2 adrenergic agonists following introduction of epitope tagged $G_s\alpha$ caused a change in the stability of the $G_s\alpha$ protein and a translocation of the polypeptide from the membrane to a cytosolic fraction [Levis & Bourne, 1992]. Given that palmitoylation may increase the membrane association of $G_s\alpha$ and that activation can cause an increase in turnover of palmitate on the protein, this may provide a molecular mechanism for this translocation [Wedegaertner & Bourne, 1994].

Of course the question of how $G_{o1}\alpha$ palmitoylation affects effector interaction and is affected by receptor occupancy cannot be answered by the C5B and D3 cell lines employed in this study, as the Rat-1 fibroblast is not known to express either the N-type calcium channel, to which the activated $G_o\alpha$ subunit couples, or any serpentine receptor which is known to activate $G_o\alpha$. The question of how palmitoylation affects the function of $G_o\alpha$ and how palmitoylation status of the protein is regulated could be addressed by

transfection of other components of the signalling cascade into these cells. Regulation of palmitoylation could be also addressed by the use of the neuronal cell line NG108-15 which are known to express $G_o\alpha$ isoforms and opioid receptors which activate them [Hamprecht *et al*, 1985; Mullancy & Milligan, 1990; **Chapter 3**].

One recent fact to emerge from studies by a number of groups is the requirement of co-translational myristoylation of G_i family members for subsequent post-translational palmitoylation. It has been demonstrated that gly2 mutants not only do not incorporate myristic acid but are not substrates for the palmitoyl transferase either [Hallak, *et al.*, 1994; Galbiati, *et al.*, 1994]. However, Parenti and co-workers have demonstrated that it is not the lack of myristate which prevents the subsequent palmitoylation, but the presence of alanine at position 2, as opposed to glycine, which causes this effect. They have demonstrated that inhibition of NMT using 2-hydroxymyristate prevents myristoylation, but not palmitoylation, of $G_{i1}\alpha$ [F. Galbiati, F. Guzzi & M. Parenti, personal communication]. The converse of this, that creation of the C3S mutant of $G_{i1}\alpha$ or $G_{o1}\alpha$ prevents myristoylation, is not true however, as the C3S mutants are still substrates for NMT [Parenti *et al.*, 1993; Galbiati *et al.*, 1994]. This observation is important for the conclusions drawn in this study. Recent observations indicate that the function of myristoylation of members of the G_i family of α subunits is to increase the hydrophobicity of the molecules just sufficiently to cause a transient association of the polypeptide with the membrane. As has previously been mentioned myristoylation provides barely enough energy to promote membrane association [Peitzsch & McLaughlin, 1993]. This transient association may then allow a membrane associated palmitoyl transferase to post translationally

add palmitate to the proteins thus facilitating full membrane interaction [Degtyarev *et al*, 1994b]. It is not the presence of myristic acid or a glycine residue at position 2, but the location of the α subunit at the plasma membrane which allows palmitoylation of $G_i\alpha$ subunits. This begs the question, what promotes the membrane association of non-myristoylated α subunits such as $G_s\alpha$ and $G_q\alpha$?

This insight calls into question much of the data concerning the effects of myristoylation on $G\alpha$ function, as many of the studies, including that of Gallego *et al.* [1992] using the *Gip2* oncoprotein, as it utilised a G2A mutation. Indeed, only the observations of Linder *et al*, [1991] can be relied upon as these workers studied $G_o\alpha$ myristoylation by expression of the $G_o\alpha$ polypeptide with or without co-expression of NMT in *E.coli* rather than creating G2A mutants. It also counsels caution in interpreting results obtained in mutagenesis studies; the correct controls must be performed in order to ensure that the observed effect is not artefactual.

This observation may also clear up a puzzle posed by van der Neut *et al.* [1993]. While $G_s\alpha$ is not subject to co-translational myristoylation, generation of a G2A $G_s\alpha$ nevertheless produces a protein with a reduced capacity to activate adenylyl cyclase. The presence of the alanine at position 2 of $G_s\alpha$ may, as it does with $G_{i1}\alpha$ and $G_o\alpha$, prevent palmitoylation of $G_s\alpha$ and it may be that this is responsible for the observed reduction in effector coupling.

If this proves to be correct, another observation may be made. Given the requirement for gly2 for the addition of palmitate to G_i and G_s

family members and the absence of this glycine residue at an analogous position in members of the G_q family may point to the presence of isozymes of the palmitoyl transferase. Analysis of the sequences of known palmitoylated proteins indicates little or no conservation which would reveal a consensus sequence for an individual transferase. Also given that the turnover of palmitate in response to activation is specific [Degtyarev *et al*, 1993b; Mumby *et al*, 1994; Wedegaertner & Bourne, 1994], the presence of isozymes of palmitoyl transferase with distinct and discrete specificities is possible. This specificity of palmitate turnover may also provide evidence for the presence of isozymes of the thio-esterase responsible for the cleavage of palmitate from G protein α subunits. Therefore, either there exists specific isozymes of the thio-esterase or one thio-esterase enzyme can be differentially regulated to produce specific effects.

This study has demonstrated that palmitoylation of $G_{o1}\alpha$ increases its avidity of interaction with the plasma membrane. It would appear that lack of palmitoylation of this α subunit does not abolish membrane interaction, as it apparently does for $G_q\alpha$, but instead affects the degree of its interaction. One thing is clear, although the palmitoylated residue lies in the proposed $\beta\gamma$ binding site, the C3S $G_{o1}\alpha$ is still able to interact with $\beta\gamma$ to a degree indistinguishable from that of the wild type protein, as assessed by their ability to act as substrates for pertussis toxin. It may be that palmitoylation increases the affinity of $G_{o1}\alpha$ for $\beta\gamma$, as myristoylation does, but unfortunately the lack of a pure source of $\beta\gamma$ prevented consideration of this point within these studies. It also remains to be seen whether palmitoylation of $G_{o1}\alpha$ affects its protein-protein interactions with either receptors or effectors as it apparently does for other α subunits.

Chapter 5.

Dual palmitoylation of murine $G_{11}\alpha$ regulates its membrane association.

Chapter 5.

Dual palmitoylation of murine $G_{11}\alpha$ regulates its membrane association.

5. 1. Introduction.

Some members of the G_i family of α subunits are dually acylated by myristic acid on gly2 and palmitic acid on cys3 [Parenti *et al.*, 1993; Hallak *et al.*, 1994; Galbiati *et al.*, 1994; Casey, 1994; Wedegaertner & Bourne, 1995]. $G_s\alpha$ is also subject to palmitoylation on cys3 [Linder *et al.*, 1993; Degtyarev *et al.*, 1993a; Wedegaertner *et al.*, 1993; Casey, 1994; Wedegaertner & Bourne, 1995]. Members of the G_q family of α subunits, which have also been shown to incorporate [^{3}H] palmitic acid [Parenti *et al.*, 1993; Wedegaertner *et al.*, 1993], do not possess a cysteine residue at this position however. Sequence analysis of $G_q\alpha$, $G_{11}\alpha$ and $G_{16}\alpha$ indicate the presence of only 2 cysteines near the N terminus [Strathmann & Simon, 1990; Amatruda *et al.*, 1991]. These cysteine residues, located at positions 9 and 10 in the primary amino acid sequences, do however align with the cys3 residue of $G_s\alpha$, $G_{i1}\alpha$ and $G_o\alpha$. Thus either cys9, cys10, or both, may be the targets for palmitoylation.

The neurite growth cone associated protein GAP-43 is dually palmitoylated on adjacent cysteine residues [Skene & Virág, 1989]. This modification has been shown to promote membrane interaction of GAP-43 [Skene & Virág, 1989; Zuber *et al.*, 1989] and to prevent the stimulation of the

GTPase activity of $G_o\alpha$ [Sudo *et al.*, 1992]. The possibility therefore exists for the dual acylation of members of the G_q family of α subunits.

Recent evidence from Wedegaertner *et al.* [1993] indeed indicated that $G_q\alpha$ is dually palmitoylated on cys9 and cys10. Production of mutant forms of the α subunit in which either cys9 or cys10 had been replaced with serine residues produced proteins with reduced ability to incorporate [^{3}H] palmitic acid. Only approximately 30% of the detectable radioactivity of the wild type protein was found in these mutants. Production of the double C9SC10S mutant abolished detectable [^{3}H] palmitate incorporation [Wedegaertner *et al.*, 1993].

This unpalmitoylated C9SC10S $G_q\alpha$ was unable to bind to the membrane at all; all the immunodetectable protein was located in the cytosol. In addition, the C9SC10S protein was unable to stimulate the phosphoinositidase C catalysed hydrolysis of phosphatidylinositol-4,5-bisphosphate, either upon stimulation with an α_2 adrenergic receptor agonist, or when the α subunit was directly activated by introduction of the constitutively active R183C mutant. The wild type and single mutant proteins were all able to stimulate phosphatidylinositol-4,5-bisphosphate hydrolysis. The ability of the palmitate negative mutant to stimulate phosphoinositidase C was restored by mutational insertion of a consensus sequence for N-myristoylation [Wedegaertner *et al.*, 1993]. Therefore lipidation of $G_q\alpha$ by palmitic acid is apparently obligatory for normal function of the polypeptide.

This chapter aimed to ascertain the effect of palmitoylation of $G_{11}\alpha$ on its membrane interaction. One problem in examining this question is

the ubiquity of both $G_q\alpha$ and $G_{11}\alpha$ in all known cell types. Some groups have utilised an influenza virus haemagglutinin epitope tag to overcome this problem [Gallego *et al.*, 1992; Wedegaertner *et al.*, 1993]. This approach was mutational, and while no difference was found between wild type and HA tagged proteins, an effect on the function of the protein cannot be ruled out. Due to the lack of a $G_{11}\alpha$ selective antisera, this study employed the concurrent detection method described in Kim & Milligan [1994] and in **Chapter 3**, using a non-selective $G_q\alpha/G_{11}\alpha$ antiserum.

Using this system, wild type, C9S, C10S and C9SC10S mutants of murine $G_{11}\alpha$ were analysed in simian COS-1 cells by transient transfection. This chapter details the production of the mutants and assesses their membrane association and ability to incorporate [^{3}H] palmitic acid. Both cys9 and cys10 of murine $G_{11}\alpha$ are targets for palmitoylation. Furthermore, there may exist a degree of co-operativity during the palmitoylation; the C9S and C10S mutants each incorporated less than 50% of the detectable radioactivity present on the wild type protein. All three mutant proteins had a decreased ability to interact with the plasma membrane and their respective abilities to interact with the membrane were found to be indistinguishable. These studies indicate a difference between $G_q\alpha$ and $G_{11}\alpha$ in terms of the effect of palmitoylation on their function.

5. 2. Results.

5. 2. 1. Subcloning of murine $G_{11}\alpha$ into pSV Sport 1.

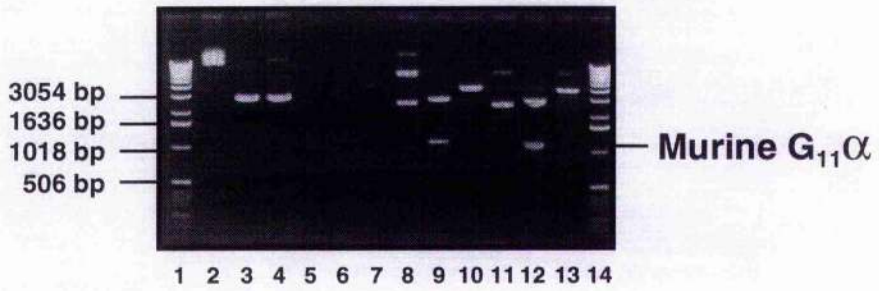
Murine $G_{11}\alpha$ was kindly donated by Dr. Melvin I. Simon, California Institute of Technology, CA, USA, in the mammalian expression vector pCMV. Lack of knowledge of this plasmid necessitated the sub-cloning of the cDNA insert into a vector for mutagenesis. To this end, the murine $G_{11}\alpha$ cDNA was excised from the pCMV vector using the restriction endonucleases Cla I and Xho I, purified from an agarose gel slice (**sections 2. 22 - 2. 24.**) and the insert ligated into the vector pGEM 7Z(f+) (**section 2. 27.**). Ligated DNA was then transformed into JM109 *E.coli* (**section 2. 16.**) Ampicillin resistant clones were tested for the presence of the murine $G_{11}\alpha$ cDNA by digestion of resultant plasmid DNA with Cla I and Xho I. A photograph of the resulting agarose gel is displayed in **figure 5. 1.**

Clone number 2 was selected for expansion and further subcloning into the vector pSV Sport 1, a full nucleotide sequence for which was available. This was achieved by initial digestion of pGEM 7Z(f+)-$G_{11}\alpha$ with Apa I, followed by treatment with Klenow DNA polymerase to create a blunt end (**section 2. 26.**). This was subsequently digested with Hind III and the fragment separated by electrophoresis on an agarose gel. Between each of these steps, the DNA was purified from enzymes and salts using Promega Clean-Up columns. The $G_{11}\alpha$ insert was then purified from the agarose gel and ligated to pSV Sport 1 which had been digested with Sma I and Hind III and purified from a gel slice. This ligated DNA was transformed into JM109

Figure 5. 1. Subcloning of murine $G_{11}\alpha$ into pGEM 7Zf(+).

1μg of murine $G_{11}\alpha$ in the vector pCMV, donated by Dr. M.I. Simon, was cut with 5 units each of the restriction enzymes Xho I and Cla I and the cDNA corresponding to $G_{11}\alpha$ was purified from an 0.8% (w/v) agarose gel using Promega PCR preps. This was then ligated into the vector pGEM 7Zf(+) which had been similarly digested and purified. Ligated DNA was then transformed into competent JM109 cells and ampicillin resistant colonies were expanded and plasmid DNA from these was assessed for the presence of murine $G_{11}\alpha$ by digestion with Xho I and/or Cla I. The resulting 0.8% (w/v) agarose gel from this digest is displayed. Lanes 1 and 14 contained 1kb DNA ladder. Lane 2, 3 and 4 contained, respectively, 200ng pGEM 7Zf(+) which was undigested, digested with Cla I and Xho I or digested with Cla I alone. Lanes 5-7 contained, respectively, 200ng of DNA isolated from clone 1 which was undigested, digested with Cla I and Xho I or digested with Cla I alone. Lanes 8-10 contained, respectively, 200ng of DNA isolated from clone 2 which was undigested, digested with Cla I and Xho I or digested with Cla I alone. Lanes 11-13 contained, respectively, 200ng of DNA isolated from clone 3 which was undigested, digested with Cla I and Xho I or digested with Cla I alone. The mobility of murine $G_{11}\alpha$ and of the DNA standards is shown.

Figure 5.1


3054 bp
1636 bp
1018 bp
506 bp
Murine $G_{11}\alpha$
1 2 3 4 5 6 7 8 9 10 11 12 13 14

E.coli and ampicillin colonies expanded, plasmid DNA isolated and the presence of $G_{11}\alpha$ cDNA detected by digestion of plasmid DNA with Cla I and Xho I. Samples of these digests are shown in **figure 5. 2.** Clone number 3 was selected for mutagenesis of the $G_{11}\alpha$ insert. These subcloning steps had one drawback in that the cDNA insert was now present in the antisense direction with respect to the SV40 early promoter of the pSV Sport 1. This necessitated, as described in the following section, the further subcloning of resulting mutant $G_{11}\alpha$ cDNAs back into the original pCMV vector to allow expression in COS-1 cells.

5. 2. 2. Generation of cys9 and cys10 mutants.

Clone 2 plasmid DNA was used as a template for mutagenesis of the murine $G_{11}\alpha$ cDNA. A pair of primers, from the list in **table 5. 1.**, were first phosphorylated as described **in section 2. 28.** before being used to amplify the entire pSV Sport 1-$G_{11}\alpha$ plasmid. *Pfu* DNA polymerase was the enzyme of choice instead of *Taq* DNA polymerase as *Pfu* contains a 5'-3' proof reading activity and lacks the adenylyl transferase-like activity of *Taq*. This not only results in an increase in fidelity of amplification but also results in the production of blunt -ended DNA.

Figure 5. 2. Subcloning of murine $G_{11}\alpha$ into pSV Sport 1.

1μg of clone 2 DNA was cut with Apa I, purified from an agarose gel, the 5' overhangs filled in with Klenow DNA polymerase, cleaned up using Promega Cleanup columns and cut with Hind III. Plasmid pSV Sport 1 was prepared by digestion with Hind III and Sma I and purified from an agarose gel. Ampicillin resistant colonies from the resulting transformation of ligated DNA were expanded and plasmid DNA from these isolated. The presence of murine $G_{11}\alpha$ was assessed by digestion of this DNA with Cla I and/or Xho I. The resulting agarose gel is shown. Lanes 1 and 14 contained 1kb DNA ladder and the mobilities of some of its components are indicated. Lanes 2-4 contained 200ng of pSv Sport 1 which was undigested, digested with Cla I alone or digested with Cla I and Xho I respectively. Lanes 5-7 contained 200ng of DNA isolated from clone 1 which was undigested, digested with Cla I alone or digested with Cla I and Xho I respectively. Lanes 8-10 contained 200ng of DNA isolated from clone 2 which was digested with Cla I alone, digested with Cla I and Xho I or was undigested respectively. Lanes 11-13 contained 200ng of DNA isolated from clone 3 which was digested with Cla I and Xho I, digested with Cla I alone or was undigested respectively. The mobility of murine $G_{11}\alpha$ is indicated.

Figure 5.2

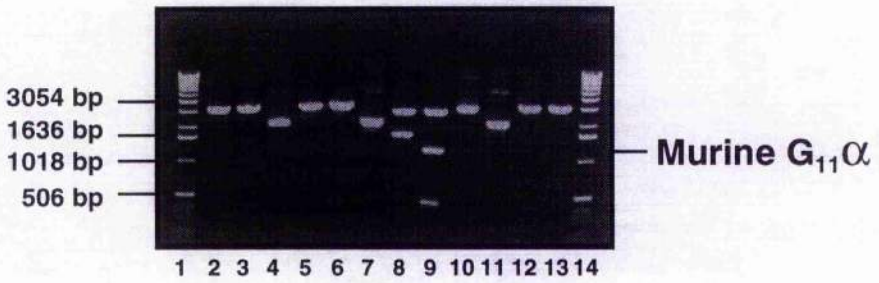

3054 bp
1636 bp
1018 bp
506 bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Murine $G_{11}\alpha$

Table 5. 1. Oligonucleotides used in the production of C9S, C10S and C9SC10S mutants of $G_{11}\alpha$.

The oligonucleotides primers used to prepare the mutants of murine $G_{11}\alpha$ are displayed. For each mutant a pair of oligonucleotides were used, one sense primer and one antisense primer. These were first phosphorylated before being used in a PCR reaction using *Pfu* DNA polymerase. Phosphorylation of these primers was required to facilitate self-ligation of the amplified DNA. The mutagenic bases are shown in bold italics.

Table 5. 1.

Mutation	Sense Oligo	Antisense Oligo
C9S $G_{11}\alpha$	5' TGCCTGAGCGACGAGGTG 3'	5' A*G*ACGCCATCATGGACTCCAG 3'
C10S $G_{11}\alpha$	5' T*C*CCTGAGCGACGAGGTG 3'	5' ACACGCCATCATGGACTCCAG 3'
C9SC10S $G_{11}\alpha$	5' T*C*CCTGAGCGACGAGGTG 3'	5' A*G*ACGCCATCATGGACTCCAG 3'

The plasmid DNA was purified on a gel slice and self-ligated. This DNA was then used to transform JM109 *E.coli* and ampicillin resistant colonies selected. Plasmid DNA from these transformants was isolated and, in the case of the C9S and C9SC10S mutants, digested with a diagnostic restriction endonuclease, BsaH I, as the creation of these mutants introduced an additional site for this enzyme. These restriction digests are displayed in **figure 5. 3.** Ampicillin resistant clones resulting from the C10S mutagenesis could not be assayed by restriction digestion as the mutagenesis did not introduce or remove any restriction sites.

All samples which were of interest were then subjected to DNA sequencing on an ABI automated sequencer to verify the integrity of amplification (see footnote*) . The relevant section of the sequences is displayed in **figure 5. 4.** To prevent sequencing the entire plasmid, a short segment, covering the mutated stretch of the $G_{11}\alpha$ cDNA and some of the pSV Sport 1 backbone was excised using the restriction endonucleases Sac I and Nhe I and subcloned back into wild type pSV Sport 1-$G_{11}\alpha$ which had not undergone PCR amplification.

The presence of the desired mutation was tracked by the diagnostic restriction digests and/or DNA sequencing. Following this, the entire $G_{11}\alpha$ cDNA was then subcloned back into the original pCMV vector by excision using Cla I and Xho I as before. Again, the presence of the required mutation was tracked by diagnostic restriction digests and/or DNA

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* Sequencing of mutant DNA in pSV Sport 1 was achieved using the T7 promoter sequencing primer (TAATACGACTCACTATAGGG). Sequencing of mutant DNA in pCMV was achieved using an internal primer, in the antisense direction, CATCGACCTCCCGGATCAGG.

Figure 5. 3. Preparation of C9S and C9SC10S mutants of murine $G_{11}\alpha$ alters its Bsa HI digestion pattern.

Following PCR amplification of pSV Sport 1-$G_{11}\alpha$ with mutagenic oligonucleotides, the DNA was self ligated and amplicillin clones selected from the resulting transformation expanded. DNA from each of these was isolated and for C9S and C9SC10S mutants, this was digested with Bsa HI. The resulting agarose gel is shown. Lanes 1 and 8 contained 1kb DNA ladder with the mobilities of some fragments indicated. Lanes 2 and 3 contained wild type pSV Sport 1-$G_{11}\alpha$ which was uncut or cut with Bsa HI respectively. Lanes 4 and 5 contained pSV Sport 1-C9S$G_{11}\alpha$ which was uncut or cut with Bsa HI respectively. Lanes 6 and 7 contained pSV Sport 1-C9SC10S$G_{11}\alpha$ which was uncut or cut with Bsa HI respectively. The different restriction patterns of pSV Sport 1-C9S$G_{11}\alpha$ and pSV Sport 1-C9SC10S$G_{11}\alpha$ are shown.

Figure 5.3

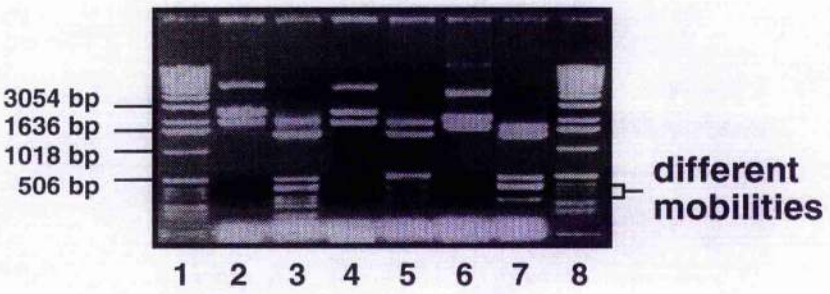
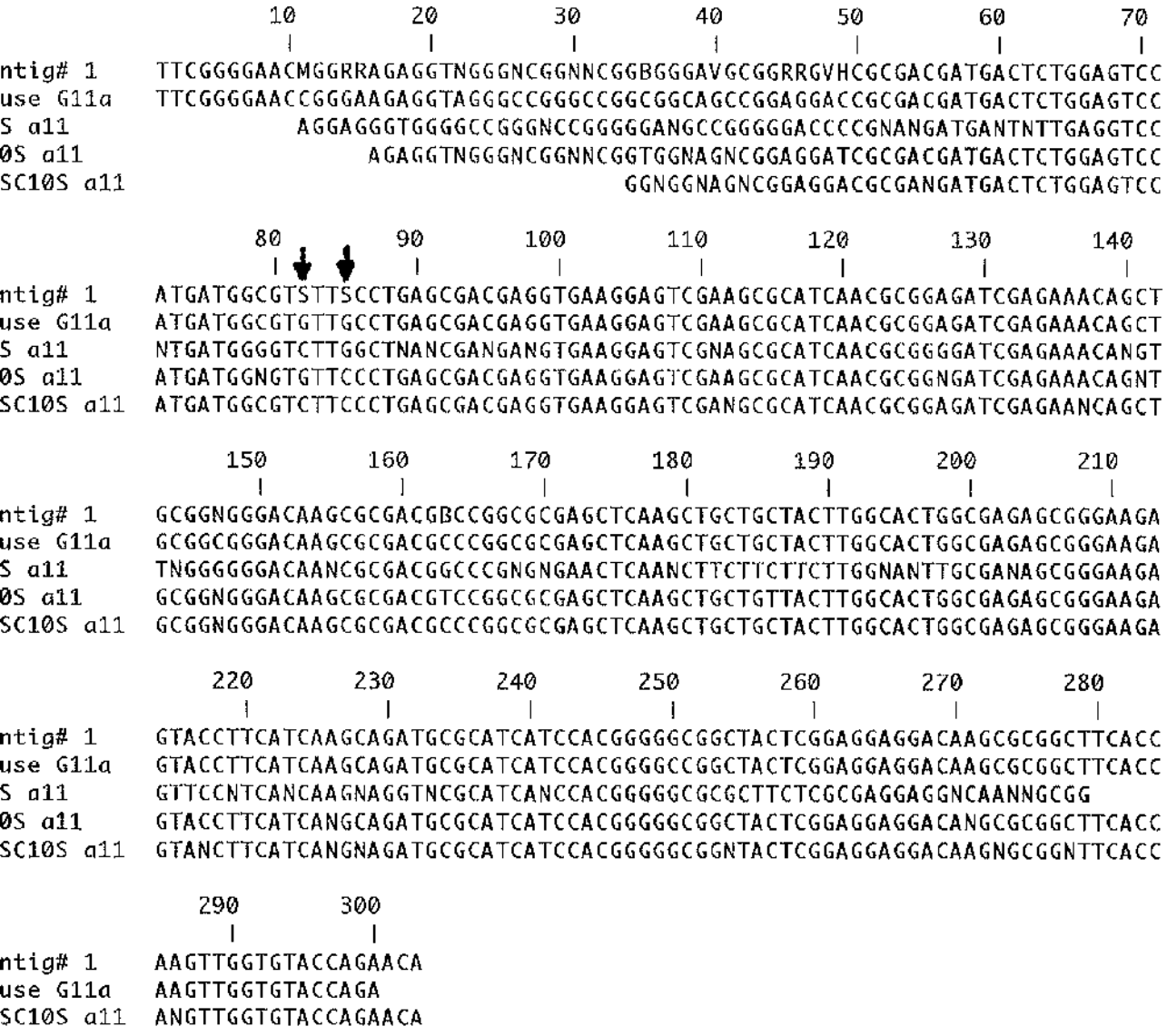

3054 bp
1636 bp
1018 bp
506 bp
different
mobilities
1 2 3 4 5 6 7 8

Figure 5. 4. Sequence of isolated clones of $G_{11}\alpha$ mutants.

Automated sequencing of pSV Sport 1-C9S$G_{11}\alpha$, pSV Sport 1-C10S$G_{11}\alpha$ and pSV Sport 1-C9SC10S$G_{11}\alpha$ was carried out on DNA from colonies with different restriction patterns (pSV Sport 1-C9S$G_{11}\alpha$ and pSV Sport 1-C9SC10S$G_{11}\alpha$) and all amplicillin resistant clones (pSV Sport 1-C10S$G_{11}\alpha$). The sequences of one clone used for further study from each mutant was aligned with wild type pSV Sport 1-$G_{11}\alpha$ using GeneJockey II on an Apple Macintosh Quadra 800. This alignment is displayed. The sites of mutagenesis of $G_{11}\alpha$ are shown as arrows.

Figure 5. 4.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use G11a TTCGGGGAACCGGGAAGAGGTAGGGCCGGGCCGGCGGCAGCCGGAGGACCGCGACGATGACTCTGGAGTCC
S a11 AGGAGGGTGGGGCCGGGNCCGGGGGANGCCGGGGGACCCCGNANGATGANTNTTGAGGTCC
0S a11 AGAGGTNGGGNCGGNNCGGTGGNAGNCGGAGGATCGCGACGATGACTCTGGAGTCC
SC10S a11 GGNGGNAGNCGGAGGACGCGANGATGACTCTGGAGTCC
80 90 100 110 120 130 140
ntig# 1 ATGATGGCGTSTTSCCTGAGCGACGAGGTGAAGGAGTCGAAGCGCATCAACGCGGAGATCGAGAAACAGCT
use G11a ATGATGGCGTGTTGCCTGAGCGACGAGGTGAAGGAGTCGAAGCGCATCAACGCGGAGATCGAGAAACAGCT
S a11 NTGATGGGGTCTTGGCTNANCGANGANGTGAAGGAGTCGNAGCGCATCAACGCGGGGATCGAGAAACANGT
0S a11 ATGATGGNGTGTTCCCTGAGCGACGAGGTGAAGGAGTCGAAGCGCATCAACGCGGNGATCGAGAAACAGNT
SC10S a11 ATGATGGCGTCTTCCCTGAGCGACGAGGTGAAGGAGTCGANGCGCATCAACGCGGAGATCGAGAANCAGCT
150 160 170 180 190 200 210
ntig# 1 GCGGNGGGACAAGCGCGACGBCCGGCGCGAGCTCAAGCTGCTGCTACTTGGCACTGGCGAGAGCGGGAAGA
use G11a GCGGCGGGACAAGCGCGACGCCCGGCGCGAGCTCAAGCTGCTGCTACTTGGCACTGGCGAGAGCGGGAAGA
S a11 TNGGGGGGACAANCGCGACGGCCCGNGNGAACTCAANCTTCTTCTTCTTGGNANTTGCGANAGCGGGAAGA
0S a11 GCGGNGGGACAAGCGCGACGTCCGGCGCGAGCTCAAGCTGCTGTTACTTGGCACTGGCGAGAGCGGGAAGA
SC10S a11 GCGGNGGGACAAGCGCGACGCCCGGCGCGAGCTCAAGCTGCTGCTACTTGGCACTGGCGAGAGCGGGAAGA
220 230 240 250 260 270 280
ntig# 1 GTACCTTCATCAAGCAGATGCGCATCATCCACGGGGGCGGCTACTCGGAGGAGGACAAGCGCGGCTTCACC
use G11a GTACCTTCATCAAGCAGATGCGCATCATCCACGGGGCCGGCTACTCGGAGGAGGACAAGCGCGGCTTCACC
S a11 GTTCCNTCANCAAGNAGGTNCGCATCANCCACGGGGGCGCGCTTCTCGCGAGGAGGNCAANNGCGG
0S a11 GTACCTTCATCANGCAGATGCGCATCATCCACGGGGGCGGCTACTCGGAGGAGGACANGCGCGGCTTCACC
SC10S a11 GTANCTTCATCANGNAGATGCGCATCATCCACGGGGGCGGNTACTCGGAGGAGGACAAGNGCGGNTTCACC
290 300
ntig# 1 AAGTTGGTGTACCAGAACA
use G11a AAGTTGGTGTACCAGA
SC10S a11 ANGTTGGTGTACCAGAACA

sequencing. The mutants were then ready for transient transfection into COS-1 cells.

5. 2. 3. The effect of mutation on the incorporation [^{3}H] palmitate to murine $G_{11}\alpha$.

80μg of either wild type, C9S, C10S or C9SC10S murine $G_{11}\alpha$ in pCMV was transfected in COS-1 cells as described in **section 2. 2. 11.** Cells were labelled with [^{3}H] palmitate as described in **section 2. 2. 8.** Immunoprecipitates (**section 2. 5. 3.**) of whole cell lysates were subsequently analysed on a 6M urea/10% acrylamide gel to separate murine $G_{11}\alpha$ from the endogenous simian protein. Gels were then dried and autoradiographed (**section 2. 11.**). This is displayed in **figure 5. 5. a.** These autoradiograms were then quantitated on a GS-670 densitometer. These show that both single mutants incorporate less than 50% of the radioactivity of wild type $G_{11}\alpha$; both incorporated around 20% of the radiolabelled [^{3}H] palmitate (**figure 5. 5. b.**). The double mutant incorporated no detectable radioactivity at all. This was not a reflection of different amounts of protein loaded onto the lanes as there was no difference in the incorporation of [^{3}H] palmitate into endogenously expressed simian $G_q\alpha/G_{11}\alpha$ (**figure 5. 5. c.**).

5. 2. 4. The effect of palmitoylation on the membrane interaction of $G_{11}\alpha$.

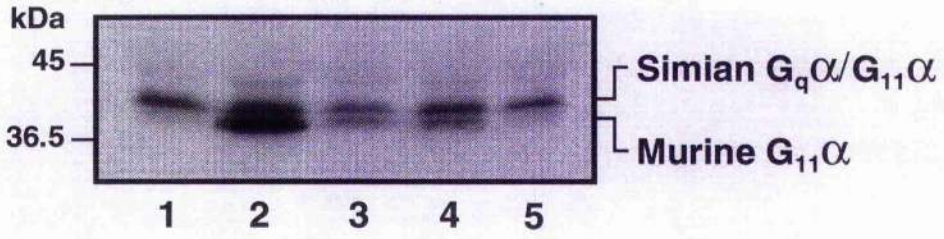
Following the attenuation of palmitate incorporation into the $G_{11}\alpha$ mutants, the effect of this on the membrane interactions of the polypeptides was assessed. Following transfection, cells were left for

approximately 72hrs before membrane and cytosolic fractions were prepared as described in **section 2. 3. 2.** These were analysed by urea containing SDS-PAGE for the presence of immunoreactive $G_q\alpha$ and $G_{11}\alpha$ in each fraction. An example of this experiment is displayed in **figure 5. 6. a.** Quantification of this experiment (**figure 5. 6. b.**) indicated that all three mutants had a decreased ability to interact with the plasma membrane; approximately 30% of the detectable murine $G_{11}\alpha$ immunoreactivity in each of the three mutant expressing transfected cells was present in the cytosolic fraction.

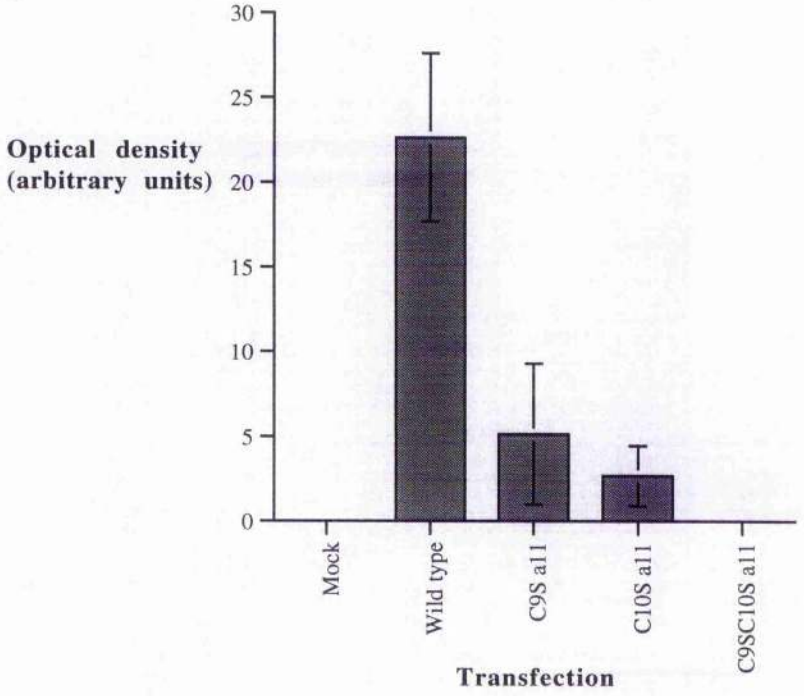
Figure 5. 5. Incorporation of [^{3}H] palmitic acid into wild type and mutant $G_{11}\alpha$.

80μg of DNA encoding either wild type $G_{11}\alpha$, C9S $G_{11}\alpha$, C10S $G_{11}\alpha$ or C9SC10S $G_{11}\alpha$ was transiently transfected into COS-1 cells for 68 hrs before being labelled with 200μCi/ml of [^{3}H] palmitic acid for a further 4 hrs. Whole cell lysates of these transfected cells were immunoprecipitated with antiserum CQ2 and these immunoprecipitates analysed by SDS-PAGE on a 6M urea containing 10 % (w/v) gel. The gel was then treated with EN^{3}HANCE and fluorographed for 50 days. this is shown in panel a. Lane 1 contained immunoprecipitates from mock transfected cells (no DNA), lane 2 contained immunoprecipitates from wild type $G_{11}\alpha$ transfected cells, lane 3 contained immunoprecipitates from C9S $G_{11}\alpha$ transfected cells, lane 4 contained immunoprecipitates from C10S $G_{11}\alpha$ transfected cells and lane 6 contained immunoprecipitates from C9SC10S $G_{11}\alpha$ transfected cells. The mobilities of endogenously expressed $G_q\alpha/G_{11}\alpha$ and of heterologously expressed $G_{11}\alpha$ are indicated. Quantitation of the amount of incorporated [^{3}H] palmitate was carried out for endogenously expressed $G_q\alpha/G_{11}\alpha$ (panel B.) and for heterologously expressed murine $G_{11}\alpha$ (panel C.) on a Bio-Rad GS670 imaging densitometer. The results are shown as the mean±half the difference of two separate experiments.

Figure 5.5



C


Optical density
(arbitrary units)
30
25
20
15
10
5
0
Mock
Wild type
C9S a11
C10S a11
C9SC10S a11
Transfection

B

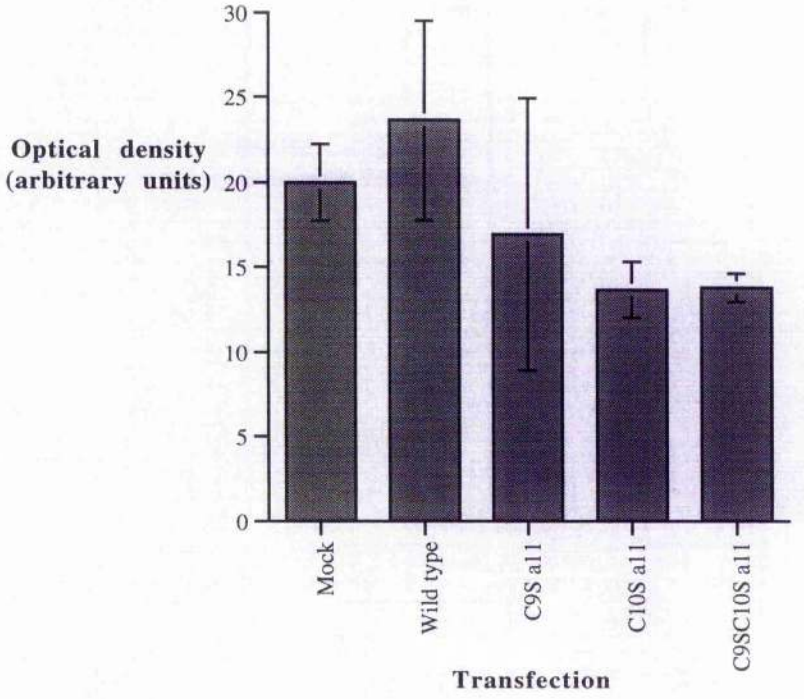
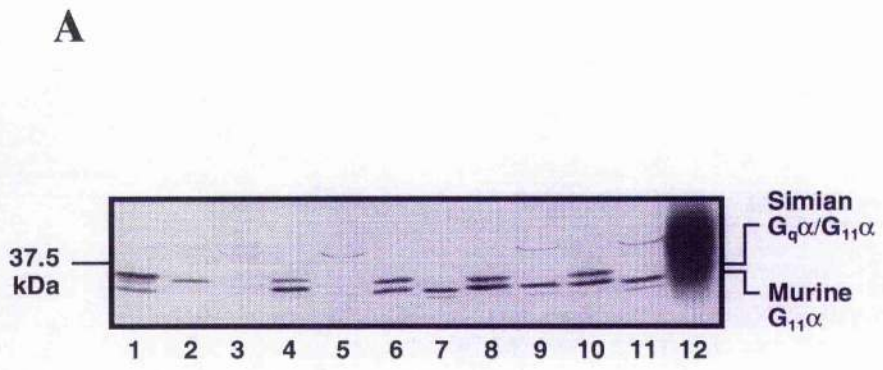

Optical density
(arbitrary units)
30
25
20
15
10
5
0
Mock
Wild type
C9S a11
C10S a11
C9SC10S a11
Transfection

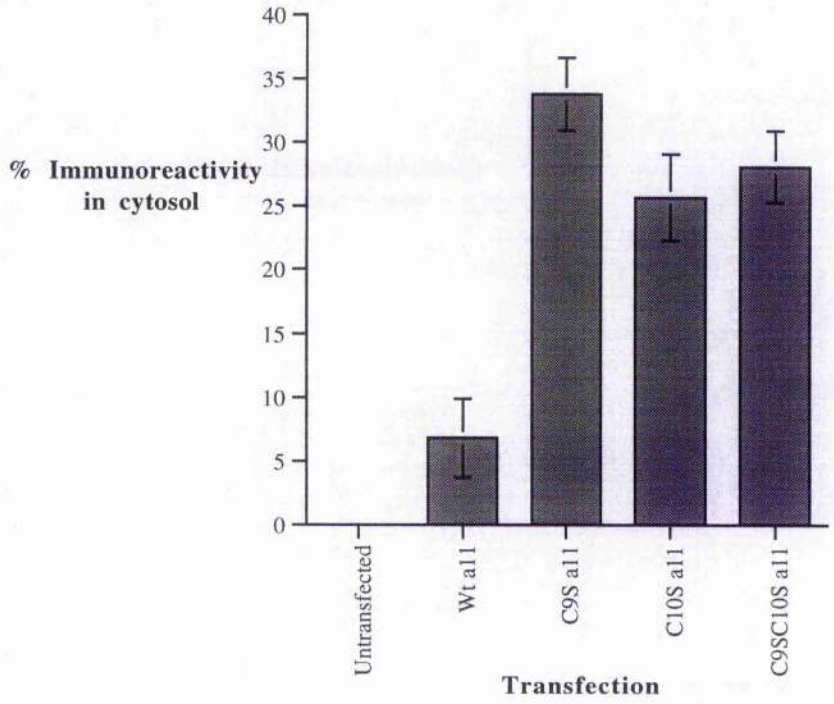
Figure 5. 6. Cellular distribution of wild type and mutant murine $G_{11}\alpha$ following transient transfection.

80μg of DNA encoding either wild type $G_{11}\alpha$, C9S $G_{11}\alpha$, C10S $G_{11}\alpha$ or C9SC10S $G_{11}\alpha$ was transiently transfected into COS-1 cells for 72 hrs before membrane and cytosolic fractions of each transfection were prepared. These fractions were analysed on a 6M urea containing 10% (w/v) polyacrylamide gel and the resulting immunoblot (panel A.) was probed with antiserum CQ2 at a dilution of 1:1000. Lane 1 contained 40μg of mouse brain membranes, Lanes 2, 4, 6, 8 and 10 contained 80μg of membranes from mock, wild type $G_{11}\alpha$, C9S $G_{11}\alpha$, C10S $G_{11}\alpha$ and C9SC10S $G_{11}\alpha$ expressing cells respectively, while lanes 3, 5, 7, 9, and 11 contained equivalent amounts of the cytosolic fractions of these transfectants. Lane 12 contained prestained lactic dehydrogenase marker. The identities of the endogenously expressed simian $G_q\alpha/G_{11}\alpha$ and the heterologously expressed murine $G_{11}\alpha$ are shown. Quantitation of the amount of murine $G_{11}\alpha$ present in the cytosol of each transfectant (panel B.) was expressed as a percentage of the total immunoreactive murine $G_{11}\alpha$ present in each transfectant. These results are shown as the mean±SEM of 4 separate experiments.

Figure 5.6


A
37.5
kDa
Simian
$G_q\alpha/G_{11}\alpha$
Murine
$G_{11}\alpha$
1 2 3 4 5 6 7 8 9 10 11 12

B


% Immunoreactivity in cytosol
40
35
30
25
20
15
10
5
0
Untransfected
Wt a11
C9S a11
C10S a11
C9SC10S a11
Transfection

5. 3. Discussion.

Wedegaertner *et al.* [1993] indicated that $G_q\alpha$ had to be palmitoylated in order to interact with the plasma membrane. Results presented here indicate that this may not be true for $G_{11}\alpha$, at least in COS-1 cells. It has been found that $G_{11}\alpha$ is only partially dependent on palmitoylation for its membrane association. This may reflect a different cellular environment from that employed by Bourne and colleagues; they utilised the human embryonic kidney cell line HEK 293 as opposed to the simian COS cell system employed here.

Evidence has also been found for co-operativity in transfer of the palmitate from the transferase to the α subunit; less than 50% of the radioactivity associated with wild type murine $G_{11}\alpha$ was found attached to either of the two single mutants. Similar levels of [^{3}H] palmitate incorporation of $G_q\alpha$ were found by Wedegaertner *et al.* [1993], who found that both C9S and C10S single mutants of $G_q\alpha$ incorporated less than 50% of the [^{3}H] palmitate found in the wild type protein.

The observed synergism of palmitoylation of $G_{11}\alpha$ may simply be due to the presence of the serine residues, as opposed to the cysteine residues normally found at these positions, causing a slight yet significant alteration in the conformation of the polypeptide preventing the palmitoyl transferase from properly recognising the intact cysteine residue. It is notable that palmitoylation of $G_z\alpha$ and $G_{i1}\alpha$ requires the presence of a glycine immediately prior to the target cysteine in order for the transfer of the palmitic acid to the α subunit [Hallak, *et al.*, 1994; Galbiati *et al.*, 1994]. The presence

of an alanine residue at this point, as has been discussed in the previous chapter, prevents palmitoylation. The transferase responsible for the palmitoylation of $G_{i1}\alpha$ is obviously very sensitive to even relatively conservative changes in the peptide environment. This of course does not preclude the possibility of synergism of palmitoylation of $G_{11}\alpha$, but caution in rushing to this conclusion should be exercised.

The recent discovery that myristoylation, or the presence of alanine at position 2, does not *per se* govern the subsequent palmitoylation of $G_{i1}\alpha$ [Degtyarev *et al.*, 1994b] but that the protein requires to be localised to the plasma membrane in order to be palmitoylated lends weight to the co-operativity theory of palmitoylation of $G_{11}\alpha$.

It would appear that however much palmitic acid is present on the mutant α subunits, the ability of these polypeptides to interact with the plasma membrane is essentially identical, perhaps indicating that a certain threshold of palmitoylation must occur to promote full membrane association. Approximately 30% of immunoreactive murine $G_{11}\alpha$ is present in the cytosolic fraction of transfected COS-1 cells, while all endogenous simian immunoreactivity is present in the membrane fraction. The value of 30% agrees well with that obtained for chemical depalmitoylation using hydroxylamine in **section 4. 2. 1.**

This system has several advantages over systems employed by other groups to study the process of palmitoylation. Many groups are now employing an epitope tagging technique to allow unambiguous detection of G protein α subunits in the absence of background levels of the particular G

protein [Gallego *et al.*, 1992; Levis & Bourne, 1992; Wedegaertner *et al.*, 1993; Wedegaertner & Bourne, 1994]. While these groups maintain that the mutagenesis of both $G_s\alpha$ and $G_q\alpha$ does not interfere with the normal functioning of the polypeptide, it may be that altering the α subunit to encode the tag sequence does indeed affect the polypeptide in some way, as yet not understood. The system employed in this study benefits from not having to introduce any mutations other than those required to study the palmitoylation status of the protein, and therefore represents a more physiologically relevant model. The endogenous $G_q\alpha$ and $G_{11}\alpha$ provided useful controls in all experiments. In the immunoblotting experiments, the endogenous $G_q\alpha$ and $G_{11}\alpha$ presented a useful control in ensuring that both the amount of protein loaded onto each lane was equivalent and that the transfection efficiency in these transiently transfected cells was the same for each murine $G_{11}\alpha$ cDNA which was introduced into the simian COS-1 cells. The endogenous proteins also ensured homogeneous labelling of the COS-1 cells with [^{3}H] palmitic acid. This was important in ensuring that the differences observed in the amounts of palmitic acid incorporated into the mutant murine cDNAs as opposed to the wild type proteins was a true reflection of the ability of these proteins to be palmitoylated and was not simply due to differences in the amounts of labelled [^{3}H] palmitate which were available for each transfected protein. This is something which other groups have not been able to demonstrate as easily [Degtyarev *et al.*, 1993b; Wedegaertner *et al.*, 1993]. Indeed, Degtyarev *et al.* [1993b] resorted to solubilisation of excised bands from their gels followed by liquid scintillation counting.

Both Degtyarev *et al.* [1993a] and Mumby *et al.* [1994] have found that mutagenesis of $G_s\alpha$ to prevent palmitoylation does not alter the

membrane association of the polypeptide. They employed a C3A mutation in order to assess this question, while this study, as well as that of Wedegaertner *et al.* [1993] have used C3S mutations. As the groups using serine mutations have found a translocation to the cytosol, it may be that the presence of alanine rather than serine increases the proteins' membrane affinity. The difference in membrane association of the various mutants may also be a reflection of the different cell types used by the various groups. While this study and Wedegaertner *et al.* [1993] used fibroblasts, Degtyarev *et al.* [1993a] and Mumby *et al.* [1994] used S49 lymphoma cells and insect Sf9 cells. Indeed Mumby *et al.* [1994] found a slight difference in the membrane association of C3A $G_s\alpha$ upon expression in Sf9 cells as opposed to S49 cells. Also recent work has shown that expression of the $G_{11}\alpha$ mutants employed in this study in HEK 293 cells are found entirely in the cytosol in agreement with the report of Wedegaertner *et al.* [1993] for $G_q\alpha$, although the $G_{11}\alpha$ located in the cytosol can still activate phosphoinositidase C [A. Wise & G. Milligan, unpublished observations].

Recent work by Degtyarev *et al.*, [1993b], Mumby *et al.* [1994] and Wedegaertner & Bourne [1994] have indicated that activation of $G_s\alpha$ leads to regulation of the palmitic acid present on the protein. Activation of $G_s\alpha$ by either incubation of cells with β_2 adrenergic agonist or by treatment with cholera toxin lead to an increase in the amount of [^{3}H] palmitic acid on the protein [Degtyarev *et al.*, 1993b]. This effect has been demonstrated to be due to an increase in the rate of turnover of the palmitate on the protein and may not reflect an actual increase in the amount of palmitate attached to the polypeptide [Mumby *et al.*, 1994; Wedegaertner & Bourne, 1994] as the stoichiometry of attachment is unknown [Mumby & Muntz, 1995]. This would

indicate that an activated $G_s\alpha$ leads to a change in its own palmitoylation status and perhaps this may give rise to a change in the subcellular location of the protein. In the present study we have not attempted to assess the rate of turnover of palmitate on $G_{11}\alpha$, due to the lack of a suitable endogenously expressed receptor, but given that receptors can alter the palmitoylation status of $G_q\alpha$ [Wedegaertner & Bourne, 1994], this phenomenon may reflect a more widespread effect on G protein α subunits.

Wedegaertner *et al.* [1993] also indicated a functional implication for depalmitoylation of $G_q\alpha$ and $G_s\alpha$. Mutated, palmitate negative forms of these proteins were found to be incapable of activating their respective effector enzymes. At least in the case of $G_q\alpha$, they employed a receptor (the α_{2A} adrenergic receptor) which was artificially introduced at high levels into the HEK 293 cells by transfection. There are several possible problems with this approach. First the α_{2A} adrenergic receptor is not thought to normally interact with $G_q\alpha$, and only overexpression of the receptor by transfection may be responsible for the activation of the G protein. A better approach to this question may have been to employ a receptor which normally activates G_q family members, such as the α_1 adrenergic receptor or the M1 muscarinic receptor.

As stated earlier, the COS-1 cells employed in this study are not known to express G_q linked receptors. Therefore it was not possible to address the questions of the effect of palmitoylation on α subunit activation of effector systems or the effect of agonist activation on the palmitoylation status of the protein.

There are two possible methods to address these questions using the mutants created in this study. One would be to co-transfect a cDNA encoding a G_q linked receptor into COS-1 cells along with the various $G_{11}\alpha$ cDNAs. Another would be to create stable transfects of cells known to express G_q linked receptors with the $G_{11}\alpha$ cDNAs. The second system would be more advantageous. The transfection would result in the production of a clonal cell line, thus all experiments would use cells which were identical, rather than the heterogeneous cell population of COS-1 cells expressing varying amounts of receptor and $G_{11}\alpha$ protein. These cells would also be utilising an endogenously expressed receptor and the system would therefore represent a more physiological model.

The use of the endogenously expressed $G_q\alpha$ and $G_{11}\alpha$ proteins may enable another important control to be used. As described, it was not possible to use these transiently transfected cells to address the questions of the effect of receptor activation on $G_{11}\alpha$ palmitoylation, due to the lack of suitable receptor. Had this been possible, either by transient co-transfection of a suitable receptor into the COS-1 cells or by stably transfecting these cDNAs into cells expressing a receptor known to activate $G_q\alpha$ and $G_{11}\alpha$, then the endogenously expressed G_q family members would have provided easily comparable controls to determine any changes in the rate of turnover of palmitic acid following agonist challenge on these endogenous proteins as compared to the heterologous proteins transfected into them. It would also be possible to compare the half-lives of the endogenous $G_q\alpha$ and $G_{11}\alpha$ proteins with the heterologously expressed wild type and mutant $G_{11}\alpha$s upon agonist treatment. Direct comparisons with the endogenous and transfected proteins

would show any changes in the ability of the cell to regulate the transfected proteins

In this study we have assessed whether murine $G_{11}\alpha$ is dually palmitoylated, as is $G_q\alpha$, on adjacent cysteine residues near its N-terminus. By a PCR based strategy, we have created site-directed mutants in which the cysteine residues thought, by sequence analysis, to be the targets for palmitoylation were altered to serines. This mutagenesis prevented all detectable [^{3}H] palmitic acid incorporation only into the mutant in which both cys9 and cys10 were changed. Single mutants, where either cys9 or cys10 was mutated, incorporated approximately 20% of the [^{3}H] palmitate of wild type $G_{11}\alpha$, which may point to a level of co-operativity in palmitoylation which has not previously been observed.

Removal of palmitic acid by this means from murine $G_{11}\alpha$ led to a release of mutant $G_{11}\alpha$ into the cytosolic fraction of cells indicating that palmitoylation increases, but does not solely govern, the affinity of $G_{11}\alpha$ for the plasma membrane. Given the apparently absolute requirement for palmitoylation of $G_q\alpha$ for membrane interaction, this may indicate a functional difference between the two α subunits, something which has been hitherto unproven. This difference of apparent affinities for the plasma membrane is not a reflection of the choice of serine for mutagenesis as the presence of alanine at this position has no effect on the resulting fractionation pattern of the $G_{11}\alpha$ mutants.

This model is only in its infancy, yet has already provided new insight into the palmitoylation of G protein α subunits. Indeed utilising this

system has demonstrated that the presence of palmitic acid on $G_{11}\alpha$ is required for the proper folding of the protein. Gel filtration carried out on extracts of COS-1 cells transfected with wild type and mutant $G_{11}\alpha$ cDNAs indicate that the C9SC10S $G_{11}\alpha$ in these cells is present as aggregated protein whereas the endogenous simian $G_{11}\alpha$ is not, indicating that the absence of palmitate may lead to a totally non-functional protein [McCallum*et al.*, 1995].

Chapter 6.

Final Discussion.

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Heterotrimeric G proteins are responsible for transmitting signals from membrane bound serpentine receptors to various effector systems which subsequently results in changes in the levels of a second messenger molecule or ion [Gilman, 1987]. The mechanism by which G proteins remain at the membrane, in close proximity to the receptor and effector systems they interact with, has been a subject for debate for several years; all three subunits, α, β and γ, are predominantly hydrophilic and contain no stretches of hydrophobic amino acids which could form putative transmembrane helices.

The discovery of isoprenoid groups at the C terminus of the γ subunit [Fukada *et al.*, 1990] prompted speculation that the $\beta\gamma$ dimer, which remains as a tightly associated complex under non-denaturing conditions, acted as an anchor for the α subunit. However agonist activation of the receptor, which subsequently causes dissociation of the α subunit from $\beta\gamma$ does not also lead to release of the α subunit from the plasma membrane. Also, overexpression of $G_{i2}\alpha$ in COS-7 cells, in excess of $\beta\gamma$, does not lead to the α subunit being found in the cytosol of such cells [Simonds *et al*, 1989]. The emergence of $\beta\gamma$ as a facilitator of G protein signals in its own right [Clapham & Neer, 1993; Sternweis, 1994] would also indicate that this original sedentary role for the dimer was incorrect.

Acylation of proteins involved intracellular signalling has been recognised for several years. Many viral proteins contain myristic acid

covalently attached via amide bonds to N terminal glycine residues and this modification is required for proper assembly of virus particles [Tashiro *et al.*, 1989; Weaver & Panganiban, 1990]. Normal cellular proteins are also modified by myristoylation, notably the catalytic subunit of PKA and calcineurin [Carr *et al*, 1982; Aitken *et al*, 1982]. The discovery of myristic acid on the α subunits of heterotrimeric G proteins was thought therefore to play a role in the membrane interactions of α subunits [Buss *et al.*, 1987; Jones *et al.*, 1990; Mumby *et al.*, 1990] However only members of the G_i family have the target glycine residue in the proper environment at their N terminus [Casey, 1994], and so another, more general mechanism must exist to explain the interactions of the other α subunits with the plasma membrane. In addition not all myristoylated proteins are membrane associated; the catalytic subunit of PKA is a soluble protein [Carr *et al*, 1982]. Work using acylated peptides throws further doubt on the theory that myristoylation is required for membrane association. It may be that myristoylation by itself cannot promote full membrane association as myristoylated peptides have insufficient free energy to stably bind to lipid bilayers [Peitzsch & McLaughlin, 1993]. The recent discovery of palmitic acid at the N terminus of G proteins [Parenti *et al.*, 1993; Linder *et al.*, 1993] and Src family [Paige *et al.*, 1993; Shenoy-Scaria *et al.*, 1993] members may provide the necessary hydrophobicity for membrane attachment of these acylated proteins.

This study has demonstrated both by chemical treatment with hydroxylamine, under conditions known to remove thio-ester linked palmitic acid from proteins [Magee *et al.*, 1984; Mage & Courtnedge, 1985], and by mutating target cysteine residues, that palmitoylation of proteins does not entirely govern the membrane association of $G_{o1}\alpha$ and $G_{11}\alpha$. It does however

contribute to their ability to interact with the membrane as approximately 30% of the protein is found in the cytosol following cell disruption or hydroxylamine treatment of isolated cell membranes. Recent work suggests that this is not an artefact caused by disruption of the cells in a hypotonic buffer as unpalmitoylated C3S $G_{o1}\alpha$ is found in the cytosol of intact D3 cells [M.A. Grassie & G. Milligan, unpublished observations]. This would suggest, given the fact that pertussis toxin can ADP-ribosylate all the C3S, non-palmitoylated $G_{o1}\alpha$ in D3 cells, that a cycling process is occuring in these cells. Whether this is simply an on/off occurrence or whether it is directed somehow by another protein which binds to non-palmitoylated $G_{o1}\alpha$ and herds it away from the membrane in unknown. The fact that myristic acid is still present [Galbiati *et al.*, 1994] and may allow transient association with the membrane [Degtyarev *et al.*, 1994b] would suggest the first hypothesis.

The role of palmitoylation of $G_{11}\alpha$ may be different. Chemical treatment of NG108-15 and Rat-1 cell membranes released approximately 30% of $G_{11}\alpha$ from the membrane. This value is similar to the amount present in the cytosolic fraction of COS-1 cells which had been transiently transfected with mutants of murine $G_{11}\alpha$, in which either cys9 or cys10 or both had been altered to serine. Thus it would seem reasonable that the function of $G_{11}\alpha$ would be intact, but it has been found that the mutated $G_{11}\alpha$ was not present as free α subunits but as aggregated protein [McCallum *et al.*, 1995]. Therefore acylation of $G_{11}\alpha$ would appear to be necessary for proper targetting of the polypeptide to specific areas of the membrane; that is prevention of polymerisation of the protein. In this state $G_{11}\alpha$ would not be able to contact other proteins and thus would be functionally dead. Recent work suggests that this aggregation of protein is due to the specific cell

background of the COS-1 cell as similar experiment in HEK-293 cells indicates that all the immunoreactive mutant $G_{11}\alpha$ present in transiently transfected HEK-293 cells is present in the cytosol and is capable of activating phosphoinositidase C [A. Wise & G. Milligan, unpublished observations].

It was also seen that the single mutants of $G_{11}\alpha$ did not incorporate [^{3}H] palmitate to 50% of the amount found in wild type $G_{11}\alpha$. This would suggest one of two things: one, that a level of cooperativity exists in palmitoylation of $G_{11}\alpha$ such that palmitoylation of one cysteine is required to promote full palmitoylation of the other cysteine or two, that mutation of one cysteine has rendered the palmitoyltransferase less able to add the acyl chain to the second cysteine due to steric considerations. In either case it would appear that the single mutants behave essentially the same as the double mutant in that all three are present in the cytosolic fraction to the same degree, and all three form aggregated clumps rather than free α subunits [McCallum *et al.*, 1995]. A similar pattern of palmitoylation has been observed for $G_q\alpha$ following transient transfection into HEK-293 cells. The two single mutants C9S and C10S incorporated [^{3}H] palmitate to less than 50% that of wild type $G_q\alpha$ and no [^{3}H] palmitate was incorporated into the C9SC10S double mutant [Wedegaertner *et al.*, 1993].

What then is the wider function of acylation and how does it affect function of target proteins. Recent work has demonstrated a high concentrations of signalling proteins in membrane invaginations termed caveolae. These areas are high in a protein, termed caveolin, which may help define the structure of caveolae by forming a cholesterol and pH dependent interaction with GPI linked proteins. Cholesterol is found in high

concentrations in caveolae, perhaps due to a "filler" function to help plug gaps in the lipid bilayer formed by lipidated proteins such as G proteins [Lisanti *et al.*, 1994]. It has been proposed that dual acylation of proteins either by myristic acid and palmitic acid (Src family members and G_i family members) or dual palmitoylation (G_q family members) may target such modified proteins to caveolae. These membrane invaginations are rich in many of the signalling proteins, including G proteins and the non-receptor tyrosine kinases. Lisanti *et al.* [1994] have speculated that the lipidation of these proteins may serve to target them to caveolae where caveolin could mesh together a complex. Recently Shenoy-Scaria *et al.* [1994] have shown that palmitoylation targets src family members to caveolae.

If dual acylation is required to target these proteins to caveolae, it is reasonable to suggest that removal of one or both of acyl chains could prevent proper association with these complexes. One problem with this theory is that $G_s\alpha$ is only singly acylated. Chemical removal of this does not affect the membrane avidity of the protein as severely as it does the other G proteins investigated in **Chapter 4**. Also several groups have attempted to ascertain the role of palmitoylation of $G_s\alpha$ in its membrane association. While one group determined that palmitoylation was absolutely required for the membrane association of $G_s\alpha$ [Wedegaertner *et al.*, 1993] this has not been the case for others who found that mutation of the palmitoylated cysteine to alanine caused no change in the ability of $G_s\alpha$ to interact with the membrane [Degtayrev *et al.*, 1993a; Mumby *et al.*, 1994], although this may simply reflect the different cell lines employed. The possibilty therefore exists that another mechanism exists to target $G_s\alpha$ to the plasma membrane and caveolae.

The addition of palmitic acid to proteins is generally via a thio-ester bond to cysteine residues. The lability of the thio-ester bond raises the possibility of regulation of protein palmitoylation and indeed the palmitoylation status several proteins has been shown to be regulated by various stimuli [Huang, 1989; James & Olson, 1989; Mouillac *et al.*, 1992; Paige *et al.*, 1993]. The palmitoylation status of $G_s\alpha$ has also been shown to be regulated by agonist activation of β_2 adrenergic receptors [Degtyarev *et al.*, 1993b; Wedegaertner & Bourne, 1994] and direct activation by cholera toxin [Degtyarev *et al.*, 1993b]. Treatment of cells with these agents caused an increase in the rate of turnover of palmitic acid on $G_s\alpha$. How this turnover of palmitic acid is facilitated and regulated is as yet unknown. A palmitoyl-protein thio-esterase has been identified [Camp & Hofmann, 1993] was thought to represent a part of this puzzle, however this enzyme has been shown to be secreted [Camp *et al.*, 1994] and so cannot be not the enzyme responsible for the regulation of palmitoylation of G proteins and other cellular signalling polypeptides. The palmitoyl-CoA:protein *S*-palmitoyl transferase responsible for addition of palmitic acid to the various proteins has not yet been identified.

Recently the possibilty of self-regulation of the palmitoylation status of a protein has been observed. Like G_i family members and ser family members, the endothelial cell nitric oxide synthase is dually acylated by myristoylation [Busconi & Michel, 1993] and palmitoylation [Robinson *et al.*, 1995; Busconi & Michel, 1995]. However it has been shown that NO inhibits the palmitoylation of GAP-43, a growth cone associated protein [Hess *et al.*, 1993], and that this causes growth cone collapse and inhibits the extension of the neurite growth cone [Hess *et al.*, 1993; Patterson & Skene, 1994]. The

palmitoylation of nitric oxide synthase would thus be inhibited by the activity of the enzyme. How this would then affect the enzyme is unclear, but it may represent a self-limiting enzyme regulation.

In conclusion, this thesis presents data which demonstrates the role of palmitoylation of rat $G_o\alpha$ and murine $G_{11}\alpha$. While using the systems described, it is apparent that the membrane association of these two G protein α subunits is not solely governed by the addition of palmitic acid. Evidence has been presented which suggests that unpalmitoylated $G_o\alpha$ is present in the cytosol of the whole cell, and that this cycles between the membrane and the cytosol. Unpalmitoylated $G_o\alpha$ is still a substrate for pertussis toxin catalysed mono ADP-ribosylation. The use of urea containing SDS-PAGE to separate species variants of $G_{11}\alpha$ has provided a system to allow unambiguous detection of a ubiquitously expressed G protein α subunit. This system differs from that used by other investigators in that the polypetide of interest was not mutationally tagged in order to detect it. This system also provides a readily accessible control in every single experiment, namely the presence of the endogenously expressed $G_q\alpha$ and $G_{11}\alpha$ as a benchmark for levels of [^{3}H] palmitate incorporation and purity of membrane and cytosolic fractions.

Much work remains to be done in the field of G protein palmitoylation and several important questions remain to be answered. Does palmitoylation affect the localistaion of G proteins to caveolae? How does palmitoylation of $G_o\alpha$ and $G_{11}\alpha$ affect their interaction with other proteins. Given that unpalmitoylated $G_o\alpha$ can still interact with $\beta\gamma$, the palmitoylation status of $G_o\alpha$ may not affect this interaction (although the observation that myristoylation of $G_o\alpha$ increases it affinity for $\beta\gamma$. [Linder *et al.*, 1991] may

indicate a more subtle affect of palmitoylation). The question of how palmitoylation affected both the abilities of $G_o\alpha$ and $G_{11}\alpha$ to interact with receptors and to activate or inhibit effectors could not be assessed due to a lack of receptors known to couple to the α subunits on the cells used in this study. These are questions which would require the transfection of the polypetides into cells which expressed these receptors or co-transfection of such receptors into cells which lacked them. These studies are currently underway in our laboratory. One intriging question is how does the process of depalmitoylation correlate with the down regulation of G protein α subunits? Is depalmitoylation a signal for degradation of the protein? One way in which to answer this question would be to examine the rate of turnover of the wild type and palmitate negative mutant $G_o\alpha$ and/or $G_{11}\alpha$ polypeptides in the basal state, and given an appropriate receptor, in the agonist activated state. The increase, or otherwise, in the rate of degradation of the α subunits may resolve this question.

Publications.

Material from this thesis has been published in the following papers:

1) Milligan, G., Mullaney, I. and McCallum, J.F. "Distribution and relative levels of expression of the phosphoinositidase-C-linked G-proteins $G_q\alpha$ and $G_{11}\alpha$: absence of $G_{11}\alpha$ in human platelets and haemopoietically derived cells" (1993) *Biochim Biophys Acta* **1197** 208-12.

2) Mullaney, I., Mitchell, F.M., McCallum, J.F., Buckley, N.J. and Milligan, G. "The human M1 acetylcholine receptor, when expressed in CHO cells, activates and downregulates both $G_q\alpha$ and $G_{11}\alpha$ equally and non-selectively." (1993) *FEBS Lett* **324** 241-5.

3) Grassie, M.A., McCallum, J.F., Parenti, M., Magee, A.I. and Milligan, G. "Lack of N terminal palmitoylation of G protein α subunits reduces membrane association." (1993) *Biochem Soc Trans* **21** 499S.

4) Grassie, M.A., McCallum, J.F., Guzzi, F., Magee, A.I., Milligan, G. and Parenti, M. "The palmitoylation status of the G-protein $G_{o1}\alpha$ regulates its avidity of interaction with the plasma membrane." (1994) *Biochem J* **302** 913-920.

5) Milligan, G., Wise, A., MacEwan, D.J., Grassie, M.A., Kennedy, F.R., Lee, T.-W., Adie, E.J., Kim, G.-D., McCallum, J.F., Burt, A., Carr, I.C., Svoboda, P., Shah, B.H. & Mullaney, I. "Mechanisms of agonist-induced G protein elimination" (1995) *Biochem Soc Trans* **23** 166-170.

6) McCallum, J.F., Wise, A., Parenti, M. & Milligan, G. "Palmitoylation negative mutants of murine $G_{11}\alpha$ have decreased ability to interact with the plasma membrane when expressed in COS-1 cells" (1994) *Biochem Soc Trans* **22** 9S.

7) McCallum, J.F., Wise, A., Grassie, M.A., Magee, A.I., Guzzi, F., Parenti, M. & Milligan, G. "The role of palmitoylation of the guanine nucleotide binding protein $G_{11}\alpha$ in defining interaction with the plasma membrane" (1995) *Biochem J.* (in press).

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