Regulation of signal transduction by RGS4

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Abstract

In the present study, the function and the mechanism of action of RGS4, a member of a family of proteins called Regulators of G protein Signalling (RGS) was investigated. A C-terminal fluorescent tag on RGS4 confirmed that transiently transfected RGS4 was predominantly cytosolic and underwent translocation to the plasma membrane of HEK293T cells following co-expression of $G\alpha_{i1}$, the α_{2A}-adrenoceptor, or agonist activated α_{2A}-adrenoceptor. This translocation of RGS4 to the plasma membrane was most pronounced with the co-expression of the constitutively active GTPase deficient $G\alpha_{i1}^{Q204L}$.

High-affinity GTPase experiments indicated that $RGS4^{S30C}$ had enhanced GAP activity towards $G\alpha_{o1}$ compared to wild type RGS4. This approach also demonstrated a simultaneous significant decrease in potency of both adrenaline and UK14304 to increase α_{2A}-arenoceptor-activated high-affinity GTPase activity of $G\alpha_{o1}$ in the presence of RGS4 and a further significant decrease in potency of both ligands in the presence of $RGS4^{S30C}$. This enhanced GAP activity and observed decrease in agonist potency was also transferable to RGS16, an RGS protein closely related to RGS4. The selectivity of the Gα subunit was also investigated. The enhanced GAP activity and simultaneous significant decrease in potency of adrenaline and UK14304 to increase α_{2A}-arenoceptor-activated high-affinity GTPase activity of $RGS4^{S30C}$ and $RGS16^{S30C}$ was selective for $G\alpha_{o1}$ over $G\alpha_{i1}$. $RGS4^{S30K}$ and $RGS4^{S30F}$ also demonstrated higher GAP activity than wild type RGS4 but no consensus side chain could be identified that conferred a specific enhancement or loss of GAP activity.

The ability to inhibit intracellular calcium release by an activated α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein was used in order to investigate the GAP activity of $RGS4^{N88S}$, $RGS4^{N128A}$ and $RGS4^{N88S,N128A}$. All three mutants had ablated GAP activity towards $G\alpha_{11}$ and therefore failed to inhibit intracellular calcium release.

A novel role for the RGS insensitive mutation G188S was also observed when despite similar expression, $G\alpha_{11}^{G188S}$ significantly reduced agonist-stimulated [^{35}S]GTPγS binding compared to wild type $G\alpha_{11}$.

RGS4 represents a novel target for pharmaceutical drug development and the study of its regulation of signal transduction is an important area of investigation. These results highlight specific areas of RGS4 research with great pharmaceutical potential.

Table of Contents

List of Tables

List of Figures

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Definitions

AC	adenylyl cyclase
AH	amphipathic helix
βCat	β-catenin interacting domain
BCA	bicinhoninic acid
$[Ca^{2+}]_i$	intracellular calcium concentration
CaM	calmodulin
CHO	chinese hamster ovary
CYS	polycysteine region
DAG	diacylgylercol
D-AKAP2	dual A kinase anchoring protein 2
DEP	disheveled EGL-10 pleckstrin
DH	dbl homology domain
DIX	dishevelled-interacting domain
eYFP	enhanced yellow fluorescent protein
ERK2	extracellular signal-regulated kinase 2
FOA	5-fluoro-orotic acid
G protein	guanine nucleotide binding protein
GAP	GTPase-activating protein
GAIP	Gα-interacting protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanine 5-diphosphate
GFP	green fluorescent protein
GGL	G-protein γ subunit-like
GIPC	GAIP-interacting protein C terminus
GIRK	G protein-gated inwardly rectifying potassium
GOF	gain-of-function
GRK	G protein receptor kinase
GoLoco	$G\alpha_{i/o}$-loco-interacting domain
GPCR	G protein coupled receptor
GSK3β	glycogen synthase kinase 3β
GST	glutathione *S*-transferase
GTP	guanine 5-triphosphate
HEK	human embyonic kidney
IP3	inositol 1,4,5-trisphosphate

LB	Luria-Bertani medium
PCR	polymerase chain reaction
PDEγ	phosphodiesterase γ
PDZ	PSD-95 Disk-Large ZO-1
PH	pleckstrin homology domain
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLCβ	phospholipase C β
PMCA	plasma membrane Ca^{2+}-ATPase
PP2A	phosphatase 2A
P. tox	pertussis toxin
PBS	phosphate-buffered saline
PTB	phospho-tyrosine binding domain
PX	phosphatidylinositol binding domain
PXA	phosphatidylinositol-associated domain
RBD	rap1/2 - or ras binding domain
RGS	regulator of G protein signalling
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SERCA	sarco/endoplasmic reticulum ATPase
SNPs	single nucleotide polymorphisms
SRC	Src family kinases
TM	transmembrane domain
TNF-α	tumour necrosis factor-α
TRH	thyrotropin-releasing hormone
TRHR-1	thyrotropin-releasing hormone receptor 1
Ura4	orotidine 5'-monophosphate decarboxylase
WGA	wheat germ agglutinin

1 Chapter 1

1.1 GPCRs

Cellular activity is regulated by a variety of receptors. The largest of these receptor families is the G protein coupled receptors (GPCRs). These are seven transmembrane domain receptors that serve to transduce signals from the extracellular to the intracellular environment. GPCRs account for more than 30% of drug targets under investigation by the pharmaceutical industry (Klabunde and Hessler, 2002;Fredriksson *et al.*, 2003). Ligands for these receptors are highly specific and include hormones, neurotransmitters, chemokines, calcium, odorants, taste and light (Pierce *et al*., 2002). Over 800 GPCRs have been revealed to be encoded by the human genome and are found throughout the body. As such, these receptors serve key regulatory functions for a large number of biological processes.

1.2 G proteins

GPCRs are associated with guanine nucleotide binding proteins (G proteins). G proteins are heterotrimeric and consist of α, β and γ subunits. To date, there are over 20 different Gα proteins encoded by over 16 different genes that are divided into four families*;* $G\alpha_s$ ($G\alpha_s$ and $G\alpha_{olf}$), $G\alpha_i$, ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_z$, $G\alpha_{t\text{-}cone}$, $G\alpha_{t\text{-}rod}$ and $G\alpha_{gust}$) $G\alpha_q$, ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{16}$) and $G\alpha_{12}$ ($G\alpha_{12}$ and $G\alpha_{13}$) based on the sequence similarity of the α subunit (Cabrera-Vera *et al*., 2003). 5 different Gβ and 12 different Gγ subunits have also been discovered (Neer, 1995;Milligan and Kostenis, 2006). It is generally accepted that ligand binding to the receptor produces G protein activation. The resulting conformational change of the three 'switch' regions of the Gα subunit promote the exchange of guanine diphosphate (GDP) for guanine triphosphate (GTP) and the dissociation of the Gα subunit from the βγ subunits. Alternatively, Bunemann *et al*., suggested that the Gα subunit and the Gβγ dimer do not physically dissociate during activation of the receptor (Bunemann *et al*., 2003). Instead, a molecular rearrangement may take place after the GDP to GTP exchange, allowing the Gβγ to stay complexed to the Gα subunit. Whichever physiological process takes place, both the Gα subunit and the Gβγ dimer are then active and can stimulate or inhibit effector proteins such as adenylyl cylcase, phospholipases and a variety of ion channels (Gudermann *et al.*, 1997;McCudden *et al.*, 2005). Intrinsic GTPase activity of the Gα subunit hydrolyses the bound GTP to GDP, resulting in the

reassociation of the G protein heterotrimer and prevention of further signalling (Figure 1.1).

The $G\alpha_{i/o}$ subfamily of G proteins are sensitive to treatment with pertussis toxin (P. tox). P.tox is one of the major virulence determinants produced by *Bordetella pertussis*, catalysing the transfer of an ADP-ribose group from NAD onto a cysteine residue four amino acids from the C-terminal of the $G\alpha_{i/o}$ subunit (cysteine^{351}) (Lochrie *et al*., 1985). The addition of a bulky side group to the $G\alpha_{i/o}$ subunit makes the Gα subunit unable to contact GPCRs, and renders it inactive.

Mutating cysteine^{351} of the P. tox sensitive Gα subunits can confer resistance to P. tox treatment. A hydrophobic residue at position 351 of the Gα subunit is crucial to give optimal interactions between GPCRs and G proteins (Bahia *et al.*, 1998). Mutation to other residues, including isoleucine, offers P. tox resistance, and following expression of the α_{2A}-adrenoceptor-$G\alpha_{i1}^{C351I}$ fusion protein, cells treated with P. tox have inactive endogenous $G\alpha_{i/o}$ proteins and agonist activation reflects only the G protein of interest in the experimental system.

1.3 GPCR desensitisation and internalisation

Following ligand binding to the GPCR, and G protein activation, GPCRs become desensitised and lose cellular activity (Ferguson, 2001). Short-term desensitisation is mediated, in part, by the phosphorylation of residues within the C-terminal tail of GPCRs, or within the third extracellular loop of GPCRs with small C-termial tails without potential phosphorylation sites, by G protein receptor kinases (GRKs). Long-term loss of cellular sensitivity can involve the down regulation of receptors by protein degradation and decreased receptor synthesis. The C-terminal tail of many GPCRs contain several serine and theronine residues that can determine the intracellular trafficking and fate by providing phosphorylation sites for several protein kinases (Smith and Scott, 2002).

There are currently seven GRK family members, and although no consensus site for GRK activity has been determined, the presence of acidic amino acids proximal to the phosphorylation site favours GRK2 mediated phosphorylation (Chen *et al.*, 1993). It is also known that GRKs preferentially phosphorylate receptors that are in the agonist-occupied conformation (Luttrell and Lefkowitz, 2002).

Phosphorylation of receptors by GRKs increase the affinity of the receptor for arrestins. Arrestin binding sterically hinders G protein coupling with the receptor, serving to uncouple the receptor from the G protein and target the receptor for endocytosis (Benovic *et al.*, 1987).

Receptor endocytosis is followed by the targeting of the receptor to either recycling pathways, for proteasomal degredation or to lysosomes for degradation (Kristiansen, 2004). Receptor resensitisation involves receptor dephosphorylation and dissociation from its ligand. The time frame over which these events occur for GPCR phosphorylation is minutes for receptor internalisation and hours for receptor down regulation.

1.4 RGS proteins

The intrinsic GTPase activity of the Gα subunit does not correlate to the physiological rate of G protein inactivation. Discovery of the product of the yeast gene S*sst2* that could negatively regulate heterotrimeric G protein signalling indicated that extrinsic factors might also regulate the G protein cycle (Dietzel and Kurjan, 1987;Dohlman *et al.*, 1996). This regulation of G proteins is not confined to *Saccharomyces cerevisiae*. Similar genes were identified in the fungal organism *Aspergillus nidulans* (*flbA*) (Lee and Adams, 1994), and the nematode *Caenorhabditis elegans* (*egl-10*) (Koelle and Horvitz, 1996). These regulatory proteins were recognised to share a novel conserved domain and mammalian genes have now also been isolated and termed regulators of G protein signalling (RGS) proteins. To date, more than 37 mammalian RGS genes have been named, all containing the novel RGS domain (Siderovski and Willard, 2005).

RGS proteins, termed GAPs due to their ability to enhance the intrinsic GTPase activity, regulate GPCR-mediated signalling through their interaction with Gα subunits. Biochemical and crystallography experiments revealed that RGS proteins preferentially bind to the transition state of the Gα protein that occurs immediately before the hydrolysis of GTP. The crystal structure of RGS4 complexed with $G\alpha_{i1}$-GDP-$AlF4^-$ (a stable mimic of Gα-GTP) revealed that the RGS domain forms a nine-alpha-helix bundle that contacts $G\alpha_{i1}$ at three distinct sites (Tesmer *et al.*, 1997;Ross and Wilkie, 2000). Two surface residues of $G\alpha_{i1}$ (threonine^{182} and glycine^{183}) appear essential for high-affinity Gα-RGS interaction, although other residues are also important (DiBello *et al.*, 1998;Posner *et al.*, 1999).

RGS activity may therefore be used to recycle Gα to increase the concentration of inactive Gα that can be reactivated by GPCRs (Zhong *et al*., 2003). Thus, RGS proteins increase the rate of GTP hydrolysis and consequently inhibit signalling. The discovery of RGS proteins shows the importance of model organisms in revealing complex signalling mechanisms which can be transferred to more complex mammalian systems.

1.5 Structure and classification of RGS proteins

Proteins containing the RGS domain or RGS-like domain have been classified into eight sub-families. A or RZ*;* B or R4*;* C or R7*;* D or R12*;* E or RA*;* F or GEF*;* G or GRK and H or SNX. Additionally, D-AKAP2 (dual A kinase anchoring protein 2) and RGS22, which contain multiple RGS domains, have not been classified. Most members of the A/RZ or B/R4 subfamily are small, 20-30 kDa proteins that contain short N- and C-terminal regions flanking the RGS domain. Members of the C/R7, D/R12, E/RA, F/GEF, G/GRK and H/SNX subfamilies (except RGS10) are much longer proteins, commonly up to 160 kDa, and contain multiple domains (Figure 1.2).

RGS21 of the B/R4 sub-family is the smallest known RGS protein consisting of little more than an RGS domain (von Buchholtz *et al*., 2004). In contrast, RET-RGS1 contains an N-terminal cysteine rich region and a putative transmembrane region. This region is likely to be involved in membrane targeting and attachment, protein-protein interaction and possible integration into the plasma membrane (Faurobert and Hurley, 1997;Jones, 2004). RGS9-2, the longer splice variant of RGS9, has a long C-terminal which functions as an 'affinity adapter' increasing the affinity of the RGS proteins to $G\alpha_o$ (Martemyanov *et al*., 2003). The shorter splice variant (RGS9-1), does not contain this long C-terminal but instead the effector enzyme phosphodiesterase γ (PDEγ) increases the affinity between the RGS protein and its retinal-specific G protein target $G\alpha_t$. Only the RGS domain seems necessary and sufficient to confer GAP activity of RGS proteins but other domains can affect RGS proteins by altering the GAP activity, increasing receptor specificity, determining the cellular localisation, acting as effector antagonists or influence signal transduction mediated by GPCR activation.

1.5.1 PDZ Domain

Of the other domains in RGS proteins, the most frequently found is the PSD-95 disk-large ZO-1 (PDZ) domain. This 90 amino acid domain facilitates protein-protein interactions

through its highly conserved glycine-leucine-glycine-phenylalanine repeated motif. Found in clusters of multiprotein signalling complexes, the PDZ domain of RGS12 has been found to selectively bind the chemokine receptor CXCR2 (Snow *et al.*, 1998b).

1.5.2 GGL Domain

The G-protein γ subunit-like (GGL) domain is found in a number of RGS proteins, including RGS6, RGS7, RGS9 and RGS11. It is a 64 amino acid domain with a high degree of similarity to the Gγ subunit. Resembling a Gγ subunit allows this domain to form dimers with a number of other G protein subunits. An RGS9-Gβ5 dimer is thought to be involved in the stabilising of the protein complex and subsequently the GAP activity of the RGS protein (Snow *et al*., 1998a).

1.5.3 GoLoco Domain

Comparable to RGS domain, the $G\alpha_{i/o}$-loco-interacting (GoLoco) domain inhibits G protein signalling. By binding directly to Gα subunits, the 19 amino acid domain stabilises the Gα-GDP form of the G protein and prevents GTP from binding. Acting as a guanine nucleotide dissociation inhibitor (GDI), G protein signalling is decreased. RGS14 contains this GoLoco domain, and signalling inhibition of $G\alpha_i$ was attained when this domain was present. However, both the RGS domain and the GoLoco domain are necessary for maximum inhibition (Mittal and Linder, 2004).

1.5.4 DEP Domain

The importance of the disheveled EGL-10 pleckstrin (DEP) domain was exemplified in the discovery that this 70 amino acid domain in RGS7 can bind synaptin. Synaptin in turn, interacts with synaptosomal-associated protein of 25 kDa, a component of

the soluble N-ethylmaleimide–sensitive factor attachment protein receptor complex, suggesting a role for RGS7 in synaptic vesicle exocytosis (Riddle *et al.*, 2005). Synaptin has also been discovered to bind to adenylyl cyclase (AC) and perhaps these complexes can come together allowing RGS7 to regulate cAMP levels (Chou *et al*., 2004).

1.6 Regulation of RGS proteins

The large number of RGS proteins and their multiple domains means that the activity of these proteins must be tightly regulated to maintain specificity within the cell. The mechanisms for this regulation are complex and developments in this area of research are still ongoing. Little is known about the functional consequences of this regulation but it is important that the direct protein-protein interactions within signalling components are elucidated for their potential as therapeutic targets.

1.6.1 Expression of RGS proteins

The transcripts of several RGS proteins have been shown to be dynamically regulated by various signals to offer feedback regulation to GPCR signalling (Ingi *et al.*, 1998;Kardestuncer *et al.*, 1998;Pepperl *et al.*, 1998). Dopamine D1 receptor agonists increase RGS2 mRNA, whereas dopamine D2 receptor agonists result in a decrease in RGS2 mRNA but increase RGS4 mRNA (Taymans *et al.*, 2003). This suggests that RGS2 and RGS4 must couple preferentially to D1 and D2 receptors respectively to exert distinct functions.

The activation of the μ- or κ-opioid receptors expressed in PC12 cells can also increase the level of RGS4 mRNA. This regulation of the opioid-signalling pathway could contribute to the desensitisation of opioid signalling and is perhaps a valuable area for future study in the combat of morphine and cocaine addiction (Nakagawa *et al.*, 2001).

Differences in expression patterns of RGS proteins in tissues add another layer of complexity to regulation of RGS proteins. Some RGS proteins have ubiquitous expression, for example, RGS5 has been detected in heart, skeletal muscle, pericyte and a variety of sub-regions within the brain (Wieland and Mittmann, 2003;Li *et al.*, 2004;Jean-Baptiste *et al.*, 2005a;Jean-Baptiste *et al.*, 2005b). RGS8 however, seems only to be expressed in the brain (Moratz *et al.*, 2004;Kurrasch *et al.*, 2004).

As mentioned previously, RGS9 has two splice variants. RGS9-1 is expressed predominantly in the retina where it regulates rhodopsin signalling (Nagata *et al.*, 2001). RGS9-2 is appropriately located in the brain to play its role in opioid signalling. Restricted tissue distribution and alternative splicing is therefore also a major factor in conferring

specificity of RGS action and together with the regulation of RGS expression levels may have a major physiological relevance for future pharmaceutical development.

1.6.2 RGS localisation

Differences in expression patterns of RGS proteins may also take place at the subcellular level. Some RGS proteins are compartmentalised with other signalling components to further regulate signalling. Limited subcellular distribution of RGS proteins has been shown in a number of instances. Surprisingly the nucleus of cells, although distant from other signalling components, has been reported to be a storage area for some RGS proteins. In fact, cytosolic localisation of RGS proteins may actually result from the competition between nuclear import and export signals located in the N-terminal of a number of RGS proteins (Chatterjee and Fisher, 2000;Heximer *et al.*, 2001). After PKA phosphorylation at its C-terminus, RGS10 translocates to the nucleus, making it unable to facilitate or impede signalling (Burgon *et al.*, 2001). RGS12TS-S is expressed in punctate nuclear foci. The underlying mechanism for this seems unclear, but many tumour suppressor proteins also have this expression pattern and for that reason it has been suggested that RGS12 may have an important role in cell cycle events (Chatterjee and Fisher, 2000). The translocation of RGS proteins from one cellular compartment to another has been suggested to very rapidly. For RGS protein to have their desired effect, this process must be rapid, to allow the RGS protein to be translocated at the plasma membrane immediately after receptor activation. Indeed, translocation of RGS4 from the cytoplasm to the plasma membrane is so fast that, to date, no researcher has been able to measure the speed of translocation. No real time footage of translocation has ever been published, although it is widely accepted that such footage would be a break through in RGS research. However, translocation from the nucleus to the cytoplasm has been reported to be slower. RGS14, for example translocates from the nucleus to the cytoplasm in less than 30 minutes (Hepler *et al.*, 2005).

1.6.2.1 Interaction with Gα

In some cases RGS proteins are preferentially located at the plasma membrane in an ideal location to allow interaction with Gα subunits and carry out their function as inhibitors of G protein signalling. In particular, upon G protein activation RGS3 is translocated from the cytosol to the plasma membrane (Cheever *et al.*, 2001). RGS2 and RGS4 also follow this translocation pattern*;* however, the activation of G proteins seems irrelevant. The G protein subtype seems particularly important in the cellular movement of RGS2 and RGS4

(Roy *et al.*, 2003). In HEK293 cells transfected with RGS2, RGS2 translocated to the plasma membrane when cells were cotransfected with $G\alpha_q$ or $G\alpha_s$ but not $G\alpha_{i1}$. In contrast, RGS4 translocated to the plasma membrane when cotransfected with $G\alpha_{i1}$ but not $G\alpha_q$ or $G\alpha_s$ (Roy *et al.*, 2003).

The large number of different RGS proteins quickly ruled out the possibility of one RGS protein interacting with only one Gα subtype. Diverse RGS proteins such as RGS1, RGS4, RGS10 and RGS19 all act on both the $G\alpha_q$ and $G\alpha_i$ class of G proteins. However, some RGS proteins are only capable of acting as GAPs on very specific Gα subunits. For example, RGSZ2 displays specificity for $G\alpha_z$ subunits (Mao *et al*., 2004) and RGS2 appears to preferentially act on $G\alpha_q$ subunits.

As mentioned previously, the threonine at position 182 in $G\alpha_i$ is particularly important for high-affinity Gα-RGS binding. In fact, this residue is highly conserved among $G\alpha_i$ and $G\alpha_q$ but not $G\alpha_s$. This may explain the specificity of some RGS proteins.

Some RGS proteins can discriminate between the two highly related $G\alpha_q$ family subunits, $G\alpha_q$ and $G\alpha_{11}$. Using a yeast based assay, Ladds *et al*., was the first to demonstrate RGS-$G\alpha_q$ selectivity (Ladds *et al.*, 2007). Despite similar Gα expression levels, RGS16 and RGS5 are unable to reduce signalling from $G\alpha_{11}$ but are able to reduce signalling from $G\alpha_q$. $G\alpha_q$ and $G\alpha_{11}$ share 98% homology and future studies should identify the regions responsible for this selective inhibition.

1.6.2.2 Interaction with GPCRs

The selective binding and consequent recruitment of RGS proteins to the plasma membrane often seems to include the involvement of GPCRs. Evidence suggests that interactions between GPCRs and all subfamilies of RGS proteins exist. One noteworthy study used confocal microscopy to show that RGS2 was translocated to the plasma membrane of HEK293 cells in response to transient expression of the angiotensin II AT1 receptor or the β_2-adrenergic receptor. Similarly, RGS4 was recruited to the plasma membrane when the M2 muscarinic receptor was also expressed (Roy *et al.*, 2003).

Subsequently, investigations have studied the direct binding of RGS proteins to receptors. Snow *et al*., demonstrated that the PDZ binding motif at the C-terminal of RGS12 interacts with CXCR2 (Snow *et al.*, 1998b). A model was proposed in which the RGS12 GAP

activity was auto-inhibited. However, the recruitment of the PDZ domain of RGS12 to the receptor stops this inhibition and allows the GAP activity of $G\alpha_{i/o}$. As mentioned previously, a number of other RGS proteins contain this PDZ domain, and it is likely that this will show binding selectivity to other relevant receptors.

Co-immunoprecipitation studies have also detailed some of the selectivity that exists between GPCRs and RGS proteins. The N-terminus of RGS2 binds to the third intracellular loop of the M3 muscarinic receptors. This binding was also seen for another member of the B/R4 family of RGS proteins, RGS16, but not for another member of this family, RGS1 (Bernstein *et al.*, 2004).

Receptor specific inhibition of signalling has also been shown for many RGS proteins. Studies in oocyte expression systems have suggested that the N-terminal of RGS8 is responsible for its ability to inhibit signalling by either the M1 muscarinic receptor or the substance P receptor but not the M3 muscarinic receptor (Saitoh *et al.*, 2002). In chinese hamster ovary (CHO) cells expressing RGS4, RGS10 or RGSZ1, there was effective inhibition in response to activation of the 5-HT_{1A} receptor but less effective inhibition against the dopmaine D2 receptor despite both receptors coupling via $G\alpha_i$ (Ghavami *et al.*, 2004). This phenomenon was also seen by Xu *et al.*, when RGS1 was a 1000-fold more potent inhibitor of $G\alpha_{q/11}$ intracellular calcium ($[Ca^{2+}]_i$) mobilisation from the muscarinic receptors than the cholecystokinin receptors (Xu *et al.*, 1999). Indeed, this study also showed receptor selectivity of RGS4. In permeabilized rat pancreatic acinar cells, cholinergic receptors showed 3- and 10-fold higher apparent affinity to RGS4 than bombesin and cholecystokinin receptors (Xu *et al.*, 1999). Localisation of RGS proteins in precise cellular compartments can increase the specificity of an RGS protein for G proteins or GPCRs. The precise mechanisms for this specificity vary but tend to involve regions outwith the RGS domain and may often involve other auxiliary proteins.

1.6.2.3 Oligomeric GPCRs

It is now generally accepted that GPCRs form dimers or higher order oligomers (Hebert and Bouvier, 1998;Park *et al.*, 2004). Several studies have demonstrated GPCR oligomerisation using biochemical and biophysical techniques (Lavoie *et al.*, 2002;Lee *et al.*, 2003;Javitch, 2004;Milligan *et al.*, 2004a;Milligan *et al.*, 2004b). Atomic force microscopy of native mouse membranes has also observed rhodopsin receptors in an oligomeric array of closely packed dimers (Liang *et al.*, 2003;Fotiadis *et al.*, 2003). This

was the first conclusive evidence that receptor dimers exist in native membranes. Four rhodopsin receptors seem to be complexed with two transducin proteins (Park *et al.*, 2004). However, the physiological relevance of oligomeric GPCRs and multiple Gα subunits on RGS regulation is still unclear.

RGS14 contains an RGS domain and a GoLoco domain, both of which inhibit G protein signalling by binding directly to Gα subunits, perhaps suggesting simultaneous regulation of multiple Gα subunits, and an involvement with oligomeric GPCRs. Hepler *et al.*, researched the activity of RGS4 when a truncated form of RGS14 containing the GoLoco domain but lacking the RGS domain was present (Hepler *et al.*, 2005). This mutant increased the GAP activity of RGS4 and it has been suggested that the binding of the truncated RGS14 to one G protein may increase the affinity of RGS4 to bind to another G protein within the signalling complex (Abramow-Newerly *et al.*, 2006). It could be implied that the recruitment of RGS proteins to the plasma membrane would be multiplicative unless the binding sites on RGS proteins for all interacting proteins were the same. The main difficulty in this area is testing these hypotheses experimentally but suggests new functional implications for the regulation of RGS proteins.

1.6.2.4 Interaction with effectors

The interactions of RGS proteins with effectors can clearly influence regulation of signal transduction. The RGS protein can act as an effector antagonist, preventing the interaction of Gα and the effector, to reduce signalling. In contrast, signalling can also be increased by the RGS-effector interaction by creating a more stable complex to allow faster signalling.

The effect of $G\alpha_s$ on AC is to increase the level of cyclic AMP. The observation that RGS2 can physically interact with $G\alpha_s$ (Ko *et al.*, 2001;Roy *et al.*, 2006) but inhibit the rate of activation by AC in the absence of $G\alpha_s$ (Sinnarajah *et al.*, 2001) suggested that RGS proteins may directly bind to AC. Evidence now shows that RGS2 binds to the cytoplasmic domain of type V AC in cell extracts (Salim *et al.*, 2003) and it has now also been demonstrated that RGS2 can translocate to the plasma membrane by expression of various AC isoforms (Roy *et al.*, 2006). The N-terminal domian of RGS2 seems particularly important for this interaction but the specific mechanism for this is still unclear. AC type V is abundant in the heart and will undoubtedly prove to be an important area for further research (Salim *et al.*, 2003).

The rapid inhibition of $[Ca^{2+}]_i$ release by RGS proteins is mediated through the interaction of RGS proteins with the $G\alpha_q$ subfamily of G proteins. $G\alpha_q$ proteins, when triggered by a receptor, activate the plasma membrane bound enzyme phospholipase C β (PLCβ). This enzyme reacts on phosphitidylinositol 4,5-bisphosphate (PIP2) in the membrane to release inositol 1,4,5-trisphosphate (IP3). The IP3 generated binds to specific receptors on the endoplasmic reticulum, which induce opening of calcium release channels. This quickly raises the concentration of Ca^{2+} ions in the cytosol. By increasing the GTPase activity of $G\alpha_q$, RGS proteins decrease the inositol signalling of PLCβ and lower the amount of Ca^{2+} ions released into the cytosol.

However, RGS4 not only binds to activated $G\alpha_q$, as predicted, but also to Gβγ and PLCβ. The affinity of RGS4 for Gβγ is much weaker than for $G\alpha_q$, but this secondary interaction may serve to keep RGS4 localised in the signalling complex and possibly in the correct orientation for $G\alpha_q$ rebinding. PLCβ, similarly to RGS4, has GAP activity for $G\alpha_q$ and the primary interaction of PLCβ has been suggested to be competitive with RGS4 interaction to $G\alpha_q$. Ternary complexes between Gα, Gβγ and PLCβ1 can form, but only at relatively high protein concentrations (Dowal *et al.*, 2001). These interactions may allow RGS4 to remain anchored to the signalling complex even in the inactive state and allow rapid cycling of activated $G\alpha_q$.

1.6.3 Cellular calcium

As an important second messenger, the cellular concentration of Ca^{2+} must be regulated for proper cell signalling. There is a very large transmembrane electrochemical gradient of Ca^{2+} driving the entry of the ion into cells and three calcium pumping ATPase systems operate to maintain cytosolic $[Ca^{2+}]$ at a low level of about 10-7 M (Lytton *et al.*, 1992). These ATPases include the sarco/endoplasmic reticulum ATPases (SERCAs) that sequester Ca^{2+} into internal release compartments, and plasma membrane Ca^{2+}-ATPases (PMCAs) located in the surface membrane of cells that extrude Ca^{2+} against a large concentration gradient (Dunham and Glynn, 1961).

These pumps are powered by the hydrolysis of ATP, with a stoichiometry of two Ca^{2+} ions removed for each molecule of ATP hydrolysed. PMCAs tightly bind Ca^{2+} ions (a high affinity, with a Km of 100 to 200 nM) but do not remove Ca^{2+} at a very fast rate (Burette and Weinburg, 2006). Thus the PMCA is effective at binding Ca^{2+} even when its concentrations within the cell are very low, thereby ideally suited for maintaining Ca^{2+}.

Ca^{2+}/calmodulin binds and further activates the PMCA, increasing the affinity of the protein's Ca^{2+} binding site twenty to thirty times. Calmodulin also increases the rate at which the pump extrudes Ca^{2+} from the cell, possibly up to ten fold (Carafoli, 1991).

These PMCAs are in contrast to the sodium-calcium exchangers (NCX), which have a low affinity and a high capacity towards Ca^{2+}. The NCX is an antiporter membrane protein which removes Ca^{2+} from cells using the energy that is stored in the electrochemical gradient of Na^{+} by allowing three Na^{+} to flow down its gradient across the plasma membrane in exchange for the countertransport of one Ca^{2+} (Yu and Choi, 1997). The NCX is considered one of the most important cellular mechanisms for removing Ca^{2+} (Dipolo and Beauge, 2006), transporting up to five thousand Ca^{2+} ions per second (Carafoli *et al.*, 2001). Therefore it requires large concentrations of Ca^{2+} to be effective, but is useful for ridding the cell of large amounts of Ca^{2+}in a short time, as is needed in a neuron after an action potential.

1.6.4 RGS proteins as scaffolding proteins

The direct and specific binding of RGS proteins to other components of the cellular signalling complex also contributes to the regulation and function of RGS proteins. Preliminary research indicates that RGS proteins can act as scaffolds to assemble signalling complexes. β-arrestins scaffold the signalling complex between GPCRs and related kinases to stabilise the MAPK signalling pathway (Miller and Lefkowitz, 2001). In a similar way, a specific interaction between RGS2 and the α_{1b}-adrenoceptor has been shown to include the scaffold, spinophillin (Wang *et al*., 2005). A more direct involvement of RGS proteins to act as scaffolds for GPCRs came from the ability of Gα-interacting protein (GAIP) to associate with the dopamine D2 receptor (Jeanneteau *et al*., 2004). This required the interaction of GAIP-interacting protein C terminus (GIPC), a protein with a history of acting as a scaffold to many signalling complexes, including TrkA nerve growth factor receptors (De Vries and Farquhar, 2002;Abramow-Newerly *et al.*, 2006). RGS19 also interacts with TrkA nerve growth factor receptor and a recent report indicated that in PC12 cells, GIPC interacts with the TrkA nerve growth factor receptor and RGS19. All three proteins co-precipitate, indicating that a trimeric complex is possible (Lou *et al*., 2001). However, to fully understand the stabilising contribution of RGS proteins, this area needs further investigation.

1.6.5 Post translational modification of RGS proteins

Direct regulation of RGS proteins can also be obtained by post-translational modifications on RGS proteins. Phosphorylation and palymitoylation have both been shown to be powerful regulatory mechanisms of cellular signalling. These modifications will only take place in living cells, stressing the importance of *in vivo* research to bring together all components of the signalling network.

1.6.5.1 Phosphorylation

Phosphorylation of RGS proteins can have the most impact on signalling. The addition or removal of a phosphate from an RGS protein has diverse and complicated patterns of effect. The phosphate group can sterically hinder or promote the interaction with other proteins in the signalling complex, increasing or decreasing GAP activity (Table 1.2).

A number of different kinases are responsible for RGS protein phosphorylation. Protein kinase C (PKC) phosphorylation of RGS7 is necessary for its interaction with the intracellular scaffold protein, 14-3-3 (Benzing *et al.*, 2000). The phosphorylation site on RGS7, serine^{434}, is within an important region for contact with $G\alpha_i$. Phosphorylation of this residue allows the RGS proteins to interact with 14-3-3 in place of $G\alpha_i$, reducing GAP activity. The complexity of the role of phosphorylation was increased when Benzing *et al.*, found that this RGS7 phosphorylation is dynamically regulated by at least tumour necrosis factor-α (TNF-α), which reduces phosphorylation, allowing uncoupling from 14-3-3 proteins and increased GAP activity (Benzing *et al.*, 2000).

Phosphorylation of RGS proteins can also influence positive feedback loops. Addition of phosphate to serine^{53} on RGS16 occurs after α_{2A}-adrenoceptor activation. Phosphorylation reduces GAP activity and further increases α_{2A}-adrenoceptor signalling (Chen *et al.*, 2001).

Transmembrane tyrosine kinases and second messenger tyrosine kinases have also been shown to phosphorylate RGS proteins (Derrien *et al.*, 2003). Recent studies have attempted to unravel this additional complexity for RGS regulation as GPCRs can themselves induce phosphorylation of tyrosine kinases (Ogier-Denis *et al.*, 2000). The network of signalling cascades is yet to be fully explored and there still seems to be no consistent effects of phosphorylation on RGS regulation.

1.6.5.2 Palmitolyation

The addition of palmitate to RGS proteins also influences regulation (Table 1.3). Palmitolyation of N-terminal cysteine residues occurs in a number of RGS proteins, including RGS3 (Castro-Fernandez *et al.*, 2002), RGS4 (Srinivasa *et al.*, 1998;Tu *et al.*, 1999;Bahia *et al.*, 2003;Osterhout *et al.*, 2003) RGS7 (Rose *et al.*, 2000;Takida *et al.*, 2005), RGS10 (Tu *et al.*, 1999;Castro-Fernandez *et al.*, 2002) and RGS16 (Druey *et al.*, 1999a). The post-translational, reversible addition of this fatty acid moiety occurs by thio-ester bonds and serves to effect the membrane attachment and cellular localisation of the modified proteins. RGS16 is palmitoylated at exposed cysteine residues 2 and 12, allowing RGS16 to become bound to the plasma membrane in close proximity to other signalling proteins. In this way, it has been suggested that RGS16 can properly regulate $G\alpha_i$ and $G\alpha_i$-linked receptors (Druey *et al.*, 1999b). The closely related B/R4 RGS protein, RGS4, also has exposed cysteine residues at position 2 and 12. However, RGS4 is also palmitolylated at reside 95 (Tu *et al.*, 1999). Cysteine^{95} is in the RGS domain and addition of palmitate reduces RGS4 binding to G proteins. Cysteine residues within the RGS domain are conserved among a number of RGS subfamilies and palmitoylation represents a common way in which post-translational modification can alter both localisation and GAP activity of RGS proteins.

1.7 RGS proteins as therapeutic targets

RGS proteins associate directly with proteins implicated in a number of diseases and consequently, RGS proteins are potential important drug targets of the future (Zhong and Neubig, 2001;Neubig and Siderovski, 2002).

1.7.1 Polymorphisms

A number of single nucleotide polymorphisms (SNPs) have been identified in RGS proteins. SNPs are a single nucleotide variation in the genome between members of a species. Non-synonymous SNPs in RGS proteins have been suggested to confer a protective genotype against tumourigenesis. For example, a polymorphic variant of RGS6 confers a reduction in the risk of bladder cancer (Berman *et al.*, 2004). Functional changes in transcript levels, alternative splicing events, and protein translation efficiency that may result from the presence of a variant cysteine to threonine allele were investigated. This SNP appears to modulate protein translation and may contribute to the protective

phenotype by increasing the level of RGS6 protein. Further research by the same investigators has shown that replacement of a serine with a glycine in PDZ-RhoGEF is associated with a reduction in lung cancer among Mexican Americans. Moreover, a combination of this SNP and the SNPs mentioned previously in RGS6 seems to have a synergistic interaction (Gu *et al*., 2006). Patients with both SNPs had significant reduction in risk of lung cancer suggesting a dose-gene dependent effect.

Many other RGS proteins have also been suggested as key regulators of tumorigenesis. For example, RGS14 binds to the Ras-related G proteins, Rap1/2 (Traver *et al*., 2000). RGS12 is a transcriptional repressor, and RGS12 overexpression in select cell lines inhibits DNA synthesis (Chatterjee and Fisher, 2002). RGS3 has been demonstrated to play a role in inducing apoptosis (Dulin *et al*., 2000). Advanced research into the genetic variation and ethnic differences of SNPs in this family of proteins may present new insights into therapeutic cancer treatments.

1.7.2 Drug development

Small molecules which modulate the RGS-Gα interaction have been proposed as novel drugs which could be used to treat numerous disease states. Up to ten individual candidate regions that could serve as targets for drugs to alter RGS function exist on RGS proteins (Figure 1.2)

One such drug has already been designed that could inhibit RGS4 from interacting with the Gα subunit (Jin *et al.*, 2004;Riddle *et al.*, 2005). RGS4 over-expression reduced ventricular hypertrophy in response to pressure overload (Rogers *et al.*, 1999). A small molecule which blocks the interaction of RGS4 would suggest that despite up-regulation, RGS4 would have limited effects on the induction of ventricular hypertrophy.

Novel drugs could also be designed for use in conjunction with existing drugs which are GPCR agonists, to maximise the signalling response. Potentiating an exogenously administered GPCR agonist could suggest a smaller therapeutic dose of the agonist would be required, reducing unwanted side effects and tolerance. Specificity of the agonist may also be enhanced due to the precise regionalised localisation of RGS proteins. This type of drug design was particularly important in a study which knocked down the levels of endogenous RGS2 and RGS9. Lower levels of RGS2 were found to make morphine less potent. However, reducing the level of RGS9 had the opposite effect, producing pain relief

at lower concentrations of morphine thus reducing tolerance and allowing multiple treatments (Garzon *et al*., 2001). Endogenous RGS9 therefore must facilitate tolerance and the potency of morphine. A small molecule designed to inhibit RGS9 has great therapeutic potential in analgesic treatment.

RGS9 has also been implicated in Parkinson's disease (Tekumalla, 2001). Significant increase in the levels of RGS9-2 was found in patients suffering from this disease. In Parkinson's disease, dopamine neurons degenerate leading to defective circuitry within the brain. Dopamine D1 and D2 receptors control movement and the equilibrium of these two receptors seems to dictate the signalling pathways. Increasing levels of RGS9-2 specifically inhibits D2 receptors, critically changing the balance of receptors (Tekumalla, 2001). Keeping the receptor signalling steady by use of a compound targeted towards RGS9-2 may prevent the progression of this disease. An RGS inhibitor might also be more specific than a D2 agonist due to the highly specific localisation of RGS9-2 in the caudate putamen (Gold *et al.*, 1997). The distinct subcellular localisation and expression pattern of RGS9-2 represent novel and potentially exciting targets for the development of new psychotropic medications**.**

Further research is also required into the possibility of using compounds that would enhance RGS function, so called RGS agonists. Blocking interactions with endogenous proteins that inhibit RGS function would stimulate GAP activity and inhibit G protein signalling. This could be beneficial in a number of disease states, for example, stimulation of RGS1 would block $G\alpha_{i1}$ signalling and reduce inflammatory responses. Blocking $G\alpha_{q}$ signalling through stimulation of the GAP activity of RGS2 could be useful in treating hypertension and vascular restenosis.

1.7.3 Transgenic animals

Despite over ten years of research, only three RGS knock-out mice have been reported, $RGS2^{-/-}$ (Tang *et al.*, 2003), $RGS4^{-/-}$ (Grillet *et al.*, 2005) and $RGS9^{-/-}$ (Chen *et al*., 2000). A novel method of studying the combined role of all endogenous RGS proteins has emerged instead. Fu *et al*., assessed the function of endogenous RGS proteins by using a knock-in strategy with a mutant Gα subunit that is unable to bind to RGS proteins (Fu *et al*., 2004). The point mutation in switch I region of $G\alpha_{o}$ (^{G184S}) or $G\alpha_{i1}$ (^{G183S}) blocks the interaction with RGS proteins but has been reported to have no effect on the G proteins ability to interact with other signalling components. Introducing these mutant G proteins

into embryonic stem cells by gene targeting allowed the function of endogenous RGS proteins in intact mice to be measured. Loss of RGS protein function potently increased the heart rate and differential use of both $G\alpha_i$ and $G\alpha_o$ were observed, again suggesting novel therapeutic potential for RGS protein regulation in cardiovascular disease.

Transgenic animals have been engineered with a double mutant approach. In this way, a specific RGS-Gα subunit interaction can be rescued (Wieland *et al*., 2000). This has been reported for both RGS16 and RGS4 where a lysine to glutamate mutation in both $G\alpha_i$ and $G\alpha_q$ renders the Gα subunit insensitive to endogenously expressed RGS proteins. On the other hand, glutamate to lysine mutants of interacting RGS proteins were able to restore the original signalling. These mutated proteins are selectively uncoupled from endogenous signal transduction at the level of RGS-Gα subunit interaction but are otherwise functionally intact. Rescuing mutant pairs may be a helpful tool to analyse RGS-Gα subunit interaction in living cells or even transgenic animals. In view of the fact that drugs targeted towards GPCRs can represent up to 30% of the portfolio of many pharmaceutical companies, RGS proteins are in a key position to become targeted for drug development.

1.8 RGS4

RGS4 is the most extensively researched RGS protein. In resting cells, RGS4 is a soluble hydrophilic protein found in the cytosol. It was one of the first RGS proteins to be discovered and the function of this RGS protein is best understood from yeast. Early studies in *S. cerevisiae* found that removal of the gene *Sst2* made the organism supersensitive to G protein directed pheromone responses (Chan and Otte, 1982;Weiner *et al.*, 1993;Dohlman *et al.*, 1995). This discovery was instrumental in detecting the presence of mammalian RGS proteins and in fact, mammalian RGS4 has now been shown to be able to directly substitute for Sst2p, showing the high level of conservation between RGS proteins in yeast and mammals.

1.8.1 N-terminal of RGS4

RGS4 has a relatively simple protein architecture. Apart from the RGS domain the only other recognisable domain is an amphipathic α helix at the N-terminal. The 33 amino acid α helix is thought to be involved in the translocation of RGS4 to the cytosolic face of the plasma membrane. In reconstituted systems using purified proteins, the N-terminus of RGS4 was found to interact with lipid vesicles (Tu *et al*., 2001). Conserved cysteine

residues in the N-terminal of RGS4 are particularly important for correctly targeting the protein within the cell. RGS4 is cytosolic, however post-translational addition of palmitate to cysteines within the N-terminus may target RGS4 to specialised lipid rafts in the plasma membrane (Druey *et al.*, 1998;Moffett *et al.*, 2000).

The lipid raft microdomain in plasma membranes is constituted of many different protein-lipid interactions thought to be involved in signal transduction. RGS4 can penetrate the inner leaflet of the plasma membrane bilayer and growing evidence suggests that phosphatidic acid within the bilayer can also inhibit RGS4 GAP activity. Use of an N-terminal truncation of RGS4 resulted in loss of both phosphatidic acid binding and lipid-mediated functional inhibition. Using single amino acid mutations, it was found that lysine^{20} is responsible for these regulatory properties in reconstituted vesicles (Ouyang *et al*., 2003).

The specificity of interaction between RGS4 and GPCRs also appears to be determined by the N-terminal of RGS4. As mentioned previously, signalling initiated by agonist binding to GPCRs bound to $G\alpha_q$ (Berridge, 1993), generates IP3 to trigger Ca^{2+} release from internal stores. In rat pancreatic acinar cells, RGS4 preferentially inhibits $G\alpha_{q/11}$-mediated signalling induced by carbachol compared to bombesin and cholecystokinin regardless of the identity of the $G\alpha_q$ subunit (Zeng *et al.*, 1998). Further use of an N-terminally truncated RGS4 exhibited no receptor selectivity but the ability to distinguish the carbachol activated muscarinic receptor was restored by re-addition of this N-terminal domain. Such selectivity suggests intact RGS4 interacts directly or indirectly with receptors, most likely through the N-terminal domain (Zeng *et al.*, 1998).

Removal of the extreme N-terminal methionine of newly synthesized RGS4 can limit RGS4 availability. The cysteine at position 2 is subsequently exposed and becomes the target for arginylation. This allows for subsequent degradation of RGS4 by ubiquitination and the 26S proteasome (Davydov and Varshavsky, 2000). Redundant proteins that are damaged or no longer required are often targeted by ubiquinitation. A mouse lacking the gene to encode the enzyme that adds arginine to the N-terminal of proteins (Arg-transferase) died with cardiovascular defects, and the unmodified substrates of this enzyme were identified to be RGS proteins. Given the ability of RGS4 to negatively regulate cardiovascular signalling pathways, RGS4 is an important target for consideration. The ubiquitin-dependent N-end rule pathway relates to the half-life of the protein. The half-life of RGS4 is approximately 1 hour but the amount of RGS4 degredaded by these means

seems to be dependent on cell type (Davydov and Varshavsky, 2000). Studies have concluded that the regulated degradation of RGS4, RGS5 and RGS16 by the N-end rule pathway is important for the correct functioning of the cardio vascular system (Lee *et al*., 2005). The ability of cysteine at postion 2 of RGS4 be a target for both palmitoylation and arginylation suggests a conflict between these two systems. Perhaps palmitoylation not only helps target RGS4 to the plasma membrane but inhibits arginylation, promoting stability of the protein.

Indeed, mutation of cysteine^2 to valine forms a degradation resistant RGS4. An epitope-tagged form of this mutant was used in co-immunoprecipitation studies in CHO cells transfected with the G protein-gated inwardly rectifying potassium (GIRK) channel (Jaen and Doupnik, 2006). RGS4^{C2V} readily co-precipitated with GIRK channels. This precipitation was shown to be RGS specific, as the short isofom of RGS3 did not interact with any of the GPCR-GIRK channel complexes. Chimeric RGS4/RGS3 constructs indicated that the N-terminal domain of RGS4 is necessary for the GIRK interaction. The N-terminus of RGS4 is therefore also important in the functional activity of the protein. The predominant expression of RGS4 is in the brain and heart so this activity may well be physiologically relevant in affecting neurotransmitter-mediated inhibitory events in the nervous and cardiovascular systems.

RGS4 has also been found to directly associate with both the μ-opioid and δ-opioid receptors (Georgoussi *et al*., 2006). GST fusion proteins of the C-terminal of both receptors were found to associate with recombinant RGS4 *in vitro*. Members of the RGS family are known to play an essential role in opioid signalling (Smrcka *et al.*, 1991;Rhee, 1991) but perhaps RGS4 has an undiscovered role in opiate action. The specific contact sites on RGS4 and the dynamics of this interaction are also still unknown. Perhaps the specificity of this interaction is also determined by the N-terminal of RGS4 and will further demonstrate that the relatively simple N-terminal of RGS4 is fundamental to the effectiveness of the protein to act as a negative regulator of G protein signalling.

1.8.2 The RGS domain of RGS4

The RGS domain of RGS4 (residues 58-177) had a normal GAP activity towards $G\alpha_o$ and appears to be able to work as a single functioning domain (Srinivasa *et al.*, 1998). Forming a bundle of nine α-helices, the domain binds $G\alpha_{i1}$ in a cleft consisting of conserved amino acids at the ends of helices 4, 7 and 8 and the loops between helices 3 and

4 and helices 5 and 6. Many other residues are important within the RGS domain of RGS4 for the folding and/or the stability of the protein. These include a pair of phenylalanine residues (phenylalanine^{79} and phenylalanine^{168}), that when either is substituted with alanine, resulted in an insoluble protein when expressed in *Escherichia coli*.

Previous studies on the role of RGS proteins on Ca^{2+} signalling led to the discovery that calmodulin (CaM) and phosphatidylinositol (3,4,5)-trisphosphate (PIP3) antagonise each others binding to the RGS domain of RGS4 (Popov *et al*., 2000). PIP3 binds RGS4 at a site within the RGS domain, distinct and opposite to the RGS/Gα contact face and inhibits the GAP activity of RGS4. When complexed with Ca^{2+}, CaM competes with PIP3 for binding to RGS4 and removes GAP inhibition and restores activity of the RGS protein. These data suggest a mechanism in which GPCR stimulation of Ca^{2+} signalling is regulated by feedback inhibition (Sierra *et al*., 2000). Interestingly, the pre-treatment of cells with the detergent methyl-β-cyclodextrin, which depletes the membrane of cholesterol and therefore disrupts the lipid rafts, and weakens the interaction of CaM with RGS4 (Ishii *et al.*, 2005). Perhaps without lipid rafts, RGS4 and CaM cannot be brought into close proximity, reducing the strength and probability of their interaction.

1.8.3 Endogenous functions of RGS4

The engineering of $Rgs4^{-/-}$ mutant mice allowed the endogenous role of RGS4 in developmental, behavioural and physiological tests to be explored (Grillet *et al.*, 2005). Subtle symptoms recorded included lower weight and poorer sensory motor coordination. More serious defects were not displayed*;* there was no alteration in neuronal differentiation or opioid signalling as mutant mice had normal tolerance to pain compared to wild type. Perhaps future studies should compare these knock-out mice with knock-down animal models. Compensation during development may take place in knock-out animals and these future experiments may reveal differences so far undiscovered.

1.8.3.1 Heart

It is increasingly clear that RGS4 acts as important negative regulator of both $G\alpha_i$ and $G\alpha_q$ signalling. Little is known about the *in vivo* role of RGS4, but one study found the overexpression of RGS4 mRNA or protein is frequently seen in patients or animals with heart dysfunction (Mittmann *et al.*, 2002). In mouse cardiomyocytes increased levels of RGS4 are thought to be associated with a reduction of smooth muscle cells of the large

vessels of the heart and coronaries activating a hypertrophic response and left ventricular dilation (Rogers *et al.*, 1999). However, the expression pattern of RGS4 in cardiomyocytes is contentious. Grillet *et al*., could not detect any RGS4 in the heart muscle itself but instead found high levels in the endothelium (Grillet *et al.*, 2005). Presumably the previous results seen in patients or animals with heart dysfunction could also have displayed this expression pattern (Mittmann *et al.*, 2002). Differences across species may account for these variations but the potential for cardiac therapeutics is low if, indeed, there is no expression of RGS4 in the heart itself.

1.8.3.2 Central Nervous System

RGS4 has also been speculated to perform physiological roles in the CNS (Gold *et al.*, 1997;Nomoto *et al.*, 1997;Erdely *et al.*, 2004). Expression in the cerebral cortex suggested regulation of dopamine, serotonin, noradrenaline, glutamate and opioid receptors makes RGS4 a candidate gene for the functional modulation of neurotransmission. Activation of opioid receptors by morphine altered the expression of RGS4 in distinct locations within the brain (Bishop *et al.*, 2002;Gold *et al.*, 2003). Factors that control opioid signalling are likely to be important to the understanding of drug abuse. Design of novel central nervous system drugs could prove useful in preventing the development of or treatment of drug dependence.

1.8.3.3 Risk Factors for Schizophrenia

The gene for RGS4 is located on chromosome 1q23 and many psychiatric genetic studies have suggested a linkage to schizophrenia (Brzustowicz *et al.*, 2000;Gurling *et al.*, 2001). The status of RGS4 as a susceptibility gene for schizophrenia first came from its high levels of expression in the cortex of the brain where it could potentially regulate dopamine and glutamate signalling.

Controversy surrounding this linkage analysis still exists, a large scale study did not find any linkage between these RGS4 and schizophrenia (Levinson *et al*., 2002) but it is still generally accepted that RGS4 is an interesting candidate gene for schizophrenia. RGS4 is not only highly expressed in brain regions implicated in the pathophysiology of schizophrenia, but also modulates the function of multiple G protein coupled neurotransmitter receptors and can exhibit robust transcriptional changes to stress (Levitt *et al*., 2006). Expression of RGS4 has been demonstrated to decrease across the cerebral

cortex in subjects with schizophrenia. Importantly, expression of other RGS proteins or levels of RGS4 in patients with an alternative major depressive disorder are not altered. A decrease in RGS4 expression may enhance $G\alpha_i$-mediated signalling, resulting in diminished AC activity as is associated with chronic stress. However, the exact Gα subunit regulated by this RGS protein in the brain *in vivo* remains unclear. Post-mortem studies of schizophrenia patients detected no associated polymorphisms in RGS4 suggesting that the principal explanation of any relationship is the decreased transcription level of RGS4 mRNA. Indeed, in the previous research which confirmed linkage, it was the upstream sequence of RGS4 which controls transcription, that linked to the schizophrenia gene locus (Chowdari *et al.*, 2002).

1.9 RGS16

RGS16, like RGS4 is a member of the B/R4 subfamily. RGS16 also has an N-terminal amphipathic α helix which is a key determinant of membrane localisation of the protein. The current model of localisation suggests that RGS16 firsts finds a docking partner at the membrane and then undergoes palmitoylation that locks the protein in place (Dunphy and Linder, 1998;Wedegaertner, 1998). Mutagenesis of key cysteine residues therefore had little effect on overall localisation, but mutation of hydrophobic resides and basic resides had a much greater effect on decreasing plasma membrane localisation and activity of RGS16 (Chen *et al.*, 1999;Bernstein *et al.*, 2000). Hiol *et al*., have suggested that cysteine^{2} and cysteine^{12} of RGS16 are palmitoylated at the plasma membrane, stabilising the protein in to lipid rafts (Hiol *et al.*, 2003). These membrane microdomains, rich in protein acyltransferase serve to rotate RGS16 exposing cysteine^{98}, potentially leading to this residue being palmitoylated, and subsequently greatly enhancing the functionality of RGS16.

RGS16 is expressed in several tissues including the liver, pituitary, retina and the pineal region of the brain (Chen *et al.*, 1996;Chen *et al.*, 1997). The pineal gland is located deep in the midbrain area near many vital structures, including the aqueduct of Sylvius, which serves as a passage allowing cerebrospinal fluid to leave the centre of the brain where it is first produced. Several types of tumours are known to originate from the pineal region and may often compress this aqueduct, causing the build-up of cerebrospinal fluid in the brain. Microarray analysis of differential gene expression patterns in tumours of the pineal region found high expression of many genes related to phototranduction in the retina, including RGS16 (Fevre-Montange *et al.*, 2006). The clinical significance of this expression needs

further investigation but perhaps implies RGS16 could be a candidate gene for use as a molecular marker for identifying patients with pineal region tumours.

Overexpression of RGS16 has also been found in megakaryocyte differentiation (Berthebaud *et al*., 2005). In these cells RGS16 negatively regulates CXCR4 signalling, and subsequently reduces downstream effectors. RGS18 is also overexpressed in megakaryocyte differentiation (Yowe *et al*., 2001) but RGS16 and RGS18 affect CXCR4 signalling differently seemingly due to RGS protein specificity of action on receptor and G protein subtypes. Experiments using RNAi interference saw reciprocal results (Airoldi *et al*., 2006) and perhaps studies on knock-out mice will further clarify the role of RGS16 in megakaryocytes.

As mentioned previously, RGS16 is a phosphoprotein. Phosphorylation of RGS16 upon stimulation of the α_{2A}-adrenoceptor by epinephrine significantly reduced its GAP function and consequently its attenuation of the MAPK pathway (Chen *et al.*, 2001). RGS16 can also undergo epidermal growth factor receptor-mediated tyrosine phosphorylation on a conserved tyrosine residue in the RGS box, tyrosine^{168}, which enhances RGS16 GAP activity in single turnover assays (Derrien *et al.*, 2003). The authors also demonstrated that tyrosine^{177}, the only other tyrosine residue in RGS16, was also phosphorylated. Phosphorylation has been suggested to induce or prevent RGS16 localisation in lipid rafts or perhaps generate a docking site for interaction with novel proteins and regulate the GAP activity of RGS16.

The high homology between RGS16 and RGS4, and the growing knowledge about the function of these proteins *in vivo* and *in vitro*, prompted the use of these RGS proteins in this study. In particular, RGS16 was employed to deduce if a mutation in RGS4 was RGS specific or RGS subtype specific.

1.10 Methods of studying RGS proteins

A current challenge is to define the function of individual RGS proteins. Cellular mechanisms of RGS proteins are coordinated to regulate a diverse range of cellular functions and methods of studying individual endogenous RGS proteins demonstrate RGS functions not yet predicted by *in vitro* assays.

1.10.1 Yeast as a model organism

The presence of multiple RGS proteins and overlapping expression in mammalian cells makes investigating the activity of one RGS protein difficult. Not every RGS protein is expressed in every cell, but various combinations are possible and create a multitude of signalling pathways (Bockaert and Pin, 1999). Yeast has a similar signalling pathway to mammalian cells but has fewer components. Yeast have therefore been previously characterised to express particular signalling components in isolation (Whiteway *et al.*, 1989;Cismowski *et al.*, 1999)

Since the identification of RGS proteins in *S. cerevisiae*, yeast has been continually used as a model organism to study the function of RGS proteins. The pheromone response pathway in yeast is a GPCR mediated signalling pathway that allows signalling between the MATa and MATα cells. The signalling components comprise of a single GPCR (Ste2p for MATa and Ste3p for MATα), a Gα protein (Gpa1p), a Gβ protein (Ste4p), a Gγ protein (Ste18p) and RGS protein (Sst2p). Their mammalian counterparts have successfully replaced many components in *S. cerevisiae*. However, the Gα subunit in *S. cerevisiae* is not equivalent Gα subunit in mammalian cells. In *S. cerevisiae* the Gα subunit is a negative regulator of signalling and the Gβγ dimer propagates the signal. Studies expressing functional mammalian Gα subunits in yeast have therefore been carried out in an alternative yeast, *Schizosaccharomyces pombe*. The Gα subunit in this yeast is a positive regulator of signalling and the pheromone response pathway in fission yeast has been previously modified to provide assays with which to analyse individual GPCR signalling pathways (Watson *et al.*, 1999;Didmon *et al.*, 2002). *Sz. pombe* mutants without Sst2p show a hyperresponsive GPCR response. Endogenous levels of RGS proteins must therefore limit the pheromone response. Overexpression of RGS proteins in this yeast strain show a reduction of this hyperresponsiveness and a reduced ability to desensitise in the continued presence of pheromone (Dohlman *et al.*, 1996). The basal level of RGS proteins in yeast is therefore the crucial rate-limiting factor in the yeast signalling pathway.

1.10.2 Mutagenesis of RGS proteins

Mutagenesis demonstrates the importance of certain amino acids in proteins. As mentioned previously, the crystal structure of RGS4 complexed with $G\alpha_i$-GDP-AlF_4^-, highlighted important amino acids at the interface between the RGS protein and the Gα subunit. Mutation of these specific residues within the Gα subunit can alter signalling. A

series of these mutations have been constructed, which are found either to be unable to release GDP (Carrillo *et al.*, 2002) or have reduced interaction with RGS (DiBello *et al.*, 1998). In addition, mutations of specific amino acids in RGS4, that directly contact the $G\alpha_{i1}$ have been reported to completely abolish RGS4 GAP activity (Srinivasa *et al.*, 1998).

The identification and characterisation of a Gα subunit mutant that specifically disrupts the interaction with RGS proteins provided a new approach to study the endogenous function of RGS proteins (DiBello *et al.*, 1998). As mentioned previously, a single amino acid change in the Gα subunit in switch region I can produce a G protein insensitive to RGS action. These RGS-insensitive G proteins can also be used *in vitro*, to study the endogenous function of RGS proteins. Following agonist activation, the μ-opioid increase in $[Ca^{2+}]_i$ was less affected when coupled through an RGS-insensitive $G\alpha_o$ as compared to a RGS-sensitive protein (Clark *et al.*, 2004). This effect caused by the inability of the RGS-insensitive protein to bind to $G\alpha_o$ suggests that the GAP activity of RGS proteins provides a control that regulates potency and maximal response of agonist activated signalling.

1.10.3 Experimental methods used

The complex and diverse structures of RGS proteins allow varied mechanisms of regulation and cellular functions. In this regard, numerous experimental methods are commonly used to study this regulation and function.

1.10.3.1 Adrenoceptors

The adrenoceptors are the target of many therapeutic agents that regulate the peripheral and central nervous system. The adrenoceptors are GPCRs by which the important neurotransmitters, noradrenaline and adrenaline function. There are nine different subtypes of adrenoceptor, classified into two types α and β. Both α- and β-adrenoceptors have now been further subdivided into two subtypes α_1, and α_2. These subtypes were at first classified by their anatomical location; α_1 is found mostly postsynaptically, whilst α_2 although typically sited presynaptically, can also occur postsynaptically. These initial subtypes were further divided into α_{1a}, α_{1b}, and α_{1d} and α_{2A}, α_{2B}, α_{2C}, and α_{2D}. This knowledge has led to the development of selective agonists and antagonists for each subtype (Guimaraes and Moura, 2001).

α_2-adrenoceptors play a key role in regulating neurotransmitter release in the central and peripheral sympathetic nervous systems. Activation of α_2-adrenoceptors on sympathetic nerve terminals leads to a reduction in sympathetic tone, with a resultant decrease in heart rate and blood pressure. These presynaptic α_2-adrenoceptors serve as autoreceptors regulating catecholamine release, inhibiting the release of noradrenaline and thus serving as an important receptor in the negative feedback control of noradrenaline release. Postsynaptic α_2 receptors are located on liver cells, platelets, and the smooth muscle of blood vessels. Activation of these receptors causes platelet aggregation, and blood vessel constriction (Starke, 2001).

Using genetically engineered mice, the α_{2A}-adrenoceptors appears to be the subtype that plays the principal role in response to α_{2A} agonists for the suppression of blood pressure, attenuation of pain perception, analgesia, anesthetic sparing, and suppression of neurotransmitter release (Limbird, 2003).

The natural ligand for α_2-adrenoceptors, adrenaline, shows no selectivity for the α_2 subtype of receptors and so for the purpose of this study the α_2 selective agonist UK14304 was also used to activate this receptor subtype. The α_{2A}-adrenoceptor couples preferentially to the $G\alpha_i$ subfamily of G proteins. The high G protein cycling of this G protein subtype and good transient transfection efficiency makes the α_{2A}-adrenoceptor ideal for using to study RGS regulation in the present study.

α_1-adrenoceptors mainly couple through the $G\alpha_q$ subfamily of G proteins (Zhong and Minneman, 1999). Activation of $G\alpha_{q/11}$ stimulates PLCβ and subsequently promotes the hydrolysis of PIP2 producing IP3 and diacylgylercol (DAG). IP3 act as second messengers to release Ca^{2+} from internal stores. DAG synergises with calcium to activate PKC which phosphorylates specific target proteins in the cell to change their function. The inhibitory action of RGS proteins on $G\alpha_q$ signalling has previously been investigated. Expression of functionally active RGS proteins reduced agonist-stimulated elevation of $[Ca^{2+}]_i$ and allowed the relative activity of individual RGS proteins on particular receptor systems to be investigated (Clark *et al.*, 2004). The previous effectiveness of the α_{1b}-adrenoceptor to study $[Ca^{2+}]_i$ promoted the use of this receptor subtype in the present study to investigate the inhibitory action of RGS proteins.

α_1-adrenoceptors are found in both the central and peripheral nervous system. In the CNS they are found mostly postsynaptically and have an excitatory function. Peripherally they

are responsible for contraction and are situated on vascular and on non-vascular smooth muscle. α_1-adrenoceptors on vascular smooth muscle are located intrasynaptically and function in response to neurotransmitter release. For non-vascular smooth muscle they can be found on the liver, where they cause hepatic glycogenolysis and potassium release. On the heart they mediate a positive inotropic effect. Cause relaxation of gastrointestinal smooth muscle and decrease salivary secretion (Marshall *et al.*, 1999).

1.10.3.2 Fusion proteins

The C-terminus of a GPCR directly fused to the N-terminal of a Gα subunit, has proved invaluable in a number of investigations. In particular, the use of these fusion proteins in high-affinity GTPase assays has been invaluable to the understanding of RGS proteins. A fusion protein defines the stoichiometry of 1:1 of receptor and Gα subunit expression and ensures the co-localisation of the two signalling proteins. In addition, preservation of interactions between GPCRs, Gα subunits and Gβγ have also been demonstrated for these fusion proteins (Wise *et al.*, 1997b;Cavalli *et al.*, 2000;Bertaso *et al.*, 2003). A number of fusion proteins have been used in the present study as a proficient method of investigating RGS proteins.

However, the use of fusion proteins creates an artificially constrained signalling cascade and is not physiological relevant. Internalisation and desensitisation of fusion proteins may be different compared to native receptors (Loisel *et al.*, 1999) and investigations using this system must be interpreted with caution.

1.10.3.3 High-affinity GTPase assay

The fusion of the α_{2A}-adrenoceptor with a P. tox resistant $G\alpha_o$ has been used successfully to study the effect on the high-affinity GTPase activity of RGS proteins (Cavalli *et al.*, 2000). Indeed, fusion proteins allow the direct measurement of regulation of the agonist-activation of a G protein, preloaded with [γ-^{32}P]GTP, by a GPCR. Monitoring the release of [γ-^{32}P]Pi, the GTPase activity of $G\alpha_o^{C351I}$ and its regulation by RGS proteins can be analysed using basic enzyme kinetics.

1.10.3.4 Measurement of $[Ca^{2+}]_i$

The α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein has previously been used effectively to study single cell Ca^{2+} mobilisation (Carrillo *et al.*, 2002). Activating cells expressing the α_{1b}-

adrenoceptor-Gα_{11} fusion protein with agonist causes a rise in $[Ca^{2+}]_i$. This effect is inhibited by the GAP activity of RGS proteins. Using a Ca^{2+} sensitive probe, RGS-mediated inhibition of Gα_{11}-mediated signalling in live cells in real time was assessed and the magnitude of signal inhibition by RGS4 was studied.

1.10.3.5 [^{35}S]GTPγS

The [^{35}S]GTPγS binding assay measures the level of G protein activation following agonist occupation of a GPCR, by determining the binding of the non-hdrolyzable analogue [^{35}S]GTPγS to the Gα subuit. The non-hydrolysable γ thiophosphate bond allows the radiolabelled Gα to accumulate and be measured. To isolate and enrich the [^{35}S]GTPγS-bound α_{1b}-adrenoreceptor-Gα_{11} fusion protein in the membrane fraction of transfected cells, Gα_{11} in the reactions was solubilised by detergents and immunoprecipitated using an anti-G protein antiserum, CQ and then counting the radioactivity. The agonism of the α_{1b}-adrenoceptor-Gα_{11} fusion protein has been well established (Carrillo *et al.*, 2002) and this method was used to measure the G protein activation of a previously identified mutant of Gα_{11}

1.11 Objectives of this study

The aim of this study was to investigate the role of RGS4 in signal transduction. To focus this general aim, particular areas of current interest were investigated. The areas explored were set into three main objectives*;*

1. To investigate the cellular localisation of RGS4

2. To examine the effect of mutating selected residues on the subcellular localisation and functional activity of RGS4

3. To investigate a potential Gα_{11} RGS-insensitive mutant

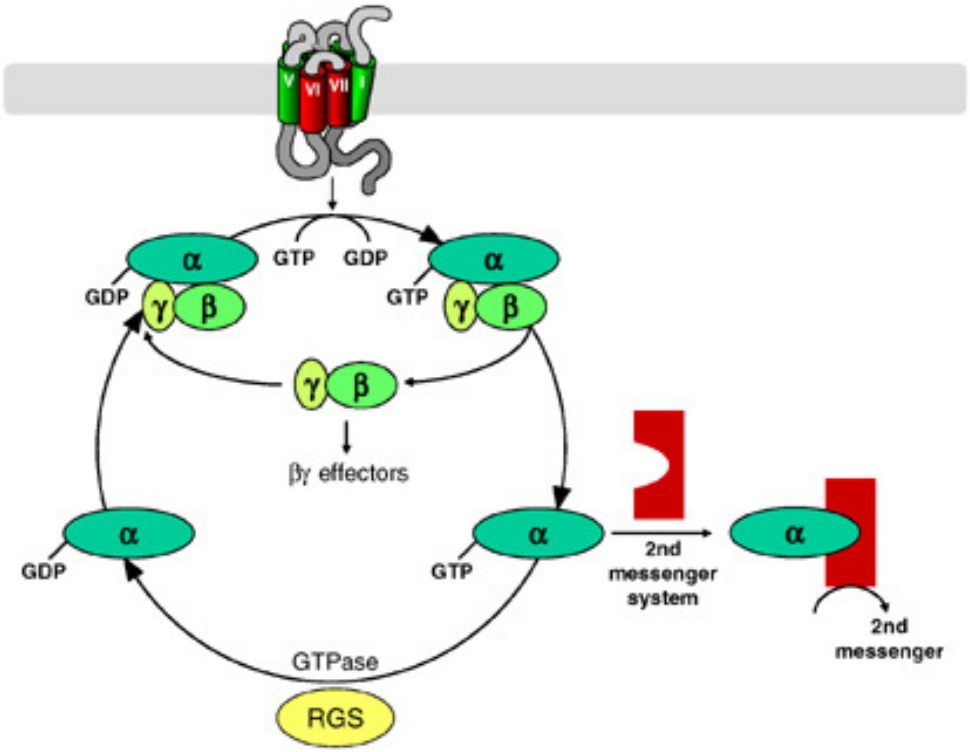


Figure 1.1 The G protein cycle

In the absence of stimulation, the Gα subunit is GDP bound and associated with the β and γ subunits. The GPCR either via constitutive activity or in response to binding of an agonist, promotes the release of GDP and its replacement with GTP. Conformational rearrangements may result in the dissociation of the Gβγ complex and these components can then interact with and regulate effectors. Intrinsic GTPase activity of the Gα subunit hydrolyses the bound GTP to GDP, this activity is accelerated by the GTPase activity of RGS proteins. The resulting reassociation of the G protein heterotrimer completes the cycle (taken from Milligan and Kostenis, 2006).

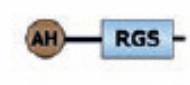
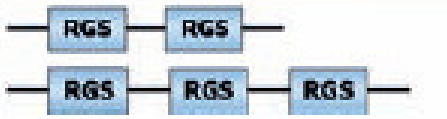
Family	Structure	Members
A/RZ		*RGS17 (RGSZ2), RGS19 (GAIP), RGS20 (RGSZ1), RET-RGS
B/R4		RGS1, RGS2, RGS3, *RGS4, RGS5, RGS8, RSG13, RGS16, RGS18, RGS21
C/R7		RGS6, RGS7, RGS9, *RGS11
D/R12		RGS10, *RGS12, RGS14
E/RA		*Axin, Conductin
F/GEF		P115-RhoGEF, PDZ-RhoGEF, *LARG
G/GRK		GRK1, * GRK2, GRK3, GRK4, GRK5, GRK6, GRK7
H/SNX		*SNX13, SNX14, SNX25
Atypical		*D-AKAP2 *RGS22

Table 1.1 Classification of RGS proteins

The RGS families of proteins. A schematic diagram depicting the structural motifs present in a representative member (shown by '*') of each of the RGS family is shown. Individual members of each sub-family do not necessarily contain all the motifs of the represented member. Abbreviations used to describe the different domains can be found in the Definitions (taken from Jean-Baptiste *et al*., 2006).

RGS Protein	Phosphorylated by	Residue	Physiological Effect	Reference
RGS2	PKC	-	< GAP activity	(Cunningham *et al*., 2001)
	PKGI-α	Serine 46/64	> GAP activity	(Tang *et al.*, 2003)
RGS3/4	PKG	-	Induces translocation from cytosol to cell membrane	(Pedram *et al*., 2000)
RGS7	PKCα	Serine 434	Promotes the binding of 14-3-3 and decreases GAP activity	(Benzing *et al.*, 2000)
RGS9	PKA	Serine 427/428	< GAP activity	(Balasubramanian *et al*., 2001)
	PKCα/PKCθ	Serine 475	Alters subcellular localisation	(Sokal *et al*., 2003)
RGS10	PKA	Serine 168	Induces nuclear translocation	(Burgon *et al.*, 2001)
RGS14	PKA	Serine 258/ Threonine 494	> GDI activity	(Hollinger *et al*., 2003)
RGS16	SRC	Tyrosine168	Promotes stability	(Derrien *et al.*, 2003)
	-	Serine 194/53	< GAP activity	(Chen *et al.*, 2001)
	-	Tyrosine 168/177	Tyrosine 168 > GAP activity	(Derrien and Druey, 2001)
RGS18	-	Serine 49		(Garcia *et al*., 2004)
RGS19	ERK2	Serine 151	> GAP activity	(Ogier-Denis *et al.*, 2000)

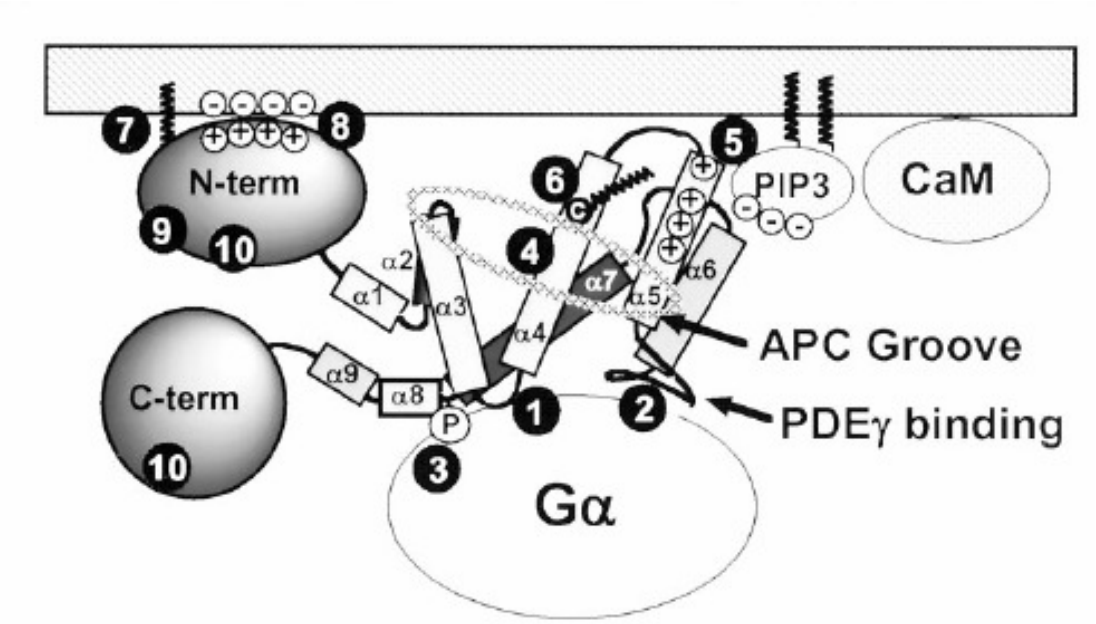
Table 1.2 Phosphorylation of RGS proteins

RGS proteins and the kinase they are phosphorylated by. If known, the amino acid phosphorylated together with the physiological effect of this post-translational modification are also shown (taken from Riddle *et al*., 2005).

RGS Protein	Residue	Subcellular Localisation	RGS activity	Reference
RGS3	-	-	-	(Castro-Fernandez *et al.*, 2002)
RGS4	Cysteine 2/12	No change	No change	(Srinivasa *et al.*, 1998)
	Cysteine 2/12/95	-	> and < activity*	(Tu *et al.*, 1999)
	Cysteine 95	-	> activity	(Osterhout *et al.*, 2003)
RGS7	Cysteine 69	Targets to membrane	No change	(Rose *et al.*, 2000)
	Cysteine 133	Targets to membrane	ND	(Takida *et al.*, 2005)
RGS10	Cysteine 66	-	> and < activity	(Tu *et al.*, 1999)
	Cysteine 60	-	> activity	(Castro-Fernandez *et al.*, 2002)
RGS16	Cysteine 2/12	No change	> activity	(Druey *et al.*, 1999c)
	Cysteine2/12/98	Targets to lipid rafts	> activity	(Hiol *et al.*, 2003)
	Cysteine 98	-	> activity	(Osterhout *et al.*, 2003)
RGS19	-	Targets to membrane	-	(De Vries *et al.*, 1996)

Table 1.3 Palmitoylation of RGS proteins

RGS proteins and the cysteine residues that they are palmitoylated by and the effect on RGS activity are shown. * dependent on assay (taken from Riddle *et al.*, 2005).



1. RGS/Gα contact site 1	2. RGS/Gα contact site 2
3. RGS/Gα contact site 3	4. Conserved APC binding groove
5. PIP3 and Ca^{2+}/CaM binding domains	6. Palmitoylation
7. Palmitoylation	8. Charged amphipathic helix
9. Undetermined regions	10. Multiple N- and C-terminal motifs

Figure 1.2 Model of potential targets on RGS proteins for drug action

Target sites 1-10 include amino acids that are essential for direct RGS/Gα binding (1, 2 and 3)*;* those that mediate RGS binding with other proteins/molecules to allosterically regulate RGS/Gα binding (4, 5 and 6)*;* amino acids required for RGS protein membrane attachment (7 and 8)*;* and those residues that mediate RGS binding to GPCR (9) and other regulatory and/or signalling proteins (10) (taken from Hollinger and Hepler, 2002).

2 Materials and Methods

2.1 Materials

2.1.1 General reagents, enzymes and kits

Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK
Full range Rainbow™ molecular weight markers, protein G, Glutathione Sepharose 4B beads

Amaxa, Gaithersburg, MD, USA

Cell Line Nucleofector kit V

BDH, Lutterworth, Leicestershire, UK

22 mm coverslips, microscope slides

Chemicon Europe Ltd., Chandlers Ford, UK
ReBlot plus solution

Eppendorf, Hamburg, Germany

96 well deepwell plates

Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK

Glacial acetic acid, glycine, HEPES, sucrose, SDS, KH_2PO_4, Na_2HPO_4, orthophosphate, $CaCl_2$, $MnCl_2$, DTT, bactotryptone, yeast extract, bactoagar, ethylene glycol, methanol, isopropanol, ethanol, NaCl, Tris

Flowgen Bioscience Ltd., Nottingham, UK
Agarose

Invitrogen Ltd., Paisley, UK
NuPage pre-cast 8-12% (w/v) Bis-tris gels, MOPS running buffer, ProLong Gold antifade reagent, Image-iT™ LIVE Plasma Membrane labelling kit

Konica Europe, Hohenbrunn, Germany

X-ray film

Merck Chemicals Ltd., Nottingham, UK

Nonidet P-40, pansorbin

New England Biolabs, MA, USA

Restriction enzymes

Perkin-Elmer Life Sciences Inc., Boston, MA, USA

384 well black walled plates, Optiplate white 96 well plates

Pierce, Perbio Science UK Ltd., Tattenhall, Cheshire, UK

Supersignal West Pico chemiluminescent substrate

Promega UK Ltd., Southhampton, UK

All restriction endonucleases, DNA ligase, Pfu polymerase, Wizard™ Plus SV mini-preps

Qiagen, Crawley, West Sussex, UK

Qiaquick PCR purification kit, Qiaquick gel extraction kit, Qiafilter maxi-prep kit

Roche Diagnostics Ltd., Lewes, East Sussex, UK

Complete™ EDTA-free protease inhibitor tablets, AppNH

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

$MgCl_2$, Tris, EDTA, bromophenol blue, Triton X-100, DMSO, glycerol, Tween 20, ethylene glycol, paraformaldehyde, ampicillin, ethidium bromide, ATP, Hoechst No. 333442, BCA solution A, Pertussis toxin, GTPγS, GDP, Fura-2 AM, $CuSO_4$, $RbCl_2$

Stratagene, Amsterdam, The Netherlands

QuikChange site directed mutagenesis kit, XL-1 Blue competent cells

Thermo Fisher Scientific, Ulm, Germany

All oligonucleotides used for PCR reactions

2.1.2 Tissue culture plasticware and reagents

Costar, Cambridge, MA., USA

5 mL, 10 mL and 25 mL pipettes, 75 cm^2 vented tissue culture flasks, 6 well plates, 100 mm dishes

Invitrogen BV, Groningen, The Netherlands

Lipofectamine™ transfection reagent, Optimem, L-glutamine (200mM)

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

DMEM, 0.25% (w/v) trypsin-EDTA, poly-D-Lysine, new born calf serum, fetal calf serum

2.1.3 Radiochemicals

Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK
[γ-^{32}P]GTP, [^{3}H]RS-79948-197

Perkin-Elmer Life and Analytical Sciences, Beaconsfield, Buckinghamshire, UK
[^{3}H]prazosin, [^{35}S]GTPγS

2.1.4 Antisera

Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK
Donkey anti-mouse IgG-HRP conjugate, donkey anti-rabbit IgG-HRP conjugate, donkey anti-hamster IgG-HRP conjugate, anti-RGS4 antiserum

A sheep polyclonal anti-GFP antiserum was generated in house.

2.2 Buffers

2.2.1 General Buffers

HEPES Buffer

130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES, 10 mM D-glucose, 0.01 mM EGTA. The pH of this solution was adjusted to 7.4 using NaOH.

Laemmli Buffer (2x)

0.4 M DTT, 0.17 M SDS, 40 mM Tris, 5 M urea, 0.01% (w/v) bromophenol blue

Phosphate Buffered Saline (10x)

137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 10.2 mM Na_2HPO_4, pH 7.4. The solution was autoclaved prior to use. This solution was diluted 1:10 prior to use.

RIPA Buffer (2x)

100mM HEPES (pH7.4), 300mM NaCl, 2% (w/v) Triton X-100, 1% (w/v) Na-deoxycholate, 0.2% (w/v) SDS, stored at 4^{o}C

Tris-EDTA (TE) Buffer (membrane)

10 mM Tris, 0.1 mM EDTA, pH 7.4

Tris-EDTA (TE) Buffer (radioligand binding)

75 mM Tris, 5 mM EDTA, pH 7.5

Tris-EDTA-Magnesium (TEM) Buffer (radioligand binding)

75 mM Tris, 5 mM EDTA, 12.5 mM $MgCl_2$, pH 7.5

2.2.2 Molecular Biology Solutions

TAE Buffer (50x)

40 mM Tris, 1 mM EDTA, 17.5% (w/v) glacial acetic acid. This solution was diluted 1:50 prior to use.

DNA loading buffer

0.25% (w/v) bromophenol blue, 40% sucrose (w/v) in H_20

LB Media (Luria-Bertani Medium)

1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.4. Sterilised by autoclaving at 126^{o}C.

Buffer 1 (for preparation of competent bacteria)

0.03M $C_2H_3O_2K$, 0.1M $RbCl_2$, 0.01M $CaCl_2$, 0.05M $MnCl_2$, 15% (w/v) glycerol. The solution was adjusted to pH 5.8 with acetic acid. The solution was filter sterilised and stored at 4°C.

Buffer 2 (for preparation of competent bacteria)

10mM MOPS pH 6.5, 0.075M $CaCl_2$, 0.01M $RbCl_2$, 15% (w/v) glycerol. The pH was corrected to pH 6.5 with concentrated HCl. The solution was filter sterilised and stored at 4°C.

2.3 Molecular Biology Protocols

2.3.1 LB agar plates

LB was prepared as detailed previously in 2.2.2. 15 g/L of bacto-agar was added to LB and autoclaved. On removal from the autoclave, the bottle was gently inverted to distribute the agar throughout the solution. The mix was cooled to 50°C prior to addition of an antibiotic. The final concentrations of antibiotics used were*;* ampicillin – 100 µg/mL or Zeocin – 50 µg/mL. The medium was mixed gently and approximately 25 mL poured into 10 cm^2 petri dishes. The dishes were left to set at room temperature before being stored at 4°C. Unused plates were disposed of three weeks following preparation.

2.3.2 Preparation of competent bacteria

XL-1 blue cells were streaked out onto a minimal agar plate and incubated overnight at 37°C. A single colony was selected, inoculated into a 5 mL culture of LB and grown in a shaking incubator at 37°C until the optical density at 550 nm was 0.48 – approximately 90 minutes of incubation. The culture was then chilled on ice for 5 minutes before being centrifuged at 3220 x g for 10 minutes at 4°C. All traces of LB were removed and then the pellet was resuspended in 20 mL of buffer 1 (as detailed above) by gently pipetting. The suspension was incubated on ice for 5 minutes prior to being centrifuged as before. The pellet was resuspended in 2 mL of buffer 2 (as detailed above) by pipetting and the suspension incubated on ice for 15 minutes. The cells were then divided in 220 µL aliquots and stored at -80°C until required.

2.3.3 Transformation

An aliquot of competent bacteria was thawed on ice and 50 µL of cells per transformation reaction aliquoted into a sterile tube. Between 1 and 10 µg of DNA was then added to the cells and incubated on ice for 15 minutes. The cells were then subjected to a heat shock at 42°C for 90 seconds and then returned to ice for 2 minutes. 450 µL of LB was added to the cells and the mix incubated for 45 minutes at 37°C in a shaking incubator. 200 µL of the mix was plated onto an LB plate containing the appropriate concentration of antibiotic and incubated inverted for 12-16 hours at 37°C.

2.3.4 Preparation of plasmid DNA

2.3.4.1 Mini-preps

Mini-prep cDNA was purified from bacterial cultures using the Wizard™ Plus SV miniprep kit. 5 mL of bacterial culture was centrifuged at 16,000 x g for 10 minutes and the supernatant removed. The pellet was resuspended in 250 µL of cell resuspension buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 µg/mL Rnase A) immediately followed by 250 µL of lysis buffer (0.2 M NaOH, 1% (w/v) SDS). 10 µL of alkaline phosphate was added to each sample and incubated for 5 minutes. 350 µL of neutralising buffer was then added (4.09 M guanidine hydrochloride, 0.76 M potassium acetate, 2.12 M glacial acetic acid, pH 4.2) to precipitate the bacterial chromosomal DNA. This was centrifuged for 10 minutes at 16,000 x g and the supernatant applied to a DNA purification column. The column was centrifuged briefly to bind the DNA and the column washed twice in wash buffer (60 mM potassium acetate, 10 mM Tris-HCl, pH 7.4, 60% (w/v) ethanol). DNA was eluted by adding 100 µL sterile water.

2.3.4.2 Maxi-preps

The Qiagen Qiafilter kit was used to produce larger scale DNA samples. As detailed previously, 5 mL of bacterial culture was grown overnight and this culture was used to inoculate a 100 mL LB culture. This was incubated for 16-18 hours at 37°C in a shaking incubator. Bacteria were pelleted by centrifugation for 30 minutes at 3220 x g at 4°C. The pellet was then resuspended in 10 mL of chilled buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/mL Rnase A) by vortexing and the cells lysed by adding 10 mL of

buffer P2 (200 mM NaOH, 1% (w/v) SDS) and incubating for 10 minutes at room temperature. 10 mL of buffer P3 (3.0 M potassium acetate, pH 5.5) was added to neutralise the reaction and the solution immediately applied to a Qiafilter cartridge. This was left for 10 minutes at room temperature to incubate. During this time a Qiagen tip 500 was equilibrated by adding 10 mL of buffer QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% (w/v) isopropanol). The plunger was then inserted into the cartridge and the lysate filtered into the equilibrated tip. The column was washed with 60 mL of buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% (w/v) isopropanol). The DNA was eluted by adding 15 mL of buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.0, 15% (w/v) isopropanol). The DNA was precipitated by the addition of 10.5 mL isopropanol and the mixture centrifuged at 12,000 x g for 30 minutes at 4°C. The pellet was washed in 5 mL of room temperature 70% (w/v) ethanol and again centrifuged for 15 minutes at 12,000 x g for 30 minutes at 4°C. The supernatant was carefully removed and the pell*et al*lowed to air dry prior to being re-suspended in 1 mL of sterile water.

2.3.5 DNA quantification

Quantification of DNA samples prepared was performed by examining the absorbance of a 1:200 dilution of sample at 260nm. A A_{260} of 1 unit was taken to correspond to 50 µg/mL of double stranded DNA.

2.3.6 Digestion of DNA with restriction endonucleases

Restriction digests of DNA were performed in order to prepare for sub-cloning of polymerase chain reaction (PCR) fragments or to ensure the successful ligation of a construct. Digests were prepared in a final volume of 20 µL using restriction enzymes as directed for each individual enzyme. Reactions were incubated at 37°C for a minimum of 1 hour before being examined using DNA gel electrophoresis.

2.3.7 DNA gel electrophoresis

Digested DNA samples were examined using gel electrophoresis to ensure correct ligation of a construct. This method was also used to isolate a plasmid vector. Samples were mixed with 2x loading buffer. A 1% (w/v) agarose gel was prepared by mixing agarose with 1x TAE buffer and heating until the agarose had melted. 2.5 µg/mL ethidium bromide was added to the gel and then poured into horizontal gel tanks. The gel was left to

set and then 1x TAE buffer added. The samples were then loaded onto the gel and were run for 20 to 40 minutes under a voltage of 50 to 100V then visualised under ultraviolet light. The sizes of the fragments were assessed by comparison with a 1Kb ladder.

2.3.8 DNA purification from agarose gels

DNA was purified from the gel by excising the band of interest and using the Qiaquick gel extraction kit in accordance with the manufacturer's instructions. Briefly, the DNA-gel fragment was dissolved in buffer QG by heating to 50°C. One gel volume of isopropanol was added to the mix and the solution transferred to a Qiaquick column. The DNA was bound to a column by centrifugation. The sample was washed using buffer PE supplemented with ethanol and then eluted from the column using sterile water.

2.3.9 Alkaline phosphatase treatment of plasmid vectors

The 5' phosphate group from plasmid vectors was removed by incubation of 200 ng of digested vector with 2 units of alkaline phosphatase for 1 hour at 37°C. The treated plasmid was then isolated from the reaction mixture by agarose gel electrophoresis and gel extraction as described previously.

2.3.10 DNA ligations

Constructs were generated by ligating digested PCR fragments into plasmid vector using T4 DNA ligase. For each construct a ratio of 1:3 and 1:6 of vector to PCR product was used in a volume of 20 µL. 1 µL of ligase was used with 2 µL of ligase buffer and the reaction incubated at 4°C for least 16 hours. The ligation reactions were transformed as detailed in section 2.3.3.

2.3.11 Polymerase Chain Reaction

PCR reactions were established in a volume of 50 µL containing 10 ng of template DNA, 0.2 mM dNTPs (dATP, dCTP, dGTP, dTTP), 25 pM of sense and anti-sense oligonucleotide primers, 1x Pfu polymerase buffer and 1 unit of Pfu polymerase. Reactions were carried out on an Eppendorf gradient Thermocycler. PCR cycles used were*;*

1. Preheating	95°C	5 minutes
2. Denaturation	95°C	1 minute
3. Annealing	50-60°C	1 minute
4. Extension	72°C	3 minutes
Repeat from step 2/	29 ×	
5. End	72°C	10 minutes
6. Hold	4°C	

The annealing temperatures were determined depending on the Tm of the primers used for each PCR.

2.3.12 QuikChange Mutagenesis PCR

The QuikChange site-directed mutagenesis PCR reactions were established in a volume of 50 µL containing: 50 ng DNA template, 15 pM of both forward and reverse primers, 0.2 mM dNTPs and 2.5 units of Pfu DNA polymerase. Reactions were cycled 30 times in an Eppendorf gradient Thermocycler system. PCR cycles used were*;*

Cycle 1:	95^{o}C for 30 seconds
Cycles 2-30:	95^{o}C for 30 seconds,
	50^{o}C for 1 minute
	68^{o}C for 1 minute per kbp of plasmid length

The product was treated with 1µl *Dpn*I restriction enzyme and incubated for 60 minutes at 37^{o}C. The digested mutated DNA and control were then transformed following the protocol described in section 2.3.3.

2.4 Generation of constructs

Wild-type human RGS4 was used as a PCR template for all RGS4 constructs.

2.4.1 RGS4-GFP^{2}

Primers were designed to amplify RGS4 and remove the stop codon*;*

Sense 5' -GGA TCC GCC ACC ATG TAT CCC TAC GAC GTC CCC GAT TAT GCG TGC AAA GGG CTT GCA GGT CTG CC-3'

Anti-sense 5' -AAA TCT AGA GGC ACA CTG AGG GAC CAG GG- 3'

The *Bam*HI and *Xba*I sites present in the sense and anti-sense primers respectively are shown underlined. The amplified fragment was digested and ligated into pcDNA3. This construct was then digested with *Kpn*I and *Apa*I and ligated in frame with GFP^2 into the vector $pGFP^2$-N1.

2.4.2 GST-RGS4-GFP^2

Primers were designed to amplify RGS4-GFP^2*;*

Sense 5' -GGA TCC GCC ACC ATG TAT CCC TAC GAC GTC CCC GAT TAT GCG TGC AAA GGG CTT GCA GGT CTG CC- 3'

Anti-sense 5' -TTT TCC TTT TGC GGC CG CTT ACT TGT ACA GCT CGT CCA TGC CGA GAG T- 3'

The *Bam*HI and *Not*I sites present in the sense and anti-sense primers respectively are shown underlined. The amplified fragment was digested and ligated downstream of GST into the vector pGEX-6P1.

2.4.3 RGS4-eYFP in pcDNA3

Primers were designed to amplify RGS4 and remove the stop codon*;*

Sense 5' -GGA TCC GCC ACC ATG TAT CCC TAC GAC GTC CCC GAT TAT GCG TGC AAA GGG CTT GCA GGT CTG CC- 3'

Anti-sense 5' -AAA TCT AGA GGC ACA CTG AGG GAC CAG GG- 3'

The *Bam*HI and *Xba*I sites present in the sense and anti-sense primers respectively are shown underlined. The amplified fragment was digested and ligated upstream and in frame with eYFP into the vector pcDNA3.

2.4.4 RGS4^{N88S}-e-YFP

Primers were designed to amplify RGS4^{N88S} and remove the stop codon*;*

Sense 5' -GGA TCC GCC ACC ATG TAT CCC TAC GAC GTC CCC GAT TAT GCG TGC AAA GGG CTT GCA GGT CTG CC- 3'

Anti-sense 5' -AAA TCT AGA GGC ACA CTG AGG GAC CAG GG- 3'

The *Bam*HI and *Xba*I sites present in the sense and anti-sense primers respectively are shown underlined. The amplified fragment was digested and ligated upstream and in frame with eYFP into the vector pcDNA3.

2.4.5 RGS4^{N128A}-eYFP

Primers were designed to amplify RGS4^{N128A} and remove the stop codon*;*

Sense 5' -GGA TCC GCC ACC ATG TAT CCC TAC GAC GTC CCC GAT TAT GCG TGC AAA GGG CTT GCA GGT CTG CC- 3'

Anti-sense 5' -AAA TCT AGA GGC ACA CTG AGG GAC CAG GG- 3'

The *Bam*HI and *Xba*I sites present in the sense and anti-sense primers respectively are shown underlined. The amplified fragment was digested and ligated upstream and in frame with eYFP into the vector pcDNA3.

2.4.6 RGS4^{N88SN128A}-eYFP

Primers were designed to amplify RGS4^{N88SN128A} and remove the stop codon*;*

Sense 5' -GGA TCC GCC ACC ATG TAT CCC TAC GAC GTC CCC GAT TAT GCG TGC AAA GGG CTT GCA GGT CTG CC- 3'

Anti-sense 5' -AAA TCT AGA GGC ACA CTG AGG GAC CAG GG- 3'

The *Bam*HI and *Xba*I sites present in the sense and anti-sense primers respectively are shown underlined. The amplified fragment was digested and ligated upstream and in frame with eYFP into the vector pcDNA3.

2.4.7 TRHR-1-Gα_{11}^{G188S}

Primers were designed to amplify rat TRHR-1 and remove the stop codon*;*

Sense 5' -AAA GGT ACC ATG GAG AAT GAA ACC GTC AGT- 3'

Anti-sense 5' -AAA GGT ACC TGT TTT CTC CTG TTT GGC- 3'

The *Kpn*I sites present in the sense and anti-sense primers respectively are shown underlined. The amplified fragment was digested and ligated upstream and in frame with $G\alpha_{11}^{G188S}$ into the vector pcDNA3.

2.4.8 GST-RGS4^{S30C}

Primers were designed to synthesise two complimentary oligonucleotides containing the ^{S30C} mutation in GST-RGS4 in the vector pGEX-6P1*;*

Sense 5' -GGT TTC CTG CAA AAA TGT CAT TCC TGT GAA CAC AAT TCT TCC- 3'

Anti-sense 5' -GGA AGA ATT GTG TTC ACA GGA ATC ACA TTT TTG CAG CAG GAA ACC- 3'

Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into BL21 competent cells.

2.4.9 RGS4^{S30C}-eYFP

Primers were designed to amplify RGS4^{S30C} and remove the stop codon*;*

Sense 5' -GGA TCC GCC ACC ATG TAT CCC TAC GAC GTC CCC GAT TAT GCG TGC AAA GGG CTT GCA GGT CTG CC- 3'

Anti-sense 5' -AAA TCT AGA GGC ACA CTG AGG GAC CAG GG- 3'

The *Bam*HI and *Xba*I sites present in the sense and anti-sense primers respectively are shown underlined. The amplified fragment was digested and ligated upstream and in frame with eYFP into the vector pcDNA3.

2.4.10 GST-RGS4^{S30A}

Primers were designed to synthesise two complimentary oligonucleotides containing the ^{S30A} mutation in GST-RGS4 in the vector pGEX-6P1*;*

Sense 5' -GGT TTC CTG CAA AAA GCT GAT TCC TGT GAA CAC- 3'

Anti-sense 5' -GTG TTC ACA GGA ATC AGC TTT TTG CAG CAG GAA ACC- 3'

Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into BL21competent cells.

2.4.11 RGS4^{S30A} – eYFP

Primers described in section 2.4.11 were designed to synthesise two complimentary oligonucleotides containing the ^{S30A} mutation in RGS4-eYFP in the vector pcDNA3. Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into XL1- competent cells.

2.4.12 GST-RGS4^{S30E}

Primers were designed to synthesise two complimentary oligonucleotides containing the ^{S30E} mutation in GST-RGS4 in the vector pGEX-6P1*;*

Sense 5' -GGT TTC CTG CAA AAA GAG GAT TCC TGT GAA CAC- 3'

Anti-sense 5' -GTG TTC ACA GGA ATC AGC TTT TTG CAG CAG GAA ACC- 3'

Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into BL21competent cells.

2.4.13 RGS4^{S30E} –eYFP

Primers described in section 2.4.12 were designed to synthesise two complimentary oligonucleotides containing the ^{S30E} mutation in RGS4-eYFP in the vector pcDNA3. Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into XL1-blue competent cells.

2.4.14 GST-RGS4^{S30F}

Primers were designed to synthesise two complimentary oligonucleotides containing the ^{S30F} mutation in GST-RGS4 in the vector pGEX-6P1*;*

Sense 5' -GGT TTC CTG CTG CAA AAA TTC GAT TCC TGT GAA CAC AAT TCT TCC- 3'

Anti-sense 5' –GGA AGA ATT GTG TTC ACA GGA ATC AAG TTT TTG CAG CAG GAA ACC- 3'

Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into BL21competent cells.

2.4.15 RGS4^{S30F} –eYFP

Primers described in section 2.4.15 were designed to synthesise two complimentary oligonucleotides containing the ^{S30F} mutation in RGS4-eYFP in the vector pcDNA3. Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into XL1-Blue competent cells.

2.4.16 GST-RGS4^{S30K}

Primers were designed to synthesise two complimentary oligonucleotides containing the ^{S30K} mutation in GST-RGS4 in the vector pGEX-6P1*;*

Sense 5' -GGT TTC CTG CTG CAA AAA AAA GAT TCC TGT GAA CAC AAT TCT TCC- 3'

Anti-sense 5' –GGA AGA ATT GTG TTC ACA GGA ATC TTT TTT TTG CAG CAG GAA ACC- 3'

Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into BL21 competent cells.

2.4.17 RGS4^{S30K} –eYFP

Primers described in section 2.4.17 were designed to synthesise two complimentary oligonucleotides containing the ^{S30K} mutation in RGS4-eYFP in the vector pcDNA3. Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into XL1-Blue competent cells.

2.4.18 GST-RGS4^{S30P}

Primers were designed to synthesise two complimentary oligonucleotides containing the ^{S30P} mutation in GST-RGS4 in the vector pGEX-6P1*;*

Sense 5' -GGT TTC CTG CTG CAA AAA CCT GAT TCC TGT GAA CAC AAT TCT TCC- 3'

Anti-sense 5' –GGA AGA GTG TTC ACA GGA ATC GGA TTT TTG CAG CAG GAA ACC- 3'

Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into BL21competent cells.

2.4.19 RGS4^{S30P} –eYFP

Primers described in section 2.4.19 were designed to synthesise two complimentary oligonucleotides containing the ^{S30P} mutation in RGS4-eYFP in the vector pcDNA3. Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into XL1-Blue competent cells.

2.4.20 GST-RGS4^{S30M}

Primers were designed to synthesise two complimentary oligonucleotides containing the ^{S30M} mutation in GST-RGS4 in the vector pGEX-6P1*;*

Sense 5' -GGT TTC CTG CAA AAA ATG GAT TCC TGT GAA CAC AAT TCT TCC-3'

Anti-sense 5' –GGA AGA ATT GTG TTC ACA GGA ATC TAC TTT TTG CAG CAG GAA ACC- 3'

Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into BL21competent cells.

2.4.21RGS4^{S30M} –eYFP

Primers described in section 2.4.20 were designed to synthesise two complimentary oligonucleotides containing the ^{S30M} mutation in RGS4-eYFP in the vector pcDNA3. Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into XL1-Blue competent cells.

2.4.22 GST-RGS16^{S30C}

Primers were designed to synthesise two complimentary oligonucleotides containing the ^{S30C} mutation in GST-RGS16 in the vector pGEX-6P1*;*

Sense 5' - CTT TCT TCA CAA ATG TGA GCT GGG CTG- 3'

Anti-sense 5' –CGC AGC CCA GCT CAC ATT TGT GAA GAA AG- 3'

Following temperature cycling the product was treated with *Dpn*I to digest parental DNA and transformed into BL21competent cells.

2.5 Cell culture

2.5.1 Cell maintenance

2.5.1.1 Human Embyonic Kidney Cells (HEK293T)

HEK293T cells were grown in 75 cm^2 flasks in Dulbecco's modified Eagle's medium containing 10% (w/v) newborn calf serum and 2 mM L-glutamine. The cells were maintained in a humidified incubator of 95% air / 5% CO_2 at 37°C.

2.5.1.2 EF88 cells

EF88 cells were grown in 75 cm^2 flasks in Dulbecco's modified Eagle's medium containing 10% (w/v) fetal calf serum and 2 mM L-glutamine. The cells were maintained in a humidified incubator of 95% air / 5% CO_2 at 37°C.

2.5.2 Cell subculture

Once confluent, cells were sub-cultured by the addition of sterile 0.25% (w/v) trypsin-EDTA solution. The medium was removed from the flask and 2 mL of trypsin solution added. The flask was gently rotated to cover the monolayer and replaced in the incubator for 3 minutes. Once the cells had detached, 3 mL of fresh medium was added to the flask and the cells resuspended by gently pipetting. The cells were then subcultured into either 75 cm^2 flasks to maintain the cell line or 10 cm^2 dishes for transfection.

2.5.3 Transient transfection of HEK293T cells

Transfection of HEK293T cells was performed when the cells had reached 60-70% confluency. Plasmid DNA was transfected using Lipofectamine in accordance with the manufacturer's instructions. Plasmid DNA was diluted to 0.1 µg/µL and the volume adjusted corresponding to the amount of DNA to be transfected and was transferred to a 15 mL tissue culture tube. A volume of Optimem was added in order to bring the volume in the tube to 600 µL. A mix of Lipofectamine and Optimem was prepared in the ratio of 20 µL Lipofectamine and 580 µL Optimem for each sample. 600 µL of this mix was gently added to the DNA mix and incubated for 30 minutes. During this time the cells were washed with Optimem. Following the incubation period, 4.8 mL of Optimem was added

to the sample tubes and the mix transferred onto the cells. The cells were returned to the incubator for 4-5 hours before removing the transfection mix and replacing with fresh media. The media was changed 24 hours later and the cells harvested approximately 36 hours post-transfection. A similar protocol was used for transfection in a 6-well plate, however, the ratio of Lipofectamine to Optimem was 1:29.

2.5.4 Pertussis toxin treatment

P. tox is secreted by *Bordella pertussis*. This toxin catalyses the addition of ADP-ribose to the α subunit of G_i and prevents receptor activation of the G protein. Transiently transfected cells were treated with P. tox (25 ng/ml) for 16 to 18 hours prior to harvesting.

2.5.5 Transient transfection of EF88 cells

Transient transfection of EF88 cells was performed using the cell line nucleofection kit V when cells were in their logarithmic growth phase. $1x10^6$ cells per nucleofection were resuspended in 100 µL room temperature Nucleofector Solution. This suspension was mixed with 1-5 µg plasmid DNA. The sample was transferred into a cuvette and inserted into an Amaxa nucleofector and programme 20 selected. 500 µL of pre-warmed medium was added to the cuvette and transferred to 6 well plates containing 1.5 mL medium. Cells were incubated in a humidified incubator with 95% air / 5% CO_2 at 37°C. Cells were used 24 hours post nucleofection.

2.5.6 Cell harvesting

Transfected cells were harvested 36 hours post-transfection. The media was removed and the cells washed 3 times in 1x PBS. Cells were scraped off the dish in 5 mL of 1x PBS using a cell scraper and transferred to a 15 mL centrifuge tube. The dish was washed again in 5 mL 1x PBS to remove additional cells before centrifuging the harvested cells for 5 minutes at 201 x g at 4ºC. The PBS was removed and the cell pellet frozen at -80ºC

2.6 Protein biochemistry and other methods of analysis

2.6.1 Cell membrane preparation

Cell pellets from transfected cells were thawed and resuspended in 1 mL of TE (membrane) buffer. The cells were homogenised using 40 strokes of a glass on Teflon homogeniser. The cells were centrifuged at 1500 x g for 10 minutes in order to separate any unbroken cells and nuclei at 4ºC. The supernatant was removed and passed through a 25 gauge needle 10 times before being transferred to ultra-centrifuge tubes and subjected to centrifugation at 50,000 x g for 30 minutes using a Beckman Optima TLX Ultracentrifuge (Palo Alto, CA). The supernatant was discarded and the pellet resuspended in 200 µL of TE buffer using a 25 gauge needle, passing through 10 times to ensure an even suspension. The protein concentration was assessed using a BCA assay (see below 2.6.2) and membranes diluted to 1 µg/µL and stored at – 80 ºC until required.

2.6.2 BCA protein quantification

Protein concentration of membrane preparations was assessed using a Bicinhoninic acid (BCA) assay. This assay utilises BCA and copper sulphate solutions in which peptide bonds reduce the Cu^{2+} ions to Cu^{+} ions in correlation with protein concentration initiating a colour change caused by BCA binding reduced Cu^{+}. This coloured solution has an absorption maximum of 562 nm, allowing quantification of the protein concentration. A standard curve is also established using standard BSA solutions allowing the concentrations of unknown samples to be established. Solutions used consisted of*;* reagent A – bicinchoninic acid solution and reagent B – 4% (w/v)$CuSO_4$. 1 part reagent A was mixed with 49 parts reagent B and 200 µL of this solution was added to 10 µL of protein standard or unknown sample in a 96 well ELISA plate. The assay was incubated at 37ºC for 25 minutes before reading the absorbance.

2.6.3 SDS-PAGE and western blotting

Protein samples were resolved using SDS-PAGE. Precast Novex Bis-tris gels were used at 4-12% (w/v) acrylamide concentration. NuPage MOPS running buffer was used for electrophoresis using the Xcell Surelock mini-cell gel tank apparatus. The gels were run at 200V for approximately 45 minutes. The proteins were transferred onto nitrocellulose

membrane using the XCell II blot module apparatus. The membrane and components of the transfer apparatus were soaked in transfer buffer (0.2 M glycine, 25 mM Tris and 20% (w/v) methanol) before initiating transfer. Proteins were transferred at 30V for approximately 1 hour. Efficient transfer was investigated by staining with Ponceau stain (0.1% (w/v) Ponceau S, 3% (w/v) trichloroacetic acid). In order to block non-specific binding sites, membranes were incubated in 5% (w/v) low fat milk, 0.1% (w/v) Tween 20/PBS (w/v) solution at room temperature on a rotating shaker for 2 hours. The membrane was incubated with primary antibody overnight in 5% (w/v) low fat milk, 0.1% (w/v) Tween 20/PBS solution at 4ºC. Approximately 16 hours later the membrane was washed three times with 0.1% (w/v) Tween 20/PBS for 5 minutes each wash. The secondary antibody which was horseradish peroxidase linked was incubated again in 5% (w/v) low fat milk, 0.1% (w/v) Tween 20/PBS solution for 1 hour at room temperature. Again the membrane was washed three times in 0.1% (w/v) Tween 20/PBS for 5 minutes each wash prior to application of ECL solution and developing of the blot. The membranes were exposed to blue Kodak film and developed using an X-Omat machine.

2.6.4 Purification of GST-tagged proteins

The cloning vector PGEX-6P1containing the appropriate cDNA fused to glutathione *S*-transferase were transformed in BL21 bacteria and the bacteria were grown up until reaching an optical density at 600 nm of 1.0. Cell pellets were lysed by resuspending in 20 mL of 1x PBS (with protease inhibitors) containing 0.5 mg/mL lysozyme followed by incubation at 4°C with rotation for 1 hour. The resuspended cells were sonicated for 1 minute each on ice. DTT was added to a final concentration of 5 mM and Triton X-100 (as a 10% (w/v) stock) to a final concentration of 1% (w/v). The lysates were then incubated at 4°C with rotation for 1 hour, and the insoluble material was removed by centrifugation at 12,000 x g for 15 minutes at 4°C. 500 µL of a suspension of washed (three times with 10 volumes of 1x PBS with protease inhibitors) glutathione Sepharose 4B beads was added to each cleared lysate and incubated at 4°C overnight with rotation. The lysates were spun at 500 x g for 5 minutes at 4°C, and the supernatant was removed from the pellet of glutathione Sepharose beads. The beads were washed three times with 10 mL of 1x PBS with protease inhibitors, and the GST fusion proteins were eluted with 5 x 1.5 mL of glutathione solution (10 mM concentration of reduced glutathione in 50 mM Tris-HCl, pH 8.0). The eluted proteins were analyzed by SDS-PAGE and BCA protein assay before being dialyzed against three changes of 1x PBS containing 5% (w/v) glycerol at 4°C over 2 days before storage at -80°C.

2.6.5 Fixed cell samples

Cells grown on coverslips were transiently transfected and washed three times with ice-cold 1x PBS. Cells were fixed for 10 minutes at room temperature using 4% (w/v) paraformaldehyde in PBS/5% (w/v) sucrose solution. The cells were washed a further three times in ice-cold 1x PBS prior to being fixed onto microscope slides with 40% (w/v) glycerol in PBS.

2.7 Assays

2.7.1 Radioligand binding

2.7.1.1 [^{3}H]prasozin binding

The expression of α_{1b}-adrenoceptor was assessed using [^{3}H]prasozin. This was performed in 96 well deepwell plates in triplicate. Total binding was initiated by adding 2 µg protein to 50µL [^{3}H]prasozin (4 nM-0.05 nM) and 100µL TEM. Non-specific binding was initiated by adding 2 µg protein, to 50 µL [^{3}H]prasozin (4 nM-0.05 nM), 50 µL 100 µM phentolamine and 50 µL TEM. Reactions were incubated for 40 minutes at 25°C. Bound ligand was separated from free by vacuum filtration through GF/B filters. The filters were pretreated with 0.3% (w/v) polyethyleneimine in TEM and washed three times with cold TE. Bound ligand was estimated by liquid scintillation spectroscopy. The specific binding was calculated by subtracting non-specific binding from total binding. Data were analysed using GraphPad Prism software (San Diego, CA). Saturation data were fitted to non-linear regression curves.

2.7.1.2 [^{3}H]RS-79948-197 binding

The expression of α_{2a}-adrenoceptor was assessed using [^{3}H]RS-79948-197. This was performed in 96 deepwell plates in triplicate. Total binding was initiated by adding by 1 µg protein to 50 µL [^{3}H]RS-79948-197 (5 nM-0.05 nM) and 100 µL TEM. Non-specific binding was initiated by adding 2 µg protein, to 50 µL [^{3}H]RS79948-197 (4 nM-0.05 nM), 50 µL 100 µM idozoxan and 50 µL TEM. The same protocol as 2.7.1.1 was followed except that the samples were incubated for 30 minutes at 30°C.

2.7.2 High-affinity GTPase assay

This assay was used to measure release of $^{32}P_i$ from [γ-^{32}P]GTP. The reaction mixture contained 0.5 μM [γ-^{32}P]GTP (≈50,000cpm), 1 mM AppNH, 1 mM ATP, 1 mM ouabain, 10 mM creatine phosphate, 5 units creatine phosphokinase, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, 12.5 mM Tris-HCl and 50 μM GTP. 50 μL of assay mix was added to deep well plates with 2 μg of protein, 1 μM purified GST-RGS protein and the final volume adjusted to 100 μL with dH_2O. Basal rate was determined in the same conditions but in the presence of 100 μM GTP. The reaction was initiated by transferring the block to a 37°C water bath for 30 minutes. Once the incubation was complete the blocks were immersed in an ice bath and 0.9 mL of 20 mM phosphoric acid (pH 2.3) containing 5% (w/v) activated charcoal was added. After centrifugation for 10 minutes at 3220 x g, radioactivity was measured in 300 μL of the supernatant using a Packard Topcount NXT™ microplate scintillation counter. All assays were performed on at least three membrane preparations derived from different transient transfections. Data expressed as the mean ± standard error of the mean (SEM).

High affinity GTPase assays were plotted to determine the K_m and V_{max} of RGS4. K_m and V_{max} are determined by incubating the RGS4 with varying concentrations of substrate (GTP) and plotting the results as a graph of rate of reaction (V) against concentration of substrate ([S]), which will normally yield a hyperbolic curve, as shown in Figure 3.3. The relationship of this curve is defined by the Michaelis-Menten equation:

$$v = V_{max} / (1 + (K_m/[S]))$$

It can be difficult to fit the best hyperbola through the experimental points, and difficult to determine V_{max} with any precision by estimating the limit of the hyperbola at infinite substrate concentration. A number of ways of re-arranging the Michaelis-Menten equation have been devised to obtain linear relationships which permit more precise fitting to the experimental points, and estimation of the values of K_m and V_{max}.

The Eadie-Hofstee plot rearranges the Michaelis-Menten equation as:

$$v = V_{max} - K_m \times v / [S]$$

plotting v against v / [S] to give a straight line as in Figure 3.4. In this graph, the y intercept is the V_{max} , the gradient is the $-K_m$, and the x intercept is the V_{max} / K_m. This plot overcomes the problem of uneven spacing of points, and undue weight given to points at low concentrations of substrate. However, it has the disadvantage that V, which is a dependent variable, is used on both axes, and hence errors in measuring the rate of reaction are multiplied, resulting in lower precision of the estimates of K_m and V_{max}.

2.7.3 [^{35}S]GTPγS binding assay

[^{35}S]GTPγS binding experiments were initiated by the addition of membranes containing between 50 and 100 fmol of receptor-G protein fusion construct to an assay buffer (20 mM HEPES pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, 1 μM GDP, 0.2 mM ascorbic acid, 50 nCi [^{35}S]GTPγS) containing the indicated concentrations of receptor ligands. Non-specific binding was determined in the same conditions but in the presence of 100 μM GTPγS. Reactions were incubated for 15 minutes at 30°C and were terminated by the addition of 0.5 mL of ice-cold buffer, containing 20 mM HEPES pH 7.4, 3 mM $MgCl_2$ and 100 mM NaCl. The samples were centrifuged at 16,000 × g for 15 minutes at 4°C, and the resulting pellets were resuspended in solubilisation buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, 1.25% (w/v) Nonidet P-40) plus 0.2% (w/v) SDS. Samples were precleared with Pansorbin followed by immunoprecipitation with an antiserum directed towards the C-terminal of the G protein. Finally, the immunocomplexes were washed twice with solubilisation buffer, and bound [^{35}S]GTPγS estimated by liquid-scintillation spectrometry.

2.7.4 $[Ca^{2+}]_i$ imaging

2.7.4.1 Single cell $[Ca^{2+}]_i$ imaging

Transfected cells were loaded with the Ca^{2+}sensitive dye Fura-2 by incubation for 20 minutes at 37°C under reduced light in growth medium containing the dye's membrane-permeant acetoxymethyl ester form (1.5 μM). Loaded cells were transferred to HEPES buffer and illuminated with an ultra high point intensity 75-watt xenon arc lamp (Optosource, Cairn Research, Faversham, Kent, UK) and subsequently imaged using a Nikon Diaphot inverted microscope equipped with a Nikon 40× oil immersion Fluor objective lens (NA=1.3) and a monochromator (Optoscan, Cairn Research), which was used to alternate the excitation wavelength between 340/380 nm and to control the excitation band pass (340 nm band pass=10 nm; 380 nm band pass=8 nm). Fura-2

fluorescence emission at 510 nm was monitored using a high resolution interline-transfer cooled digital CCD camera (Roper Scientific/Photometrics, Tucson, AZ). MetaFluor imaging software (version 4.6.8, Universal Imaging Corp., Downing, PA) was used for control of the monochromator, CCD camera, and for processing of the cell image data. Sequential images (2×2 binning) were collected every 2 seconds; exposure to excitation light was 100ms/image. Agonist was added after 60 seconds for 60 seconds using a perfusion system. Sequential images were collected for a total of five minutes and MetaFluor software was used for analysis. Pooled average ratio values measured from single cells were expressed as the mean ± SEM of at least 10 cells.

2.7.5 Fluorescence microscopy

2.7.5.1 Multiple fluorescence imaging

Paraformaldehyde fixed HEK293T cells, expressing the appropriate fusion protein were imaged using an inverted Nikon TE2000-E microscope equipped with a 60 x, (NA=1.4), oil-immersion Plan Fluor Apochromat lens and a CCD camera. Fluorescence excitation light was generated by an ultra high point intensity 75-watt xenon arc lamp coupled to a computer controlled Optoscan monochromator.

To visualize the plasma membrane, cells were treated, (as specified by the manufacturer), with the reagents in the Image-iT™ plasma membrane and nuclear labelling kit, in which the plasma membrane is specifically labelled with wheat germ agglutinin (WGA)-Alexa Fluor 594 (red) and nuclei are stained simultaneously with Hoechst 33342 (blue). The monochromator was set to 360/10, 500/8 nm and 575/12 nm for the sequential excitation of Hoechst, eYFP and WGA-Alexa Fluor 594 respectively. Hoechst, eYFP and Alexa Fluor 594 excitation light was reflected through the objective lens using the following single pass dichroics: 400DCLP for Hoechst, Q515LP for eYFP and Q595LP for Alexa Fluor 594. Hoechst, eYFP and fluorescence emission was controlled via a high-speed filterwheel device (Prior Instruments, MA, USA) containing the following emitters: HQ480/40 nm for Hoechst; HQ535/30 nm for eYFP, and HQ645/75 nm for WGA-Alexa Fluor 594. Using these filter sets, the fluorophores were easily separated with no bleed through. Sequential 12 bit images, (2 x 2 binning, 200-400 milli-second exposure/image), were collected using a CCD camera operated in 12-bit mode. Computer control of all electronic hardware and camera acquisition was achieved using Metamorph software.

2.7.5.2 Multiple fluorescence analysis

The plasma membrane was identified and segmented into pixels by treating the cells with the membrane marker WGA-Alexa Fluor 594. Images were deconvolved using an iterative and constrained algorithm (Autodeblur software, version 9.3.6**,** Autoquant Imaging, Watervliet, NY). Surface and cytosolic masks were created and superimposed. Expressed RGS-eYFP fluorescence pixel intensity values located at the plasma membrane and cytoplasm of the cell were quantified from each generated mask and were expressed as a percentage of the total eYFP intensity. The number of cells analysed from each experimental group was 3 and the statistical significance of any difference between mean values was determined using a Student's *t* test.

3 Chapter 3

3.1 Introduction

The intracellular localisation of RGS proteins is crucial for their regulatory action. A commonly used fluorescent tag was introduced to RGS4 in order to investigate this localisation. It was particularly important to establish that the presence of this fluorescent protein did not interfere with functional activity of RGS4.

Green fluorescent protein (GFP) has become a valuable tool in the study of cellular localisation of proteins (Cubitt *et al.*, 1995;Baumann *et al.*, 1998). GFP is an auto-fluorescent protein of 27 kDa from the jellyfish *Aequoria victoria*. Fusing the protein of interest to GFP allows direct visualisation of the protein in living cells without the need for immunocytochemistry. Several modified variants of GFP with distinct spectral properties have been isolated (Htun *et al.*, 1996;Stauber *et al.*, 1998). One such variant, GFP^2, was originally developed to maximally absorb the energy released by the oxidation of the substrate DeepBlueC by *Renilla* luciferase for use in Bioluminescence Resonance Energy Transfer^2 (Packard Bioscience), a methodology widely used to study protein-protein interactions (Jensen *et al.*, 2002;Ramsay *et al.*, 2002).

A red-shifted variant of GFP that has also been widely used in research is enhanced yellow fluorescent protein (eYFP). The spectral properties of eYFP allow a stronger fluorescence emission compared to wild-type GFP (Baumann *et al.*, 1998). Both GFP^2 and eYFP were individually ligated to the C-terminal of RGS4.

The pGEX glutathione *S*-transferase (GST) system has been used extensively for high-level expression of recombinant proteins (Smith and Johnson, 1988;Frangioni and Neel, 1993). This previously successful strategy was therefore employed to produce recombinant RGS4 proteins. RGS4 and RGS4-GFP^2 were inserted into the multiple cloning site of the pGEX plasmid, fusing the N-terminus of the predicted protein to GST. GST, from the parasite *Schistosoma japonicium*, is a 26 kDa enzyme which binds reversibly but with high affinity to glutathione, allowing recombinant GST-RGS4 or GST-RGS4-GFP^2 to be purified.

Functional activity of the recombinant RGS proteins was measured in high-affinity GTPase assays. Such GTPase activity can be successfully measured by the use of fusion

proteins between GPCRs and G protein α subunits. In addition, the use of G protein α subunits mutationally modified to be resistant to P. tox-catalysed ADP-ribosylation (^{C351I} in $G\alpha_{o1}$) allows the GTPase activity of the exogenous GPCR and G protein to be measured in isolation (Moon *et al*., 2001). The 1:1 stoichiometry of the GPCR to the Gα subunit in such fusion proteins permits ligand binding to determine the precise expression level of not only the receptor but also of the G protein. The expression level and the measured GTPase activity allow the calculation of the turnover number of the G protein. It has previously been published that transient expression of the α_{2A}-adrenoceptor fused to $G\alpha_{o1}{}^{C351I}$ in cells with the subsequent addition of a recombinant RGS protein to cell membrane preparations, results in a concentration-dependent increase in the high-affinity GTPase activity (Bahia *et al.*, 1998;Cavalli *et al.*, 2000). Using this methodology the functional activity of fluorescently modified, recombinant RGS4 was compared to wild-type.

Studies on the distribution patterns of RGS proteins have revealed diverse patterns of cellular localisation (Chatterjee and Fisher, 2000). However, RGS proteins must ultimately localise near or at the plasma membrane in close proximity to G proteins to produce their effects as a GAP. RGS4 has been reported to be located in the cytoplasm then translocated to the plasma membrane indirectly by G protein activation or by directly binding to G proteins at the plasma membrane. Translocation of RGS4 to the plasma membrane has also been reported by co-expression of specific G proteins, or corresponding receptors that activate these specific G proteins (Roy *et al.*, 2003). Defective G proteins, apparently unable to interact with RGS proteins, have also been reported conflictingly to be both able (Druey *et al.*, 1998) and unable (Roy *et al.*, 2003) to translocate RGS4 to the plasma membrane. Following expression, immunoblotting and microscopy were used to study the intracellular localisation of RGS4-eYFP in the presence and absence of other co-expressed proteins.

The aim of this chapter was to investigate the cellular localisation of transiently expressed RGS4 in HEK293T cells. Confirmation that a fluorescent tag tethered to the C-terminal of RGS4 did not affect function was crucial before the levels of protein expression and the cellular localisation of RGS4 were investigated.

3.2 Results

3.2.1 Purification of recombinant RGS4 and RGS4-GFP^2

Purified recombinant GST-fused proteins were resolved by SDS-PAGE and stained with Coomassie Blue. Figure 3.1 shows similar induction and elution of purified (A) GST-RGS4 and (B) GST-RGS4-GFP^2. Quantitative evaluation of the pooled, eluted GST-fused proteins allowed determination of the concentration of each purified protein.

3.2.2 Functional activity of recombinant RGS4 and RGS4-GFP^2

In order to ensure that a C-terminal fluorescent tag on RGS4 did not disrupt the GAP activity of this RGS protein, it was important to compare the functional activity of the recombinant proteins. Activity of GST-RGS4 and GST-RSG4-GFP^2 were compared using high-affinity GTPase assays. A fusion protein, in which the C-terminus of the porcine α_{2A}-adrenoceptor and the N-terminus of rat $G\alpha_{o1}$, with a P. tox-insensitive mutation, were linked in frame was expressed in HEK293T cells. Cells were pre-treated for 16 hours with an amount of P. tox (25 ng/mL) sufficient to cause ADP-ribosylation of endogenous P. tox-sensitive G proteins (Wise *et al.*, 1997b). The amount of transiently expressed α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein was routinely determined by performing saturation, specific binding studies using the α_2-adrenoceptor antagonist [^{3}H]RS-79948-197 (Figure 3.2). Levels of expression of the receptor-G protein varied between individual transfections, although typically were between 10-20 pmol/mg of membrane protein.

High-affinity GTPase activity was then measured at a range of GTP concentrations in membranes of these cells (Figure 3.3). Basal and adrenaline-stimulated (100 μM) GTPase activity were measured in the presence and absence of purified, recombinant RGS4 (1 μM). Non-linear regression analysis showed that in the absence of RGS4, adrenaline-stimulated high-affinity GTPase was greater than basal activity. However, in the presence of RGS4 there was a strong enhancement of adrenaline-stimulated activity.

To estimate the kinetic parameters (K_m and V_{max}) for RGS4, linear regression analysis was applied to determine the linear correlation between V and V/[GTP]. In this Eadie-Hofstee transformation (Figure 3.4), V is the rate of GTPase activity; V_{max} represents the maximum rate of GTPase activity and K_m is the affinity constant of GTP (the slope of the line). Analysis of basal and adrenaline-stimulated GTPase activity showed that the effect

of adrenaline was to increase the V_{max} without altering the K_m for GTP (Table 3.1). However, addition of recombinant RGS4 (1 μM) to transfected membranes significantly increased adrenaline-stimulated V_{max} of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein and the K_m for GTP ($p<0.05$). These studies, as has been noted previously (Cavalli *et al.*, 2000), clearly show that RGS4 functioned as a GAP for the receptor-activated G protein as is reflected by an effect on both V_{max} and K_m.

It should be noted however, that when assaying the relative increase in both V_{max} and the K_m for GTP of GST-RGS4, a more suitable experiment to show that the presence of RGS4 was solely responsible for the increase in V_{max} and K_m would have been the addition of purified GST. The control used here, no addition of a GST fusion protein, does not rule out the possibility that the presence of GST is somehow responsible for any differences in V_{max} and K_m .

With knowledge of the levels of expression of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein from [^{3}H]RS-79948-197 binding studies, adrenaline-stimulated GTPase turnover number was calculated to significantly increase from 2.3 ± 0.5 minute^{-1} to 10.2 ± 1.2 minute^{-1} in the presence of 1 μM RGS4 (mean ± SEM, from three individual experiments, $p<0.05$).

Following purification of recombinant RGS4-GFP^2, the GAP activity of this protein (0.8 μM) was directly compared to wild-type recombinant RGS4 (0.8 μM). Using 0.5 μM GTP as substrate, addition of a maximally effective concentration of the α_{2A}-adrenoceptor agonist adrenaline (100 μM) (Carr *et al*., 1998) caused a parallel increase in high-affinity GTPase activity for both RGS4 and RGS4-GFP^2 (Figure 3.5). Importantly, the turnover number for each GST-fusion proteins was not significantly different from each other (Figure 3.6) ($p>0.05$). These studies indicate that GFP^2 attached to the C-terminus of RGS4 does not affect the GAP activity.

3.2.3 Intracellular localisation of RGS4

The intracellular localisation of RGS4 was examined by transfection of HEK293T cells with C-terminally eYFP-tagged RGS4. Figure 3.7 shows microscope images of cells transiently expressing RGS4-eYFP. The blue colour (A) represents Hoechst 33342 DNA staining that was used to identify the nuclei in these cells. The green colour (B) represents eYFP fluorescence from expressed RGS4-eYFP. The red colour (C) represents Wheat

Germ Agglutinin (WGA) -Alexa Fluor 594 used to label the plasma membrane. To identify any RGS4-eYFP localised at the plasma membrane, an overlay image is also shown (D). In these overlay images a yellow colour represents RGS4-eYFP that is co-localised with WGA-Alexa Fluor 594 at the plasma membrane. These images clearly demonstrate that the vast majority of transiently transfected RGS4-eYFP in HEK293T cells is localised within the cytoplasm.

These images were then used to compare the percentage redistribution of transiently expressed RGS4-eYFP fluorescence at the plasma membrane versus the cytoplasm. Images were deconvolved and the surface and cytosolic masks were created and superimposed. Total fluorescence pixel intensity values corresponding to eYFP located at the membrane and cytoplasm of the cell were quantified and were expressed as a percentage of the total fluorescent eYFP intensity. This comparison confirmed that RGS4 in HEK293T cells is localised predominantly within the cytoplasm. Figure 3.7(E) illustrates a significant difference ($p<0.05$) between fluorescence intensity of RGS4-eYFP within the cytoplasm (83.7 ± 2.0 %) compared to fluorescence intensity of RGS4-eYFP at the plasma membrane (16.3 ± 2.0%). Using an antiserum specifically raised against RGS4, an immunoblot further demonstrated the predominant cytoplasmic localisation of transiently expressed RGS4-eYFP in HEK293T cells (Figure 3.7 (F)).

3.2.4 Effect of G proteins on intracellular localisation of RGS4

HEK293T cells endogenously express many G proteins including a number of P. tox-sensitive subtypes such as $G\alpha_{i1}$. However, over-expressing exogenous $G\alpha_{i1}$ has previously been shown to promote the translocation of RGS4 from the cytoplasm to the plasma membrane (Roy *et al.*, 2003). In this study, microscopy and immunoblotting of transiently co-transfected RGS4-eYFP and $G\alpha_{i1}$ in HEK293T cells were used to confirm data by Roy *et al*., (2003) (Figure 3.8). The overlay image of cells co-expressing both RGS4-eYFP and $G\alpha_{i1}$ illustrates that over-expressing exogenous $G\alpha_{i1}$ promotes the translocation RGS4-eYFP from the cytoplasm to the plasma membrane in HEK293T cells.

Figure 3.8 (D) shows the comparison of percentage eYFP fluorescence of transiently expressed RGS4-eYFP at the plasma membrane (38.6 ± 3.1%) and in the cytoplasm (61.4 ± 3.1 %) in the presence of exogenous $G\alpha_{i1}$. When compared to HEK293T cells transiently transfected with RGS4-eYFP alone (Figure 3.7 (E)), over-expression of $G\alpha_{i1}$ in HEK293T cells was sufficient to cause translocation of ~22% of RGS4-eYFP to the

plasma membrane. Immunoblots using an antiserum raised against GFP, which also detects YFP, allowed comparison of RGS4-eYFP expression in both membrane and cytosolic fractions of HEK293T cells transiently co-transfected with $G\alpha_{i1}$ (Figure 3.8(E)). When compared to the immunblot of HEK293T cells transiently transfected with RGS4-eYFP alone, over-expression of exogenous $G\alpha_{i1}$ clearly resulted in a translocation of RGS4-eYFP from the cytoplasm to the plasma membrane.

Previous studies have shown that constitutively active forms of $G\alpha_{i2}$ cause RGS4 to become localised at the plasma membrane (Druey *et al.*, 1998;Roy *et al.*, 2003). The conservation of residues between $G\alpha_{i2}$ and $G\alpha_{i1}$ allowed the constitutive mutation to be transferred. Figure 3.9 (A-E) shows transiently co-transfected RGS4-eYFP and the constitutively active G protein, $G\alpha_{i1}^{Q204L}$, in HEK293T cells. The co-localisation of RGS4-eYFP and WGA-Alexa Fluor 594 in the overlay image (Figure 3.9 (C)) illustrates that expression of $G\alpha_{i1}^{Q204L}$ promotes the translocation RGS4-eYFP from the cytoplasm to the plasma membrane in HEK293T cells. In the presence of $G\alpha_{i1}^{Q204L}$ the percentage of RGS4-eYFP fluorescence at the plasma membrane was $57.3 \pm 6.4\%$ compared to RGS4-eYFP fluorescence within the cytoplasm, $42.7 \pm 6.4\%$. Interestingly, the constitutively active G protein mutation increased the percentage of RGS4-eYFP fluorescence at the plasma membrane compared to wild-type $G\alpha_{i1}$. Figure 3.9 (E) shows an immunoblot of membrane and cytosolic fractions of HEK293T cells transiently co-transfected with RGS4-eYFP and $G\alpha_{i1}^{Q204L}$. Expression of $G\alpha_{i1}^{Q204L}$ resulted in translocation of RGS4-eYFP to the plasma membrane. Again, the constitutively active G protein mutation increased the translocation of RGS4-eYFP to the plasma membrane compared to wild-type $G\alpha_{i1}$

It was thought perhaps that a greater expression of transiently transfected $G\alpha_{i1}^{Q204L}$ compared to $G\alpha_{i1}$ could be responsible for the constitutively active G protein increasing the translocation of RGS4-eYFP to the plasma membrane. Loading an equal volume of cell lysates transiently expressing $G\alpha_{i1}$ (lane 2) or $G\alpha_{i1}^{Q204L}$ (lane 3), Figure 3.10 shows a greater intensity of the $G\alpha_{i1}$ band compared to $G\alpha_{i1}^{Q204L}$. This suggests that the constitutively active G protein is expressed at lower levels when directly compared to expression of wild-type $G\alpha_{i1}$. The increased expression of $G\alpha_{i1}^{Q204L}$ is therefore not responsible for the increased translocation of RGS4-eYFP to the plasma membrane. Instead, properties of the constitutively active G protein must influence the translocation. Activation of $G\alpha_{i1}$ by receptor is therefore not required for translocation of RGS4-eYFP from the cytoplasm to the plasma membrane in HEK293T cells, however constitutive activity of $G\alpha_{i1}$ causes the translocation of RGS4-eYFP to be more distinct.

3.2.5 Effect of receptors on cellular localisation of RGS4

It has been reported that that co-transfection of both RGS4-GFP and the M2 muscarinic receptor promotes translocation of RGS4 to the plasma membrane (Roy *et al.*, 2003). Receptors assist G protein activation and therefore may modulate RGS-G protein interaction. The $G\alpha_{i1}$ coupled, α_{2A}-adrenoceptor was therefore co-transfected with RGS4-eYFP in HEK293T cells and microscopy and immunoblotting were used to study the effect of the α_{2A}-adrenoceptor expression on the cellular localisation of RGS4-eYFP (Figure 3.11). Microscopy images illustrate that RGS4-eYFP becomes localised to the plasma membrane in the presence of the α_{2A}-adrenoceptor, in a similar pattern to the effect of the cognate G protein (Figure 3.11 (A), (B), (C)). The majority of RGS4-eYFP fluorescence was still localised ($p<0.05$) within the cytosol (76.6 ± 2.4 %) (Figure 3.11 (D)). However, ~ 7% of total RGS4-eYFP was still translocated from the cytosol to the plasma membrane in the presence of the α_{2A}-adrenoceptor when the percentage fluorescence of co-expressed RGS4-eYFP and the α_{2A}-adrenoceptor (23.4 ± 2.4 %) were compared to RGS4-eYFP transfected alone (16.27 ± 2.05 %). (Figure 3.11 (E)) clearly shows that there is an increase in RGS4-eYFP at the plasma membrane in the presence of the receptor. RGS4-eYFP is translocated from the cytosol to the plasma membrane in the presence of the α_{2A}-adrenoceptor but this translocation in HEK293T cells appears to be less than in the presence of $G\alpha_{i1}$ (Figure 3.8 (D)). This pattern of RGS4-eYFP translocation was also reflected in immunoblots of membrane and cytosolic cell lysate fractions. Following co-transfection of RGS4-eYFP and the α_{2A}-adrenoceptor, immunoblots (Figure 3.11 (E)) showed an increase in the intensity RGS4-eYFP located in the membrane compared to when RGS4-eYFP was transfected alone (Figure 3.7 (F)) but less than when compared to co-transfection of RGS4-eYFP and $G\alpha_{i1}$ (Figure 3.8 (E)).

Agonist activation of the α_{2A}-adrenoceptor with UK14304 (100 μM) was hypothesised to further activate the receptor and therefore cause a greater percentage translocation of RGS4-eYFP to the plasma membrane (Figure 3.12). However, the overlay image (Figure 3.12 (C)) showed no further co-localisation of RGS4-eYFP and the WGA-Alexa Fluor 594-labelled plasma membrane marker. Similarly, the percentage fluorescence showed no difference compared to unstimulated cells transfected with RGS4-eYFP and the α_{2A}-adrenoceptor; cytosolic RGS4-eYFP (22.4 ± 1.9%) and plasma membrane associated RGS4-eYFP (77.6 ± 1.9%). The immunoblots also showed comparable intensity of the RGS4-eYFP in the membrane and cytosolic HEK293T cell lysates in agonist-activated

α_{2A}-adrenoceptor (Figure 3.12 (E)) and unstimulated α_{2A}-adrenoceptor expressing cells (Figure 3.11 (E)). Agonist-stimulation of the α_{2A}-adrenoceptor did not further increase RGS4-eYFP translocation to the plasma membrane.

These immunblots showing the relative fractions of RGS4 in the membrane and cytosolic fractions were not stripped and reprobed with appropriate marker proteins. Reprobing with an antibody raised against a defined membrane and cytosolic protein would have more clearly defined the fractions as "membrane" or "cytosol" to show no cross contamination of samples.

Measuring RGS4-eYFP fluorescence in HEK293T cell membranes further compared the effects of co-transfection of G protein, receptor and activated receptor on the cellular localisation of RGS4-eYFP. Figure 3.13 shows the co-transfection of $G\alpha_{i1}$ has the greatest percentage effect (235.4 ± 20.7%) on RGS4-eYFP translocation to the plasma membrane. The presence of the α_{2A}-adrenoceptor or UK14304 (100μM) stimulated α_{2A}-adrenoceptor translocated RGS4-eYFP to the plasma membrane to a similar degree*;* 169.9 ± 10.0%, 152.8 ± 12.3% respectively.

Again, however, with retrospect, it can be noted that a parallel data set to that of Figure 3.13 should have been included, whereby eYFP was expressed with the indicated receptor/G protein. This set of experiments would have conclusively shown that RGS4 was solely responsible for the increase translocation of the fluorescent protein.

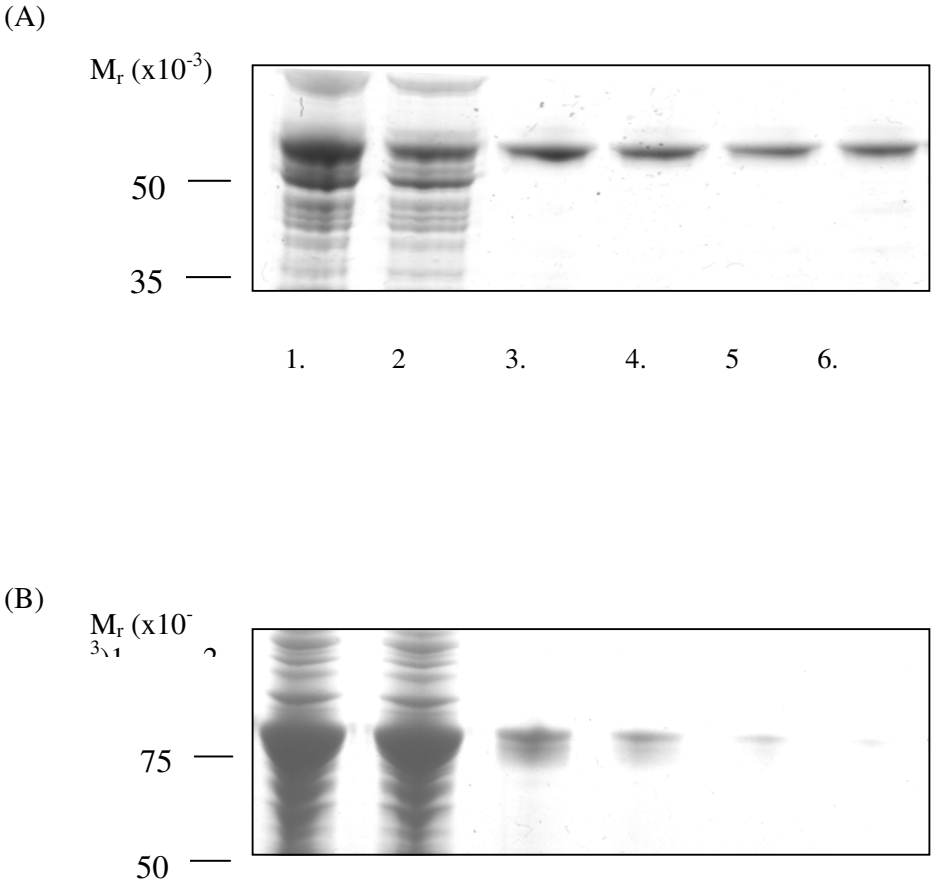


Figure 3.1 Coomassie Blue staining for purified GST-RGS4 and GST-RGS4-GFP^2

BL21 bacteria were transformed with the plasmid pGEX 6P-1 encoding (A) GST-RGS4 or (B) GST-RGS4-GFP^2. GST-fused proteins were induced and purified and equal volumes of each induced fraction or purified elution fraction were resolved by SDS-PAGE and stained with Coomassie Blue. Induced cell lysates of BL21 bacteria before addition of glutathione sepharose beads (lane 1). Induced cell lysates of BL21 bacteria after incubation with glutathione sepharose beads (lane 2). Elution 1 (lane 3). Elution 2 (lane 4). Elution 3 (lane 5). Elution 4 (lane 6).

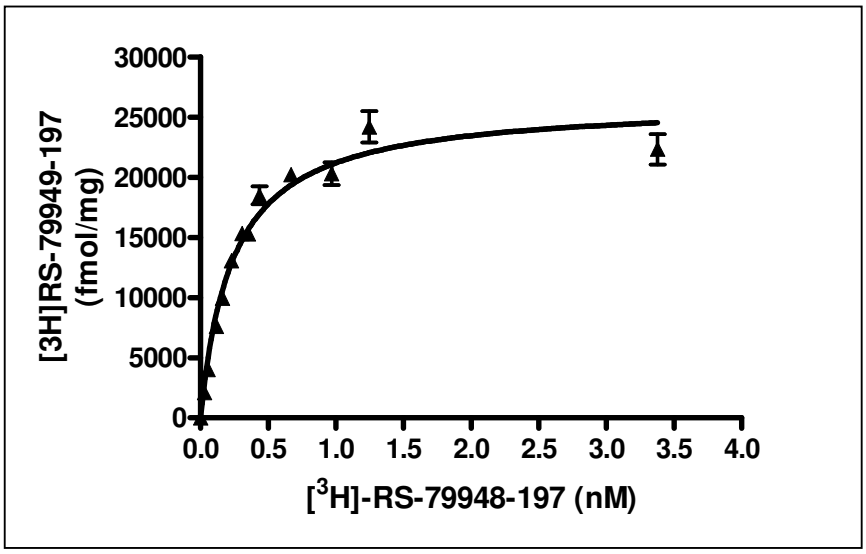


Figure 3.2 Specific binding of [^{3}H]RS-79948-197 to the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein

HEK293T cells were transfected to express the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein. Cells were treated with P.tox (25 ng/ml for 16 hours) and then membranes were prepared. Expression levels of the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein were detected by the binding of 0.00-3.4 nM [^{3}H]RS-79948-197 to 1 μg of membranes. Non-specific binding was determined in the presence of 100 μM idozoxan in parallel assays. Data shown are from triplicate determinations (mean ± SEM.) and are representative of six individual experiments performed.

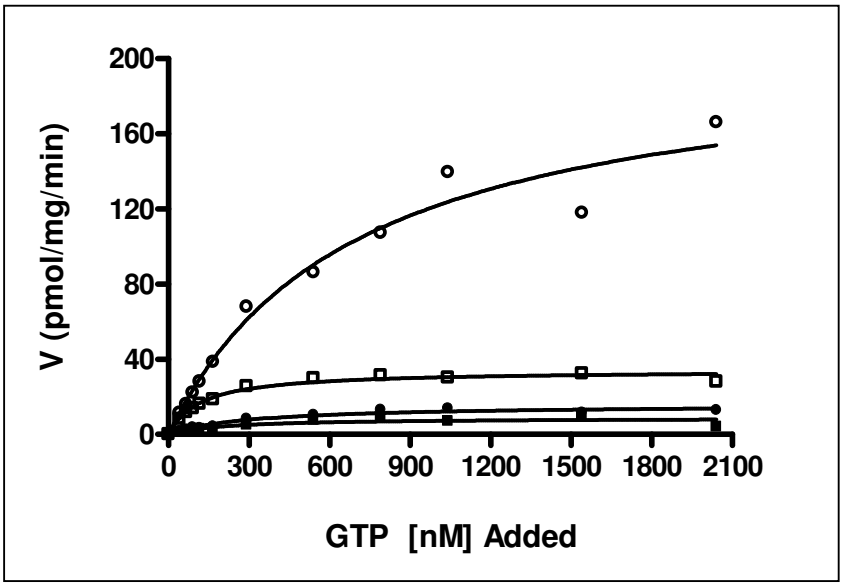


Figure 3.3 GTPase activity in cell membranes expressing the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein in the presence and absence of GST-RGS4

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein. Cells were treated with P. tox (25 ng/ml for 16 hours) and then membranes prepared. High-affinity GTPase activity in the absence (filled) or presence of 100 μM adrenaline (open) was measured at various concentrations of GTP in the presence (circles) or absence (squares) of GST-RGS4. Data shown are from triplicate determinations and are representative of three individual experiments performed.

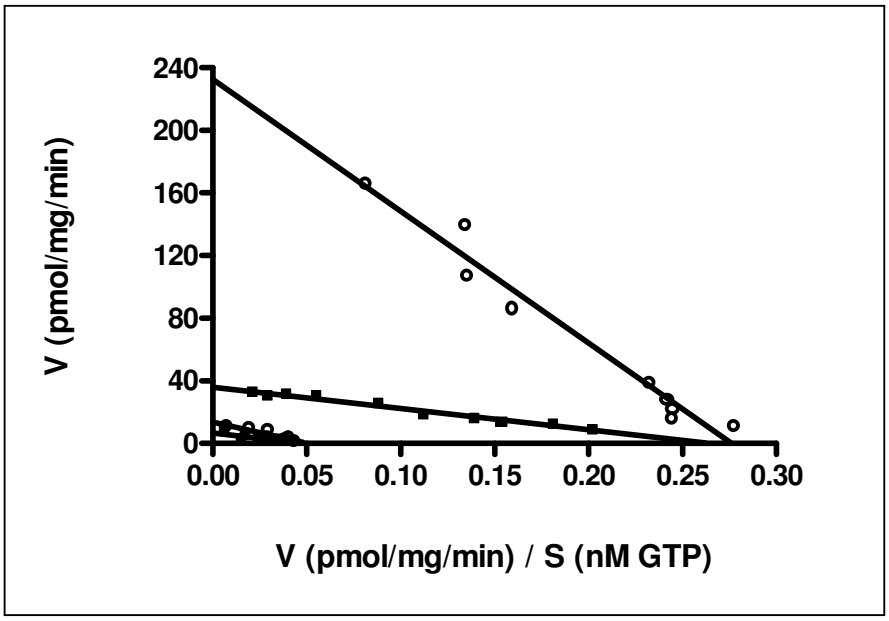


Figure 3.4 Kinetic analysis of GTPase activity in cell membranes expressing the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein in the presence and absence of GST-RGS4

Data generated from Figure 3.3 shown in Eadie-Hofstee transformation to show K_m and V_{max}. High-affinity GTPase activity in the absence (filled) or presence of 100 μM adrenaline (open) was measured at various concentrations of GTP in the presence (circles) or absence (squares) of GST-RGS4. Data shown are from triplicate determinations and are representative of three individual experiments performed.

		V_{max} (pmol/mg/min)	K_m (nM)
No RGS	Basal	**7 ± 1**	**137 ± 49**
	+ Adrenaline (100 μM)	**36 ± 1 ***	**136 ± 7**
+ 1 μM RGS4	Basal	**15 ± 1**	**325 ± 52**
	+ Adrenaline (100 μM)	**233 ± 13 *, ****	**843 ± 61*, ****

Table 3.1 Enzyme kinetics of GAP activity of RGS4

Data generated from Figure 3.4 are tabulated for comparison of enzyme kinetic results. Values of V_{max} and K_m are compared in the presence and absence of adrenaline (100 μM) and presence or absence of RGS4 (1 μM) in cell membranes expressing the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein. Data shown are mean ± SEM from three individual experiments. * Denotes significantly different from absence of agonist, $p<0.05$. ** Denotes significantly different from adrenaline-stimulated kinetics in absence of 1 μM RGS4, $p<0.01$.

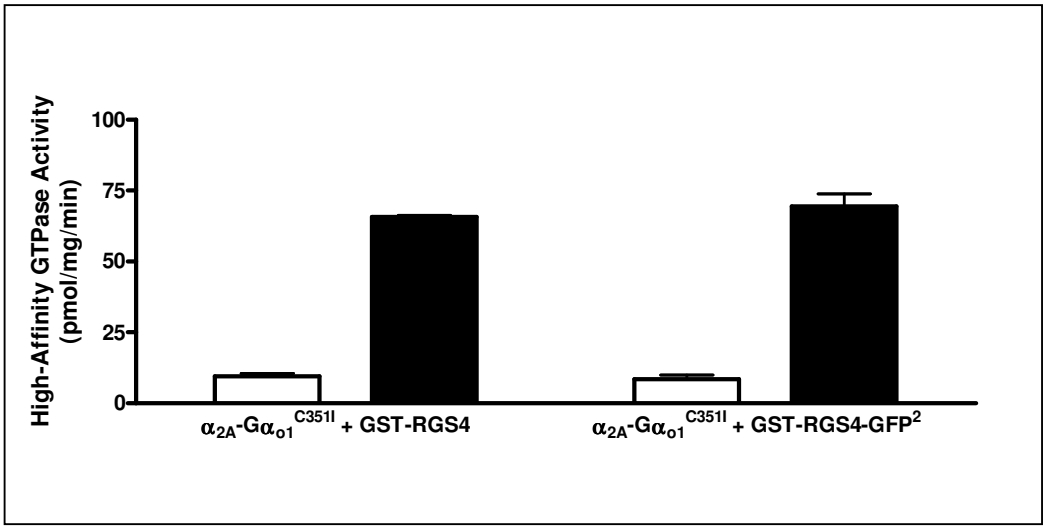


Figure 3.5 Comparison of GAP activity of GST-RGS4 and GST-RGS4-GFP^2

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-G$\alpha_{o1}{}^{C351I}$ fusion protein. Cells were treated with P.tox (25 ng/ml for 16 hours) and then membranes prepared. High-affinity GTPase activity (at 0.5 μM GTP) in the presence of 0.8 μM GST-RGS4 or presence of 0.8 μM GST-RGS4-GFP^2 was measured in the absence (open bar) or presence of 100 μM adrenaline (filled bar). Data shown are mean ± SEM of three individual experiments performed.

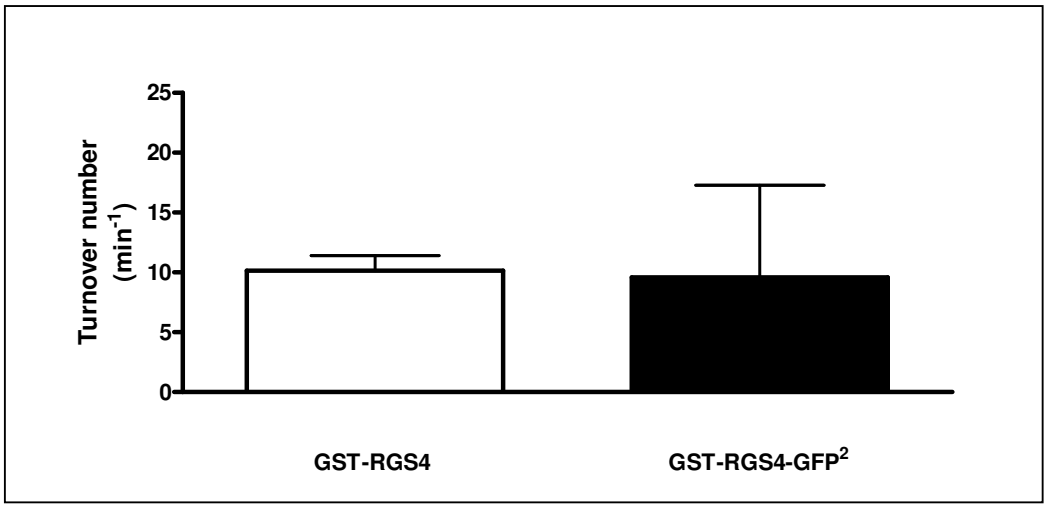
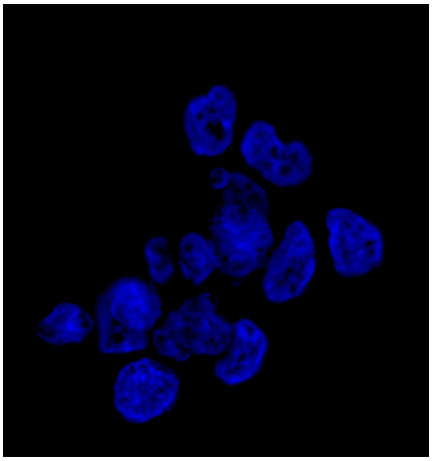


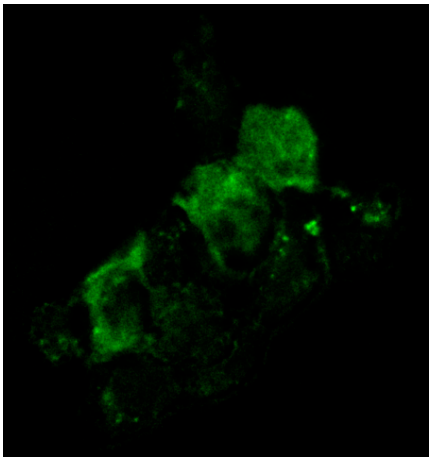
Figure 3.6 Comparison of turnover number of $G\alpha_{o1}$ in the presence of GST-RGS4 or GST-RGS4-GFP^2

The turnover number of $G\alpha_{o1}$ in the presence of GST-RGS4 (open bar) or GST-RGS4-GFP^2 (filled bar) is shown. The turnover number was calculated from expression level of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein and the high-affinity GTPase activity of the GST fusion protein in these membranes. Data shown are mean ± SEM. of three individual experiments performed.

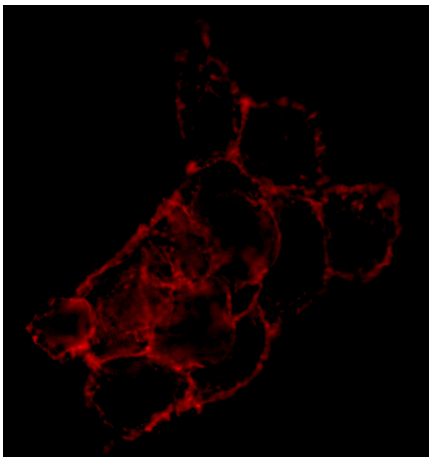
(A)



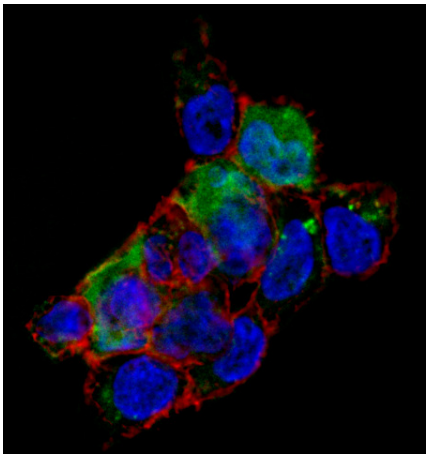
(B)



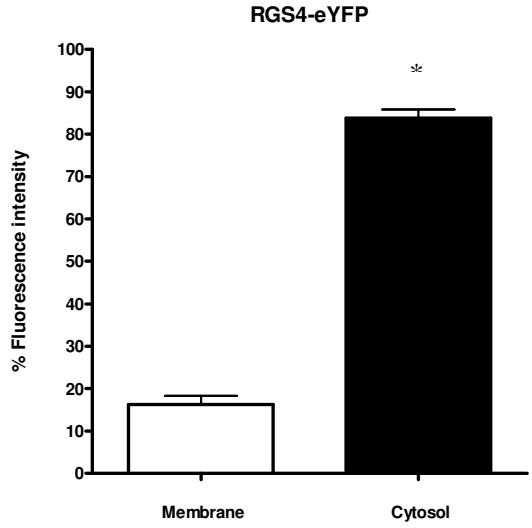
(C)



(D)



(E)


RGS4-eYFP
% Fluorescence intensity
100
90
80
70
60
50
40
30
20
10
0
*
Membrane
Cytosol

(F)

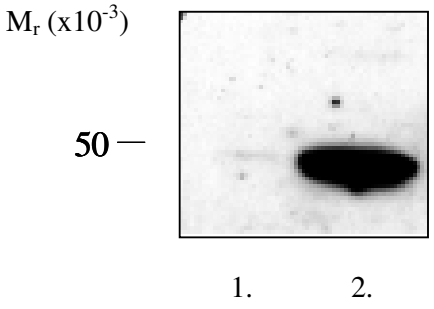


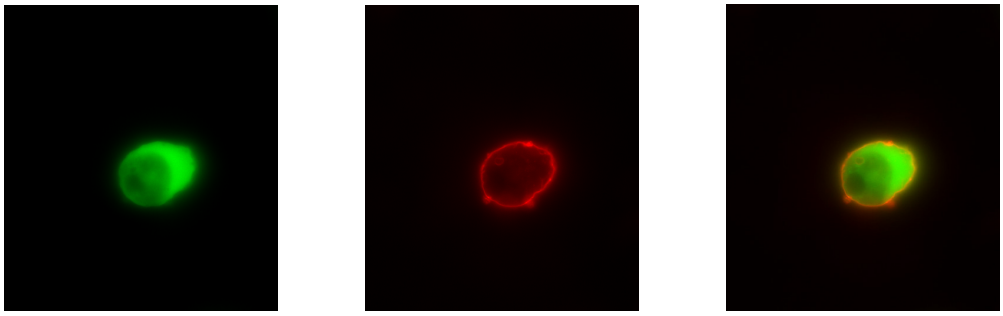
Figure 3.7 The cellular localisation of RGS4-eYFP

HEK293T cells grown on coverslips were transiently transfected with RGS4-eYFP and visualised with (A) Hoechst 33342 nuclei staining (blue) (B) RGS4-eYFP (green) or (C) WGA-Alexa Fluor 594 plasma membrane staining kit (red). Images were colour combined to create merged images (D). Results shown are of a single experiment and are representative of three experiments performed.

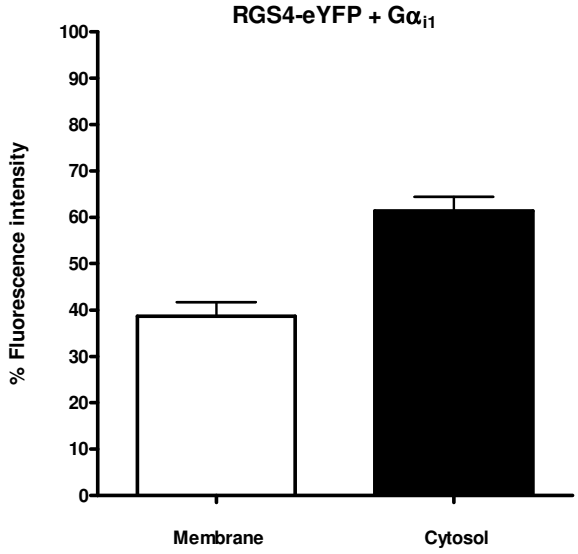
(E) HEK293T transfected cells were also measured for RGS4-eYFP fluorescence intensity at the plasma membrane (open bar) and cytoplasm (closed bar). Images were deconvolved using an iterative and constrained algorithm. Surface and cytosolic masks were created and superimposed and fluorescence pixel intensity values corresponding to RGS4-eYFP located at the plasma membrane and cytoplasm of the cell were quantified from each generated mask. Fluorescence was expressed as a percentage of the total RGS4-eYFP intensity using a mean of 3 cells ± SEM. Statistical significance was determined using a Student's *t* test, * $p<0.01$.

(F) Cells transiently transfected with RGS4-GFP[2] were separated into membrane (lane 1) and cytosolic (lane 2) fractions, resolved by SDS-PAGE and immunoblotted with an anti-RGS4 antiserum. Results shown are of a single experiment and are representative of three experiments performed.

(A) (B) (C)



(D)


RGS4-eYFP + Gαi1
% Fluorescence intensity
100
90
80
70
60
50
40
30
20
10
0
Membrane
Cytosol

(E)

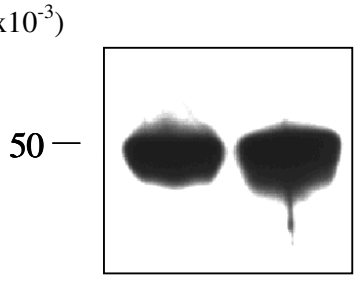

Mr (x10-3)
50
1.
2.

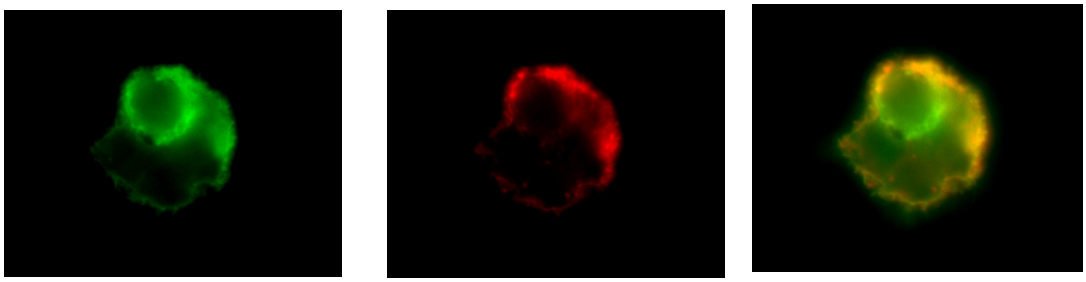
Figure 3.8 The cellular localisation of RGS4-eYFP when co-expressed with $G\alpha_{i1}$

RGS4-eYFP is translocated to the plasma membrane when transiently co-transfected with $G\alpha_{i1}$. (A) HEK293T cells grown on coverslips transiently transfected with RGS4-eYFP and $G\alpha_{i1}$. (B) HEK293T cells grown on coverslips transiently transfected with RGS4-eYFP and $G\alpha_{i1}$ with WGA-Alexa Fluor 594 plasma membrane staining kit. Images colour combined to create merged images (C). Results shown are of a single experiment and are representative of three experiments performed.

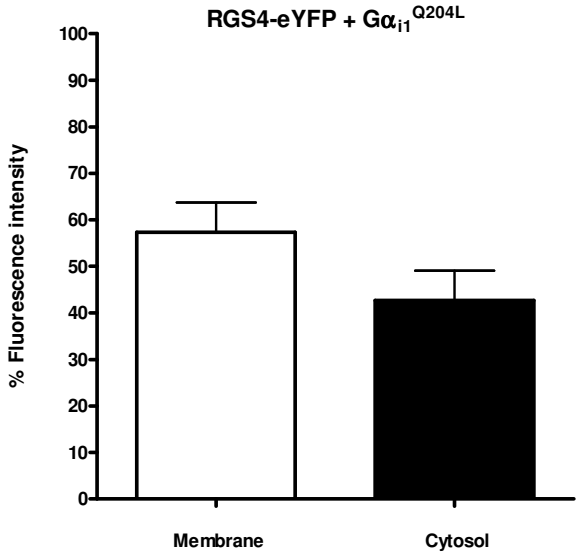
(D) HEK293T cells co-transfected with both RGS4-eYFP and $G\alpha_{i1}$ were also measured for RGS4-eYFP fluorescence intensity at the plasma membrane (open bar) and cytoplasm (closed bar). Images were deconvolved using an iterative and constrained algorithm. Surface and cytosolic masks were created and superimposed and fluorescence pixel intensity values corresponding to RGS4-eYFP located at the plasma membrane and cytoplasm of the cell were quantified from each generated mask. Fluorescence was expressed as a percentage of the total RGS4-eYFP intensity using a mean of 3 cells ± SEM. Statistical significance was determined using a Student's *t* test, * $p<0.01$.

(E) HEK293T cells transiently transfected with RGS4-eYFP and $G\alpha_{i1}$ were separated into membrane (lane 1) and cytosolic (lane 2) fractions and resolved by SDS-PAGE and immunoblotted with an anti-GFP antiserum. Results shown are of a single experiment and are representative of three experiments performed.

(A) (B) (C)



(D)


RGS4-eYFP + Gα$_{i1}^{Q204L}$
% Fluorescence intensity
100
90
80
70
60
50
40
30
20
10
0
Membrane
Cytosol

(E)

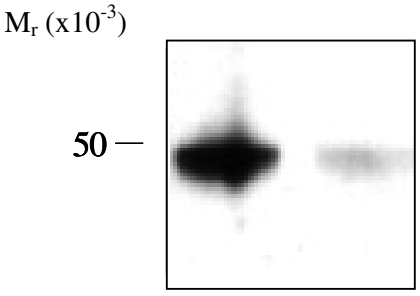

M$_r$ (x10^{-3})
50
1.
2.

Figure 3.9 The cellular localisation of RGS4-eYFP when co-expressed with $G\alpha_{i1}^{Q204L}$

RGS4-eYFP is translocated to the plasma membrane when transiently co-transfected with $G\alpha_{i1}^{Q204L}$. (A) HEK293T cells grown on coverslips transiently transfected with RGS4-eYFP and $G\alpha_{i1}^{Q204L}$. (B) HEK293T cells grown on coverslips transiently transfected with RGS4-eYFP and $G\alpha_{i1}^{Q204L}$ with WGA-Alexa Fluor 594 plasma membrane staining kit. Images colour combined to create merged images (C). Results shown are of a single experiment and are representative of three experiments performed.

(D) HEK293T cells co-transfected with both RGS4-eYFP and $G\alpha_{i1}^{Q204L}$ were also measured for RGS4-eYFP fluorescence intensity at the plasma membrane (open bar) and cytoplasm (closed bar). Images were deconvolved using an iterative and constrained algorithm. Surface and cytosolic masks were created and superimposed and fluorescence pixel intensity values corresponding to RGS4-eYFP located at the plasma membrane and cytoplasm of the cell were quantified from each generated mask. Fluorescence was expressed as a percentage of the total RGS4-eYFP intensity using a mean of 3 cells ± SEM. Statistical significance was determined using a Student's *t* test, * $p<0.01$.

(E) HEK293T cells were transiently co-transfected with RGS4-GFP[2] and $G\alpha_{i1}^{Q204L}$. Membrane (lane 1) and cytosolic (lane 2) fractions were separated and resolved by SDS-PAGE and then immunoblotted with an anti-GFP antiserum. Results shown are of a single experiment and are representative of three experiments performed.

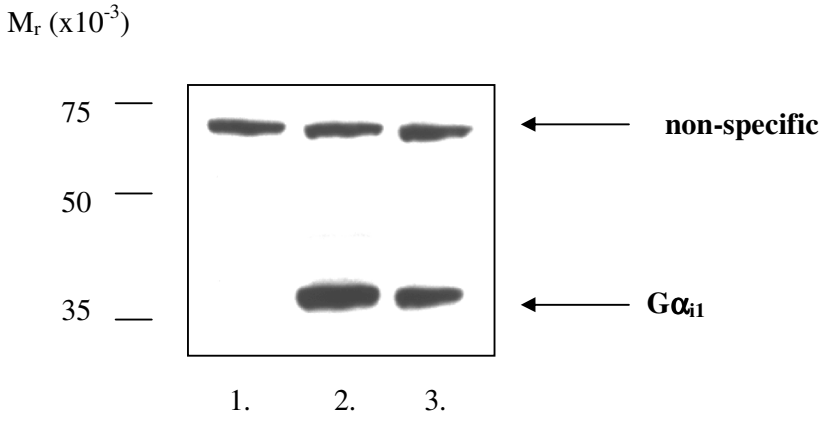
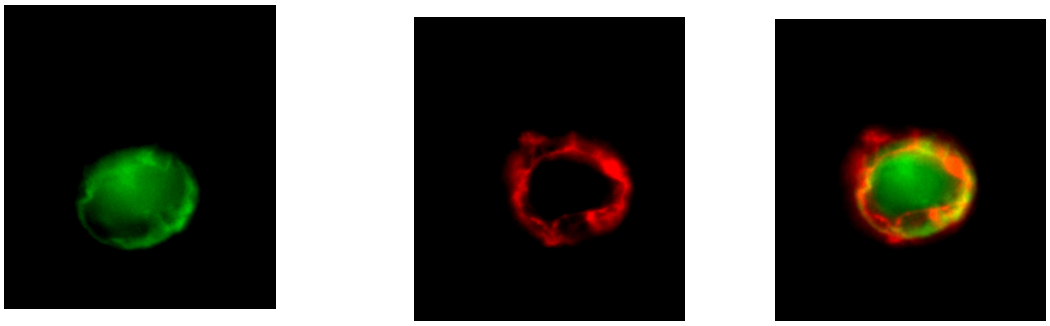


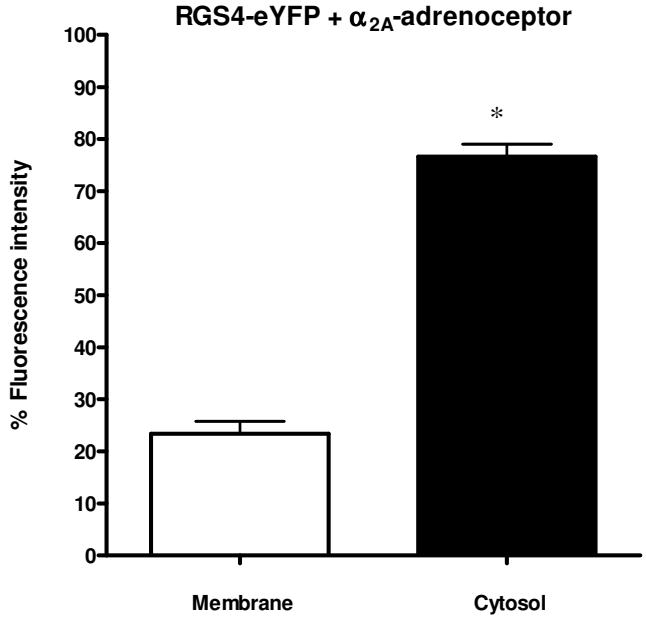
Figure 3.10 Expression and immunological detection of $G\alpha_{i1}$ and $G\alpha_{i1}^{Q204L}$

HEK293T cells were transfected to transiently express $G\alpha_{i1}$ (lane 2) or $G\alpha_{i1}^{Q204L}$ (lane 3). Untransfected HEK293T cell lysates were included as a control (lane1). Cell lysates were resolved by SDS-PAGE and then immunoblotted with an antiserum that identifies $G\alpha_{i1}$. Results shown are of a single experiment and are representative of three experiments performed.

(A) (B) (C)



(D)


RGS4-eYFP + α2A-adrenoceptor
% Fluorescence intensity
100
90
80
70
60
50
40
30
20
10
0
*
Membrane
Cytosol

(E)

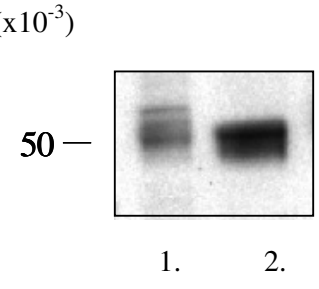

Mr (x10-3)
50
1.
2.

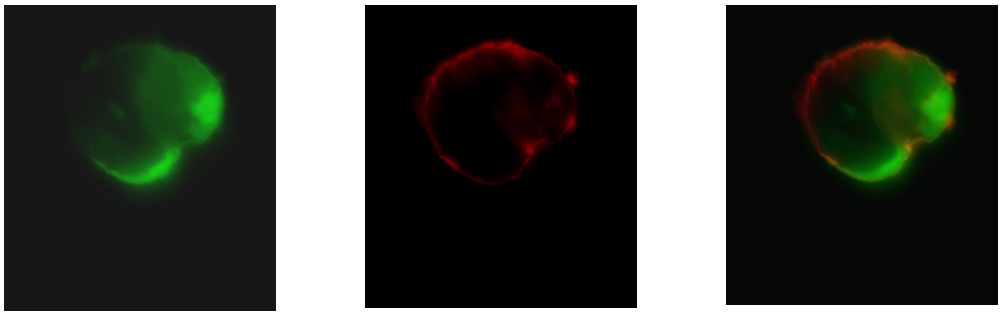
Figure 3.11 The cellular localisation of RGS4-eYFP when co-expressed with the α_{2A}-adrenoceptor

RGS4-eYFP is translocated to the plasma membrane when transiently co-transfected with RGS4-eYFP and α_{2A}-adrenoceptor. (A) HEK293T cells grown on coverslips transiently transfected with RGS4-eYFP and α_{2A}-adrenoceptor. (B) HEK293T cells grown on coverslips transiently transfected with RGS4-eYFP and α_{2A}-adrenoceptor with WGA-Alexa Fluor 594 plasma membrane staining kit. Images colour combined to create merged images (C). Results shown are of a single experiment, representative of three experiments performed.

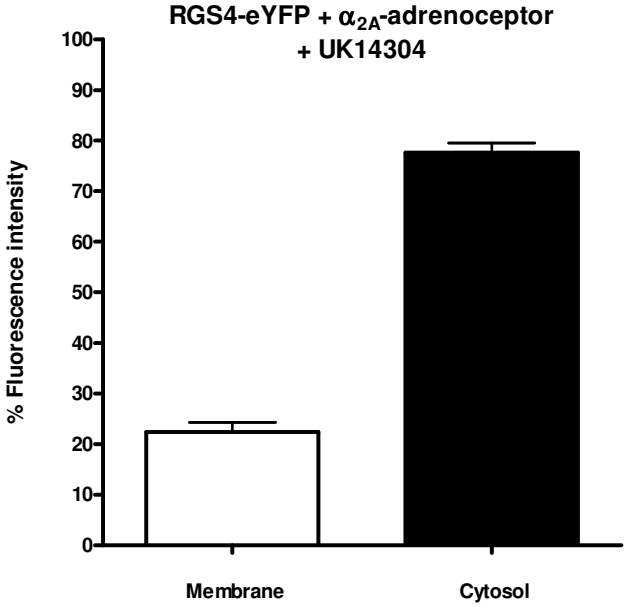
(D) HEK293T cells co-transfected with both RGS4-eYFP and the α_{2A}-adrenoceptor were also measured for RGS4-eYFP fluorescence intensity at the plasma membrane (open bar) and cytoplasm (closed bar). Images were deconvolved using an iterative and constrained algorithm. Surface and cytosolic masks were created and superimposed and fluorescence pixel intensity values corresponding to RGS4-eYFP located at the plasma membrane and cytoplasm of the cell were quantified from each generated mask. Fluorescence was expressed as a percentage of the total RGS4-eYFP intensity using a mean of 3 cells ± SEM. Statistical significance was determined using a Student's *t* test, * $p<0.05$.

(E) HEK293T cells were transiently co-transfected with RGS4-eYFP and the α_{2A}-adrenoceptor. Membrane (lane 1) and cytosolic (lane 2) fractions were separated and resolved by SDS-PAGE and then immunoblotted with an anti-GFP antiserum. Results shown are of a single experiment, representative of three experiments performed.

(A) (B) (C)



(D)


RGS4-eYFP + α2A-adrenoceptor
+ UK14304
% Fluorescence intensity
100
90
80
70
60
50
40
30
20
10
0
Membrane
Cytosol

(E)

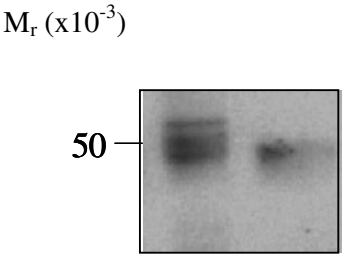

Mr (x10-3)
50
1.
2.

Figure 3.12 The cellular localisation of RGS4-eYFP when co-expressed with the α_{2A}-adrenoceptor and stimulated with UK14304

RGS4-eYFP is translocated to the plasma membrane when transiently co-transfected with the α_{2A}-adrenoceptor then stimulated with UK14304 (100 μM) for 30 minutes. (A) HEK293T cells were grown on coverslips and transiently transfected with both RGS4-eYFP and the α_{2A}-adrenoceptor then subsequently stimulated with UK14304 (100 μM) for 30 minutes. (B) HEK293T cells grown on coverslips, transiently transfected with both RGS4-eYFP and the α_{2A}-adrenoceptor were stimulated with UK14304 (100 μM) for 30 minutes, with WGA-Alexa Fluor 594-labelled plasma membrane staining kit. Images colour combined to create merged images (C).

(D) HEK293T cells transiently transfected with RGS4-eYFP and the α_{2A}-adrenoceptor then subsequently stimulated with UK14304 (100 μM) for 30 minutes were measured for RGS4-eYFP fluorescence intensity at the plasma membrane (open bar) and cytoplasm (closed bar). Images were deconvolved using an iterative and constrained algorithm. Surface and cytosolic masks were created and superimposed and fluorescence pixel intensity values corresponding to RGS4-eYFP located at the plasma membrane and cytoplasm of the cell were quantified from each generated mask. Fluorescence was expressed as a percentage of the total RGS4-eYFP intensity using a mean of 3 cells ± SEM. Statistical significance was determined using a Student's *t* test, * $p<0.05$.

(E) HEK293T cells transiently transfected with RGS4-eYFP and the α_{2A}-adrenoceptor then subsequently stimulated with UK14304 (100 μM) for 30 minutes were separated into membrane (lane 1) and cytosolic (lane 2) fractions and resolved by SDS-PAGE and immunoblotted with an anti-GFP antiserum. Results shown are of a single experiment and are representative of three experiments performed.

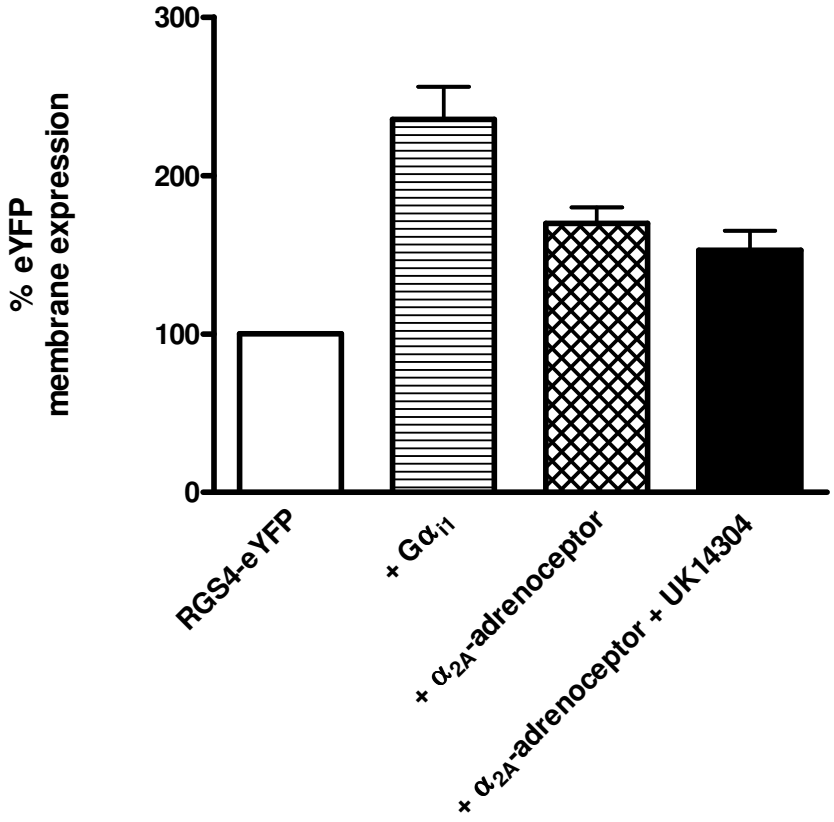


Figure 3.13 Comparison of percentage RGS4-eYFP fluorescence at the plasma membrane

HEK293T cells were transiently transfected to express RGS4-eYFP, RGS4-eYFP co-transfected with $G\alpha_{i1}$, RGS4-eYFP co-transfected with the α_{2A}-adrenoceptor or RGS4-eYFP co-transfected with the α_{2A}-adrenoceptor and stimulated with 100 µM UK14304 for 30 minutes. The membrane fraction of these cells was measured for eYFP fluorescence and expressed as a percentage compared to RGS4-eYFP (defined as 100%). Data shown are from triplicate determinations (mean ± SEM.) and are representative of three experiments performed.

3.3 Discussion

The introduction of N- and C-terminal modifications on RGS4 could potentially impair function or cellular localisation of the protein. The *in vitro* function of several B/R4 RGS proteins, including RGS4, appears to be uncompromised by the addition of an N-terminal tag (Berman *et al.*, 1996b;Chen *et al.*, 1997). Similarly, the addition of a C-terminal tag has also been shown to have no effect on the functionality or the localisation of RGS4 (Chatterjee and Fisher, 2000). In this study, addition of GFP^2 to the C-terminal of RGS4 did not change the GAP activity of the protein compared to wild-type RGS4.

High-affinity GTPase assays measure the rate of GTP hydrolysis to GDP (McKenzie and Milligan, 1990;Wise *et al.*, 1997a;Wise *et al.*, 1997b). G proteins fused to the C-terminal of GPCRs were first used in high-affinity GTPase assays by Wise *et al.*, (1997a). The expression of such fusion proteins defines a 1:1 stoichiometry of receptor and G protein. Each receptor is in equivalent proximity to the G protein and furthermore, receptor-G protein fusion proteins function effectively as agonist-activated GTPases. The α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein has previously been used to characterise RGS4 GTPase activity (Cavalli *et al.*, 2000;Bahia *et al.*, 2003). A mutagenic alteration in the G protein four amino acids from the C terminal at a conserved cysteine residue (^{C35Il} in $G\alpha_{o1}$) defines that the G protein cannot be ADP-ribosylated and therefore becomes resistant to P. tox treatment. P. tox catalyses ADP-ribosylation on a sensitive G protein α subunit, therefore making it unable to exchange GDP for GTP in response to receptor stimulation. Thus, P. tox treatment abolishes signalling through the endogenous pool of inhibitory G proteins. G protein families endogenously expressed in HEK293T cells such as, $G\alpha_s$, $G\alpha_q$ and $G\alpha_{12}$, which are not sensitive to P. tox, produce too limited a signal to be detected with the high-affinity GTPase assay. The expression of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein and P. tox treatment of cells therefore allows the GTPase signal to be attributed solely to the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein.

To study the enzyme kinetics of the high-affinity GTPase assay, various concentrations of GTP acting as a substrate on the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein can be used (Cavalli *et al.*, 2000;Bahia *et al.*, 2003). Such a successful strategy was also employed in this study. As expected, in the absence of recombinant RGS4, the rate of GTPase activity was increased by adrenaline but did not alter the K_m. RGS family members act catalytically*;* relatively small amounts of RGS protein can substantially increase the K_m and the V_{max} of GTP hydrolysis. It could therefore be deduced by the lack of alteration of

K_m in the absence of added RGS4, that the endogenous levels of RGS proteins present in the HEK293T cell membrane preparation are low.

Addition of recombinant RGS4 to the high-affinity GTPase assay resulted in an increase in both V_{max} and the K_m for GTP as anticipated by the basic catalytic mechanism of RGS function . As such a single concentration of GTP was initially sufficient to demonstrate that the fluorescently modified RGS4 was functionally comparable to wild-type. Further investigations using various concentrations of GTP acting as a substrate on the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein allowed the determination of the turnover number of RGS4-GFP^2 and showed that addition of GFP^2 to the C-terminal of RGS4 did not change the GAP activity of the protein compared to wild-type RGS4.

RGS proteins have been reported to physically interact with Gα subunits, stabilising the transition state conformation of the G protein and increasing the intrinsic GTPase activity (Berman *et al.*, 1996b). RGS4 is thought to be recruited from cytosol to the plasma membrane by Gα subunits particularly those of the $G\alpha_i$ and $G\alpha_q$ subfamilies (Druey *et al.*, 1998;Srinivasa *et al.*, 1998). The cytoplasmic localisation of RGS4 allows access to the inner face of the plasma membrane to interact with G proteins and receptors and effectors. However, the mechanisms of plasma membrane association are still uncertain.

The present results sought to clarify the cellular localisation and trafficking of RGS4 in mammalian cells. Many studies have observed that transiently transfected RGS4 is localised in the cytoplasm (Druey *et al.*, 1998;Chatterjee and Fisher, 2000). As mentioned previously, the N-terminus of RGS4 is extremely important for cellular localisation. Indeed, a sequence element at the N-terminus of the RGS domain has been identified to cause cytoplasmic retention of RGS4. A retroviral-like sequence has been shown to mediate nuclear-cytoplasmic export of RGS4 by interaction with the exportin-1-RanGTP complex (Chatterjee and Fisher, 2000). However, it was noticed in the present study that although the vast majority of RGS4-eYFP is cytoplasmic, a relatively small proportion of RGS4-eYFP is membrane associated at any given time (Figure 3.7).

Previously, it has been shown that the presence of melatonin causes nuclear exclusion of the androgen receptor. The generally accepted mechanism for this nuclear exclusion involves the $G\alpha_i$ mediated increase of intracellular cyclic guanosine monophosphate (cGMP) concentrations, calcium entry into the cell and protein kinase C (PKC) activation. Importantly, this mechanism also leads to the enhanced cytoplasmic distribution of RGS2

and RGS10 in prostate carcinoma cells. It was also discovered that in these cells stably expressing the androgen receptor, RGS4 surprisingly accumulated in the nucleus and showed no recruitment from the cytoplasm to the plasma membrane in the presence of melatonin (Rimler *et al.*, 2006). This has lead to speculation that in addition to the regulation of membrane bound G proteins, RGS4 may perhaps have other undiscovered regulatory functions including modulating the intensity and the duration of signal transduction.

Numerous studies have provided evidence that RGS proteins interact directly with Gα subunits. In a yeast two-hybrid screen, GAIP was found to interact specifically with members of the $G\alpha_i$ subfamily, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{iz}$, and $G\alpha_o$ (De Vries and Farquhar, 2002). A co-immunoprecipitation study found that RGS2 binds $G\alpha_s$ protein *in vitro* (Tseng and Zhang, 1998). It has been demonstrated that RGS3 binds to $G\alpha_q$, but not $G\alpha_s$ (Neill *et al.*, 1997) and that RGS1 and RGS4 interacts with $G\alpha_i$, but not $G\alpha_s$ (Watson *et al.*, 1996;Berman *et al.*, 1996b). However, recent studies failed to prove binding between RGS2 and $G\alpha_s$ *in vivo* (Chen *et al.*, 1997;Heximer *et al.*, 1997) despite the understanding that RGS2 is recruited to the plasma membrane after co-expression of $G\alpha_s$ (Roy *et al.*, 2006) and inhibits cAMP signalling (Sinnarajah *et al.*, 2001). Thus, the interaction between RGS and heterotrimeric G proteins may possibly be cell specific and further investigation is required.

The RGS-Gα protein interaction may also be increased by receptors (Ingi *et al.*, 1998;Zeng *et al.*, 1998;Xu *et al.*, 1999). GPCRs could influence RGS orientation, promoting localisation at the plasma membrane (Hollinger and Hepler, 2002), or perhaps expression of GPCRs could induce a greater pool of endogenous RGS proteins (Druey *et al.*, 1996;Tseng and Zhang, 1998). Importantly, a recent study found that RGS2 binds selectively to the third intracellular loop of the M1 muscarinic receptor (Bernstein *et al.*, 2004) and to the third intracellular loop of the α_{1a}-adrenoceptor (Hague *et al.*, 2005). There is a high degree of selectivity of interactions between RGS proteins and receptors but to date no corresponding binding regions have been identified (Zeng *et al.*, 1998). It may be reasoned that the selectivity of RGS proteins for receptors must regulate very specific physiological responses. In this study, cytoplamsic RGS4 was recruited to the plasma membrane following co-expression with the α_{2A}-adrenoceptor in HEK293T cells. No further recruitment of RGS4 to the plasma membrane was observed when the α_{2A}-adrenoceptor was stimulated with agonist. Endogenous G proteins in HEK293T cells are perhaps sufficiently activated by presence of the receptor, or are able to serve as better

RGS targets when coupled to receptors enabling the RGS4-eYFP to be translocated to the plasma membrane in the absence of agonist. This translocation of RGS4-eYFP in the presence of the α_{2A}-adrenoceptor suggested that further study using cells transfected with the α_{2A}-adrenoceptor and over expressing exogenous $G\alpha_i$ was not required. Perhaps further investigation may reveal that there is a simple physical association between the RGS4 and the α_{2A}-adrenoceptor, which allows RGS4-eYFP to be translocated to the plasma membrane.

Mutation of glutamic acid 204 of $G\alpha_{i1}$ to leucine abolishes the hydrolysis of GTP to GDP on the Gα protein. Glutamic acid 204 stabilises the negative charge on the phosphate leaving group in the transition state of the reaction complex and orientates the attacking water molecule to allow GTP hydrolysis (Graziano and Gilman, 1989). The expression of the constitutively active $G\alpha_{i2}$ mutant ($G\alpha_{i2}^{Q205L}$) showed translocation of cytosolic RGS4 to the plasma membrane (Druey *et al.*, 1998). Constitutively activated forms of the G protein, $G\alpha_q$ ($G\alpha_q^{Q209L}$ and $G\alpha_q^{R183C}$) have also been researched and co-expression with RGS4 also show the translocation of RGS4 to the plasma membrane (Heximer *et al.*, 2001;Roy *et al.*, 2003). The use of constitutively active G proteins was also used in the present study. The corresponding constitutively active mutant of the $G\alpha_{i1}$ G protein ($G\alpha_{i1}^{Q204L}$) showed increased recruitment of RGS4 to the plasma membrane compared to wild-type $G\alpha_{i1}$. This was not caused by a higher expression of $G\alpha_{i1}^{Q204L}$ compared to $G\alpha_{i1}$ suggesting that the conformation of the constitutively active G protein is more favourable for the recruitment of RGS4-eYFP to the plasma membrane.

The data shown in this chapter confirm that the over-expression of exogenous $G\alpha_{i1}$ recruits RGS4-eYFP to the plasma membrane in HEK293T cells. When a cognate receptor that activates this G protein was co-transfected in place of the G protein, corresponding patterns of translocation were observed. The ability of a transfected receptor, unstimulated by agonist, to increase levels of RGS4 at the plasma membrane implies that RGS4 is recruited to the plasma membrane almost certainly in response to an increased number of RGS4 protein binding targets. However, more RGS binding sites at the plasma membrane may be sufficient to trigger translocation of RGS4 in intact cells, spontaneously activating endogenous G proteins to cause RGS4 translocation. The transient transfection of RGS4-eYFP may have concealed any differences in cellular translocation patterns. Perhaps future technical advances and investigation on endogenous RGS4 will reveal currently unrecognised localisation patterns of RGS proteins.

4 Chapter 4

4.1 Introduction

Previously, a deletion of the fission yeast *S. pombe* RGS gene, *rgs1*, produced an increased sensitivity of pheromone signalling as measured by pheromone-dependent transcription (Watson *et al.*, 1999). In fact, loss of *rgs1* not only made the yeast cells more sensitive to pheromone stimulation but also increased production of the reporter gene, β-galactosidase, in the absence of mating factor. These changes were due to the loss of *rgs1*, as they were overcome by expression of an exogenous Rgs1p (Didmon *et al.*, 2002). It was reasoned that if such loss-of-function mutations in yeast amplify pheromone signalling, gain-of-function (GOF) mutations that block signalling could also be obtained. Thus, a GOF mutation in an RGS gene would be anticipated to increase RGS activity to further inhibit cellular signalling. Indeed, RGS GOF mutations were detected in *S. cervisiae* by growth on plates containing very high (normally lethal) concentrations of α-factor (6 μM) (Dohlman *et al.*, 1995).

The simplicity of the yeast pheromone signalling cascade has allowed large scale screens to identify RGS GOF mutants. *Sz. pombe* consists of three main signalling components, a GPCR, a Gα subunit and a single RGS protein. The use of a *Sz. pombe* screen to identify RGS4 mutants by Hill *et al*., (personal communication) was the first description of a GOF mutant in a mammalian RGS. $RGS4^{S30C}$ showed decreased production of β-galactosidase and decreased sensitivity of pheromone signalling. Residue 30 in RGS4 is located in the N-terminal domain of RGS4. Traditionally, the RGS box was thought to exclusively confer GAP activity to RGS proteins. It was therefore initially surprising that mutation of a single amino acid, especially in the N-terminal domain of RGS4, decreased sensitivity of pheromone signalling and pheromone-dependent transcription.

A series of deletion mutants have previously been used to define particular regions of RGS4 that confer specific functions (Zeng *et al.*, 1998). RGS4 is most easily split into three such deletion mutants, allowing functions to be attributed to the RGS box, the flanking N-terminal, or C-terminal domains. Clearly, all three domains must work cooperatively to regulate selective inhibition of G protein signalling but findings have shown that the flanking regions of RGS4 were essential for optimal GAP activity in receptor-G protein signalling. In particular, it was shown that the N-terminal domain of

RGS4 is essential for high potency inhibition of G protein signalling and receptor selectivity.

Further studies of the N-terminal of RGS4 used a 33 amino acid N-terminal peptide of RGS4 (P_{1-33}). This peptide was shown to effectively inhibit $G\alpha_q$-mediated Ca^{2+} signalling. Importantly, this inhibition was selective for the M3 muscarinic receptor over the cholecytokinin receptor (Zeng *et al.*, 1998). The N-terminus of RGS4 may therefore help position RGS4 between the receptor and the G protein optimally positioning the RGS box for effective GAP activity.

RGS4 has been implicated in regulating a number of signalling pathways. A mammalian GOF mutant in RGS4 could therefore produce new insights into the physiological relevance of RGS4. The aim of the studies in this chapter was to investigate $RGS4^{S30C}$ *in vitro* and in intact mammalian cells. High-affinity GTPase assays measured the sensitivity of the mutant to agonist and moreover, the rate of GTPase activity. The cellular localisation of $RGS4^{S30C}$ was also investigated. Importantly, the ability of the GOF mutation to be transferred into another member of the B/R4 RGS family, RGS16, and the selectivity of these RGS proteins for different Gα subtypes was also studied. The G protein selectivity, potency and GAP activity of other selected residue 30, RGS4 mutants were also investigated.

4.2 Results

4.2.1 Identification of RGS4 mutants

Mutations within the RGS4 open reading frame resulting in increased activity were identified by Hill *et al*., (personal communication). A previously identified *Sz. pombe* yeast strain (JY731) was employed which incorporates a pheromone-inducible reporter, sxa2>ura4, but lacks the endogenous RGS protein, Rgs1p (Didmon *et al.*, 2002). Signalling responses were assayed utilising agar plates containing 5-fluoro-orotic acid (FOA) and varying concentrations of the yeast pheromone, P-factor. Exposure of the *ura4* reporter strain to P-factor results in pheromone-dependent production of orotidine 5'-monophosphate decarboxylase (Ura4). Ura4 subsequently converts FOA into a toxic product, killing signalling cells (Boeke *et al*., 1984). GOF mutants were isolated by identifying yeast colonies capable of reducing signalling of the strain JY731, permitting growth at 10 nM of P-factor. A further round of selection was carried out on agar plates

lacking uracil but containing 1 μM of P-factor. This second round of selection eliminated any false positives colonies, *e.g.* colonies that contained spontaneous mutations in the *ura4* gene rendering the Ura4 protein inactive. 25 such RGS4 GOF mutant plasmids were identified in this yeast-based screen. Sequence analysis showed that mutations included point mutations, base pair deletions and or duplications of the open reading frame. The most abundant of the mutants was a serine to cysteine mutation at residue 30 ($RGS4^{S30C}$). This specific mutation was remade by site-directed mutagenesis in both pcDNA3 and pGEX6-P1 vectors. Residue 30 in RGS4 was subsequently mutated to alanine, glutamic acid, lysine, methionine, phenylalanine or proline. For comparison, these amino acid structures are shown in Figure 4.1

Figure 4.2 shows SDS-PAGE gels of purified GST-fused recombinant (A) RGS4 and (B) $RGS4^{S30C}$ stained with Coomassie Blue. Similar induction and purification of each protein is shown at around 50 kDa. Quantitative evaluation of the pooled eluted proteins using the BCA assay allowed the concentration of each eluted protein to be determined.

4.2.2 Functional activity of $RGS4^{S30C}$

High-affinity GTPase assays were used to compare the functional activity of $RGS4^{S30C}$ and wild-type RGS4. The GTPase activity of the $G\alpha_{o1}$ subunit was determined following transient expression of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein in HEK293T cells as before (Chapter 3 Section 3.3), and it was therefore important to establish if the presence of either RGS protein disrupted the binding of adrenaline to the α_{2A}-adrenoceptor.

The α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein was transiently expressed in HEK293T cells and cells were pre-treated with P. tox (25 ng/ml, 16 hours) before membranes were prepared. 2 μg of membranes transiently expressing the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein were used to compare the ability of adrenaline to compete with [^{3}H]RS-79948-197 for binding to α_{2A}-adrenoceptor in the absence of an RGS protein or in the presence of 1 μM GST-RGS4 or 1 μM GST-$RGS4^{S30C}$ (Figure 4.3). The total [^{3}H]RS-79948-197 bound specifically in the absence of RGS protein was represented as 100%. The presence of either RGS protein did not alter the affinity of adrenaline for α_{2A}-adrenoceptor (Figure 4.4).

These membranes expressing the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein were challenged with varying concentrations of adrenaline (1 mM – 0.1 nM) in the absence of

RGS protein or in the presence of 1 μM GST-RGS4 or 1 μM GST-RGS4^{S30C}, and high-affinity GTPase activity measured (Figure 4.5). The intrinsic rate of $G\alpha_{o1}$ GTPase activity (in the absence of RGS protein) was stimulated in a concentration-dependent manner with pEC_{50} 7.18 ± 0.07. The presence of GST-RGS4 did not significantly increase the basal high-affinity GTPase activity but increased the maximal adrenaline-stimulated GTPase activity of $G\alpha_{o1}$ and significantly decreased the potency of adrenaline to stimulate high-affinity GTPase activity (pEC_{50} 6.72 ± 0.08, $p<0.01$) (Figure 4.6).

The addition of purified GST-RGS4^{S30C} to HEK293T membranes expressing the α_{2A}-adrenoceptor-$G\alpha_{o1}$Cys^{351}Ile fusion protein resulted in an increase in the basal GTPase activity ($p<0.05$) and a larger increase in the maximal adrenaline stimulated GTPase activity of $G\alpha_{o1}$ than was produced by addition of purified GST-RGS4. The potency of adrenaline was further significantly decreased (pEC_{50} 6.46 ± 0.11, $p<0.01$) (Figure 4.6). This decrease in pEC_{50} is consistent with an increase in activity of the RGS.

A similar pattern of increased GTPase activity by GST-RGS4^{S30C} was also seen when the α_{2A}-adrenoceptor-$G\alpha_{o1}{}^{C351I}$ fusion protein expressing membranes were challenged with the α_2-adrenoceptor selective agonist UK14304 (Figure 4.7). The intrinsic rate of $G\alpha_{o1}$ GTPase activity (in the absence of RGS protein) was stimulated in a concentration dependent manner with pEC_{50} 7.71 ± 0.15. The presence of GST-RGS4 did not significantly increase the basal high-affinity GTPase activity but increased the maximal UK14304 stimulated GTPase activity of $G\alpha_{o1}$ and significantly decreased the potency of UK14304 to stimulate high-affinity GTPase activity (pEC_{50} 7.23 ± 0.12, $p<0.01$). Compared to addition of wild-type RGS4, addition of purified RGS4^{S30C} resulted in a larger increase in the maximal UK14304-stimulated GTPase activity of $G\alpha_{o1}$ and the potency of UK14304 was further significantly decreased (pEC_{50} 6.76 ± 0.18, $p<0.01$) (Figure 4.8). The enhanced high-affinity GTPase activity of $G\alpha_{o1}$ by RGS4^{S30C} seen is therefore not restricted to the action of a single ligand.

In contrast, when the G protein fused to α_{2A}-adrenoceptor was replaced with $G\alpha_{i1}$, addition of purified GST-RGS4 displayed little capacity to increase the high-affinity GTPase activity and GST-RGS4^{S30C} did not act as a GOF mutant (Figure 4.9). Furthermore, comparison of the addition of purified RGS4^{S30C} and addition of purified wild-type RGS4, shows no difference in the potency of adrenaline-stimulated GTPase activity of $G\alpha_{i1}$ (Figure 4.10) ($p>0.05$). This indicates that RGS4 is G protein selective and this selectivity is preserved in RGS4^{S30C}.

From the increase in functional activity of $G\alpha_{o1}$ produced by the presence of $RGS4^{S30C}$, it was thought necessary to examine the effect this mutated RGS protein had on the activity of downstream signalling. Expression of the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein elevates intracellular Ca^{2+} in response to the α-adenoceptor agonist phenylephrine (Stevens *et al.*, 2001). This effect was inhibited by the co-expression of an RGS protein and therefore, co-expression of a more active RGS protein might be able to further inhibit $[Ca^{2+}]_i$ release. The stop codon of RGS4 and $RGS4^{S30C}$ were removed and both proteins were independently fused in-frame to eYFP. The presence of this fluorescent tag allowed detection of cells positively transfected with these proteins. Co-expression of the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein and RGS4-eYFP resulted in a decrease in the maximal extent of agonist mediated elevation of $[Ca^{2+}]_i$ compared to expression of α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein alone. However, expression of $RGS4^{S30C}$-eYFP did not result in any further inhibition in extent or alter the kinetics of the signalling response compared to RGS4 (Figure 4.11).

4.2.3 Functional activity of $RGS16^{S30C}$

Sequence alignment of RGS proteins in the B/R4 subfamily revealed that the serine residue at position 30 in RGS4 was conserved in RGS2, RGS8 and RGS16 (Figure 4.12). To determine if the GOF mutation present in RGS4 was transposable to RGS16, high-affinity GTPase assays were performed as before. It was observed that the potency for adrenaline on the addition of purified GST-RGS16 was pEC_{50} 5.68 ± 0.3 (Figure 4.14). Addition of purified GST-$RGS16^{S30C}$ increased the maximal adrenaline stimulated GTPase activity of $G\alpha_{o1}$ and significantly decreased the potency for adrenaline compared to GST-RGS16 (pEC_{50} 5.39 ± 0.36, $p<0.01$) (Figure 4.14). Importantly, however, there was no difference in the GTPase activity (Figure 4.15) or the potency for adrenaline (Figure 4.16) when α_{2A}-adrenoceptor was fused to $G\alpha_{i1}$ ($p>0.05$). In addition to altering the potency for adrenaline, addition of purified GST-$RGS16^{S30C}$ increased the basal GTPase activity of α_{2A}-adrenoceptor-$G\alpha_{o1}$ but not of α_{2A}-adrenoceptor-$G\alpha_{i1}$. This increase in basal activity did not seem to be agonist mediated, as the increase was apparent even without the presence of adrenaline. Thus, the $RGS4^{S30C}$ mutation is transposable between the B/R4 subfamily to preserve the enhanced $G\alpha_{o1}$ activity and G protein selectivity.

4.2.4 Expression of RGS4^{S30C}–eYFP

The effect of the RGS4^{S30C} mutation on expression was determined. RGS4-eYFP and RGS4^{S30C}-eYFP were transiently expressed in HEK293T cells and cell lysates were separated by SDS-PAGE and expression levels detected with an antiserum directed against GFP (Figure 4.17). Bands corresponding to RGS4-eYFP (lane 2) and RGS4 ^{S30C}-eYFP (lane 3) were of comparable intensity indicating similar expression of the two proteins. Cell lysates of vector alone transfected cells were included as a control (lane 1). Increased expression of RGS4^{S30C} is therefore not responsible for the GOF properties of RGS4^{S30C}.

4.2.5 Localisation of RGS4^{S30C} –eYFP

It was hypothesised that a change in the subcellular localisation of RGS4^{S30C} may enhance the activity of RGS4. Increased localisation of RGS4^{S30C}-eYFP at the plasma membrane compared to RGS4-eYFP might allow the mutated protein to be in closer proximity to other signalling complexes located at the plasma membrane subsequently enhancing the apparent activity of the RGS protein. The subcellular localisation of transiently expressed RGS4^{S30C}-eYFP in HEK293T cells was visualised by microscopy (Figure 4.18). Panel (A) demonstrates that analogous to RGS4-eYFP, RGS4^{S30C}-eYFP was predominantly cytosolic. This was confirmed by pixel by pixel analysis of the distribution of eYFP fluorescence intensity (Figure 4.19). The RGS4^{S30C} mutation did not affect RGS4 localisation.

As before (Chapter 3, Section 3.5 and Section 3.6), the intracellular localisation of RGS4^{S30C}-eYFP was examined by co-expression of other signalling proteins (Figure 4.18). RGS4^{S30C}-eYFP co-expressed with Gα_{i1} (Figure 4.18 (B)), the α_{2A}-adrenoceptor (Figure 4.18 (C)), or the α_{2A}-adrenoceptor stimulated with UK14304 for 30 minutes (Figure 4.18 (D)). All these conditions show the translocation of RGS4^{S30C}-eYFP to the plasma membrane. No obvious difference between RGS4 and RGS4^{S30C} translocation could be detected using this methodology.

The subcellular localisation and translocation pattern of RGS4^{S30C} was also confirmed by determining the percentage fluorescence of RGS4^{S30C}-eYFP in the plasma membrane of the transfected HEK293T cells. Percentage fluorescence of RGS4^{S30C}-eYFP in the plasma membrane (defined as 100%) was directly compared to the percentage fluorescence of RGS4^{S30C}-eYFP co-expressed with other signalling proteins, Gα_{i1}, α_{2A}-adrenoceptor, or

agonist stimulated α_{2A}-adrenoceptor (Figure 4.20). The co-expression with other signalling proteins all increased the percentage fluorescence of RGS4^{S30C}-eYFP in the plasma membrane. In a similar pattern to RGS4-eYFP (Chapter 3, Section 1.6), RGS4^{S30C}-eYFP co-transfected with $G\alpha_{i1}$ had the greatest percentage effect (188 ± 12%) on RGS4^{S30C}-eYFP translocation to the plasma membrane. The presence of the α_{2A}-adrenoceptor or the α_{2A}-adrenoceptor stimulated by UK14304 (100 μM) both translocated RGS4-eYFP to the plasma membrane, 161 ± 81% and 157 ± 33% respectively. This further confirmed that the RGS4^{S30C} mutation did not affect localisation as the percentage fluorescence of RGS4^{S30C}-eYFP in the plasma membrane was not different from the translocation of RGS4-eYFP.

4.2.6 Importance of residue 30 in RGS4

The presence of serine 30 in RGS4 is clearly important for wild-type functioning of RGS4 and mutating this residue to cysteine increases the activity of this protein. To assess what other amino acid side chains did to the function of this protein, residue 30 was mutated to various other amino acids including*;* alanine, glutamic acid, lysine, methionine, phenylalanine, or proline.

4.2.6.1 Purification of GST-RGS4^{S30X}

All RGS4^{S30X} mutations (where X denotes one of the above amino acids) fused to GST were made by PCR based site-directed mutagenesis. SDS-PAGE gels of induced and purified recombinant GST-fused proteins stained with Coomassie Blue are shown, RGS4^{S30A} (Figure 4.21), RGS4^{S30E} (Figure 4.22), RGS4^{S30K} (Figure 4.33), RGS4^{S30M} (Figure 4.24), RGS4^{S30F} (Figure 4.25), RGS4^{S30P} (Figure 4.26). Similar induction and purification of each GST fused protein is shown. The exact protein concentration of each eluted protein was routinely determined using the BCA assay.

4.2.6.2 Functional activity of RGS4^{S30X}

The ability of GST-RGS4^{S30X} mutants to increase the high-affinity GTPase activity of the α_{2A}-adrenoceptor-$G\alpha_{o1}Cys^{351}Ile$ fusion protein challenged with varying concentrations of adrenaline (1 mM – 0.1 nM) was determined. All assays were done in the absence of RGS protein or addition of purified RGS4 or RGS4^{S30X}: RGS4^{S30A} (Figure 4.27), RGS4^{S30E} (Figure 4.29), RGS4^{S30K} (Figure 4.31), RGS4^{S30M} (Figure 4.33), RGS4^{S30F} (Figure 4.35),

RGS4^{S30P} (Figure 4.37). The maximal adrenaline stimulated GTPase activity of $G\alpha_{o1}$ of each mutant demonstrated that RGS4^{S30A}, RGS4^{S30F} and RGS4^{S30K} all showed a significant increases in high-affinity GTPase activity compared to wild-type RGS4 ($p<0.01$). Addition of purified RGS4^{S30E} had no effect on the maximal adrenaline-stimulated GTPase activity of $G\alpha_{o1}$ compared to wild-type RGS4 and, interestingly, the addition of either RGS4^{S30M} or RGS4^{S30P} both decreased the maximal adrenaline-stimulated high-affinity GTPase activity of $G\alpha_{o1}$ compared to wild-type RGS4.

The ability of these mutants to change the value of V_{max} has also been considered. However, to do this, all high-affinity GTPase graphs were normalised and high-affinity GTPase activity expressed as a percentage. This analysis disguised differences in basal activity and it was thought not to show a true representation of the effect of the mutation on high-affinity GTPase activity.

The potency of adrenaline to stimulate high-affinity GTPase activity of the α_{2A}-adrenoceptor-$G\alpha_{o1}Cys^{351}Ile$ fusion protein was significantly decreased for RGS4^{S30A} compared to wild-type RGS4 (RGS4 pEC_{50} 6.81 ± 0.06, RGS4^{S30A} pEC_{50} 6.36 ± 0.20, $p<0.01$) (Figure 4.28), RGS4^{S30F} (RGS4 pEC_{50} 6.48 ± 0.05, RGS4^{S30A} pEC_{50} 6.11 ± 0.24, $p<0.01$) (Figure 4.36) and RGS4^{S30K} (RGS4 pEC_{50} 6.48 ± 0.05, RGS4^{S30A} pEC_{50} 6.21 ± 0.04, $p<0.01$) (Figure 4.32). See Table 4.1 for summary and statistics. Surprisingly, the addition of purified RGS4^{S30M} to the membranes expressing the α_{2A}-adrenoceptor-$G\alpha_{o1}Cys^{351}Ile$ fusion protein although decreasing the maximum GTPase activity, increased the pEC_{50} (Figure 4.34). In contrast RGS4^{S30P} (Figure 4.38) decreased the GTPase activity of $G\alpha_{o1}$ but did not affect the pEC_{50}. The calculated pEC_{50} values for each assay are shown in Table 4.1. Student's *t* tests were performed to compare the significance of the pEC_{50} values compared to WT RGS4. From these results, RGS4^{S30C} and RGS4^{S30F} ($p<0.05$), RGS4^{S30A}and RGS4^{S30K} ($p<0.005$) were shown to significantly decrease the pEC_{50} value compared to wild-type RGS4. These GOF mutants increase the maximal activity of $G\alpha_{o1}$ and increase the concentration of adrenaline required to produce half maximal GTPase activity.

It should be noted however, that there may be some limitations to the statistical analyis. The experiments performed to produce the date in Table 4.1 for RGS4^{S30K}, RGS4^{S30FM}, RGS4^{S30F} and RGS4^{S30P} were done using the same wild type controls. The data presented here may not be as significantly different, as reported here.

4.2.6.3 Expression of RGS4^{S30X} –eYFP

All RGS4^{S30X} mutations were C-terminally tagged with eYFP in pcDNA3. HEK293T cells were transfected with constructs containing RGS4-eYFP, RGS4^{S30A}-eYFP or RGS4^{S30E}-eYFP. To determine expression levels, cell lysates were separated by SDS-PAGE and detected using an antiserum directed against GFP. Relative expressions of RGS4-eYFP mutants (lane 3) were compared to RGS4-eYFP (lane 2) and vector only control (lane 1). RGS4^{S30A} (Figure 4.39), RGS4^{S30E} (Figure 4.40) show bands equal intensity of mutant RGS4 and wild-type RGS4 indicating equivalent expression of the two proteins.

4.2.6.4 Localisation of RGS4^{S30X} –eYFP

To determine the cellular localisation of RGS4^{S30X} of HEK293T cells transiently expressing RGS4^{S30A}-eYFP (Figure 4.41), RGS4^{S30E}-eYFP (Figure 4.42), RGS4^{S30K}-eYFP (Figure 4.43), RGS4^{S30M}-eYFP (Figure 4.44), RGS4^{S30F}-eYFP (Figure 4.45), RGS4^{S30P}-eYFP (Figure 4.46) were visualised by microscopy. Panel (A) shows all RGS4^{S30X}-eYFP mutants were localised predominantly within the cytoplasm. As before, the intracellular localisation of all mutants was examined by co-expression of (B) $G\alpha_{i1}$, (C) α_{2A}-adrenoceptor or (D) α_{2A}-adrenoceptor and stimulated with UK14304 for 30 minutes. The yellow co-localisation of RGS4^{S30X}-eYFP with WGA-Alexa Fluor 594-labelled membrane staining in image (iv) in panels (B), (C) and (D) suggest that co-expression with other signalling proteins translocates all RGS4^{S30X}-eYFP to the plasma membrane. It was demonstrated that all of the tested RGS4^{S30X} mutations did not vary the cellular localisation of the protein compared to wild-type RGS4.

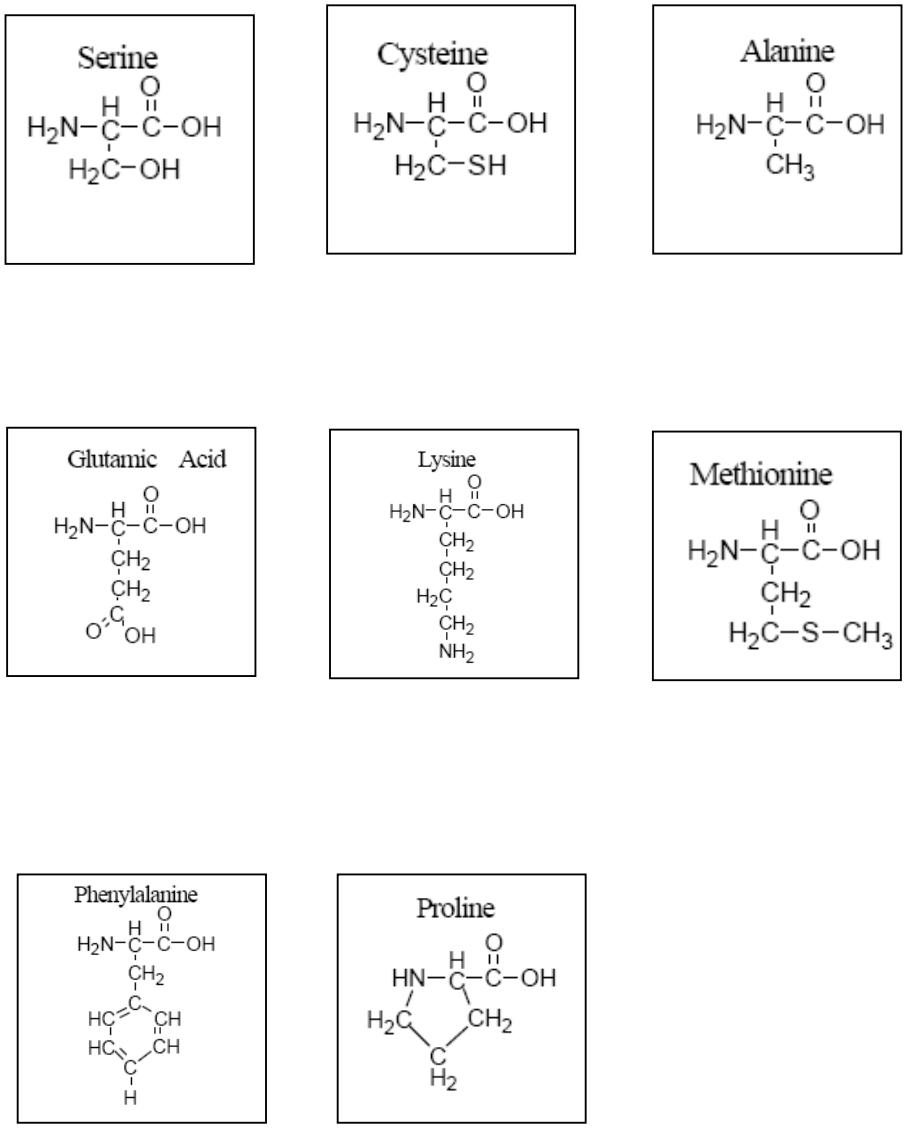
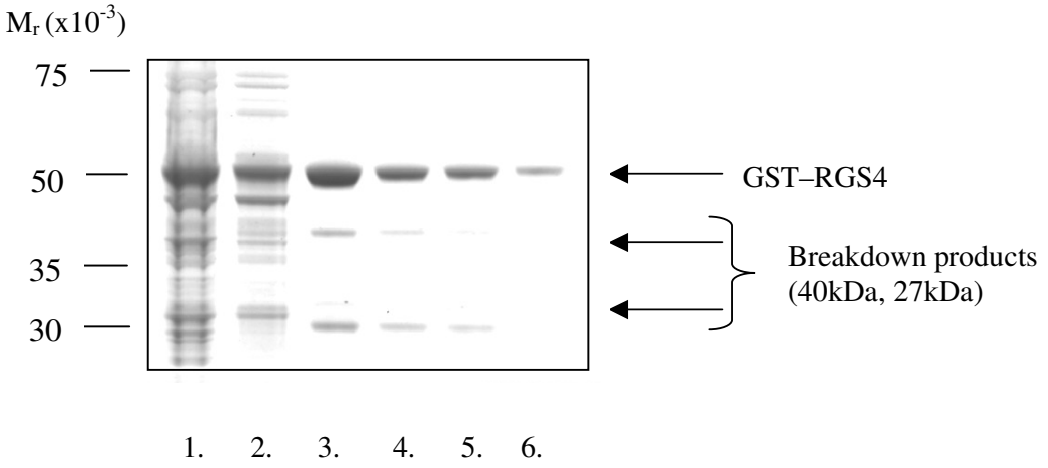


Figure 4.1 Amino acids selected for substitution of serine 30 in RGS4

Serine 30 in RGS4 was selected for site directed mutagenesis. An amino acid with side chain (X) is shown where X is serine, cysteine, alanine, glutamic acid, lysine, methionine, phenylalanine or proline.

(A)



(B)

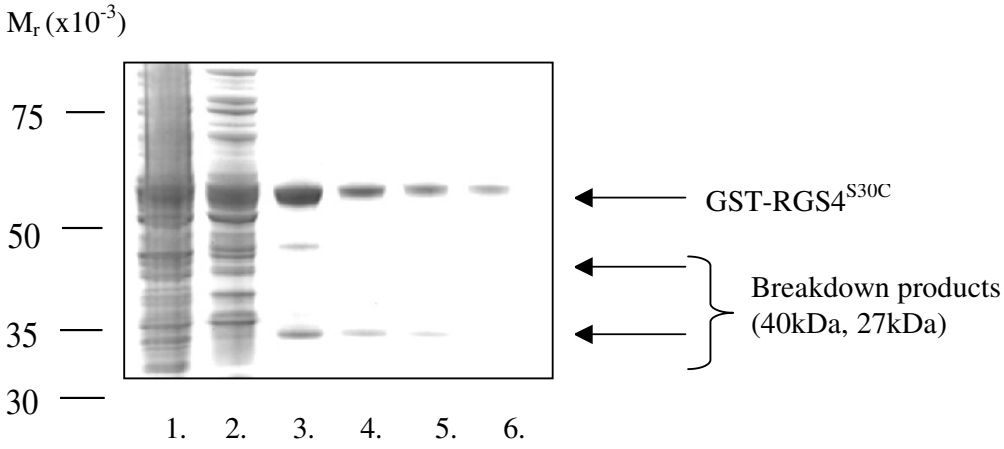


Figure 4.2 Coomassie Blue staining of purified GST-RGS4 and GST-RGS4^{S30C}

BL21 bacteria were transformed with the plasmid pGEX 6P-1 encoding (A) GST-RGS4 or (B) GST-RGS4^{S30C}. GST-fused proteins were induced and purified and equal volumes of each fraction were resolved by SDS-PAGE and stained with Coomassie Blue. Cell lysates of BL21 before addition of glutathione sepharose beads (lane 1), cell lysates of BL21after over-night incubation with glutathione sepharose beads (lane 2), elution 1 (lane 3), elution 2 (lane 4), elution 3 (lane 5), elution 4 (lane 6).

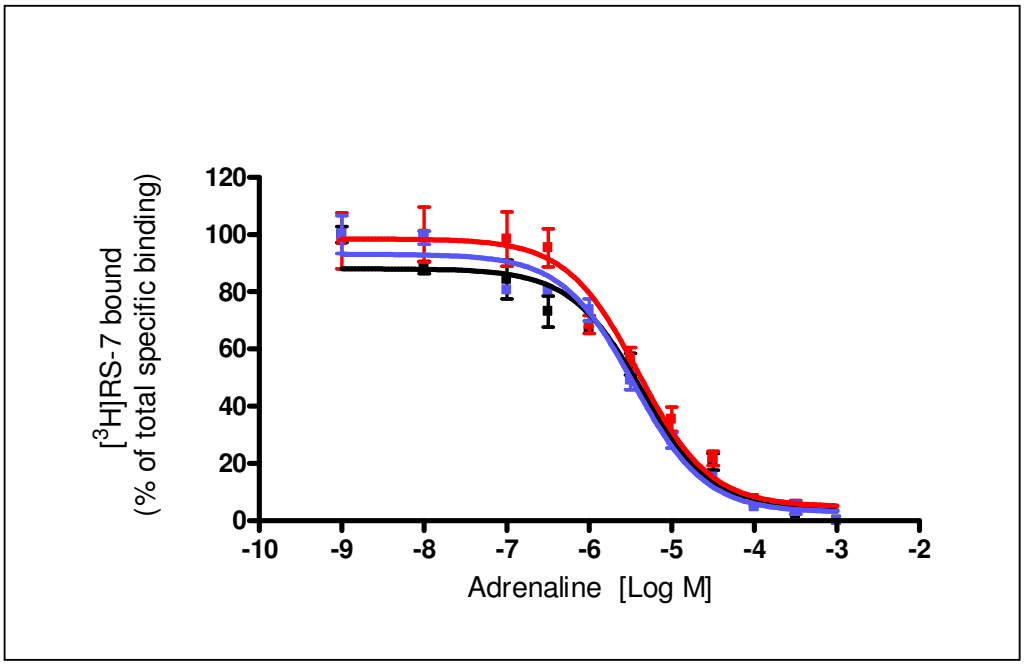


Figure 4.3 The ability of adrenaline to compete with [^{3}H]RS-79948-197 for binding to the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein in the presence and absence of RGS4 or RGS4^{S30C}

HEK293T cells were transfected to express the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein. Cells were treated with P. tox (25 ng/ml for 16 hours) then membranes were prepared. The ability of adrenaline (1 mM - 1 nM) to compete with 0.5 nM [^{3}H]RS-79948-197 was determined in the absence (black) or presence of 1 µM GST-RGS4 (red) or 1 µM GST-RGS4^{S30C} (blue). Data shown are from triplicate determinations (mean ± SEM) and are representative of three individual experiments performed**.**

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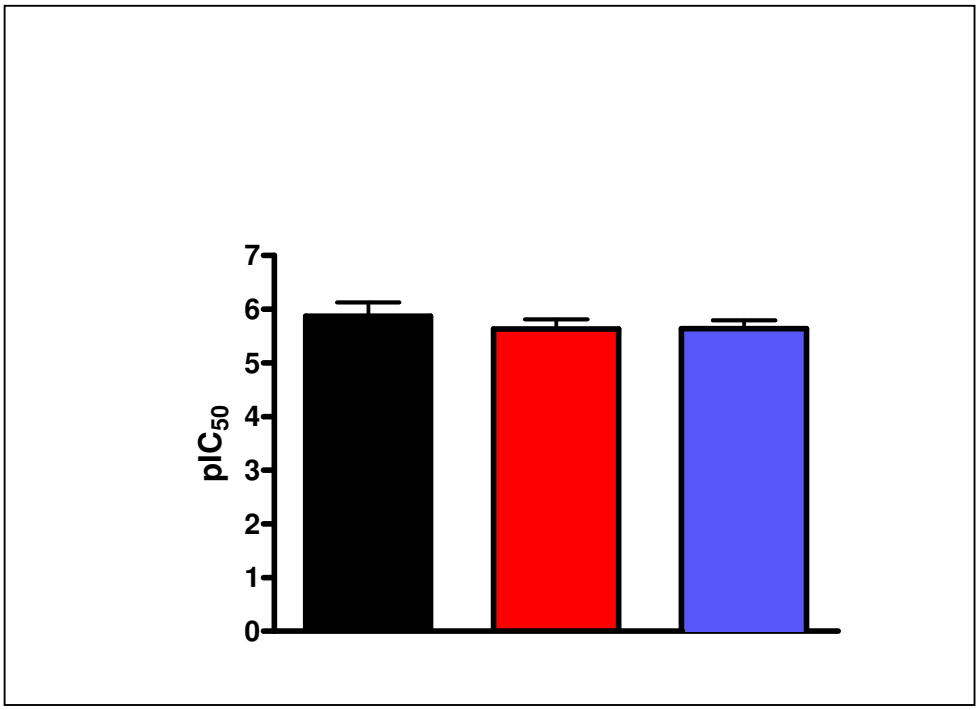


Figure 4.4 Comparison of GST-RGS4 or GST-RGS4^{S30C} on the ability of adrenaline to compete with [^{3}H]RS-79948-197 for binding to the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein

The ability of adrenaline to compete with 0.5 nM [^{3}H]RS-79948-197 was determined in the absence (black) or presence of 1 µM GST-RGS4 (red) or 1 µM GST-RGS4^{S30C} (blue). The IC_{50} results from Figure 4.3 are presented for comparison of results. Results shown are mean ± SEM. from three individual experiments performed.

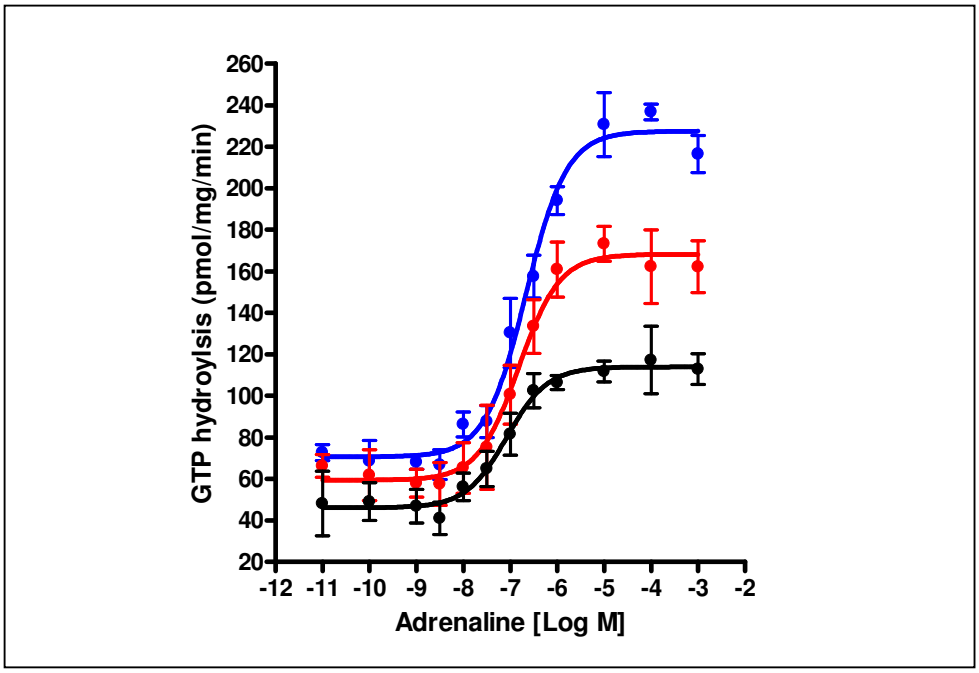


Figure 4.5 Adrenaline-stimulated GTPase activity of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein in the presence and absence of GST-RGS4 or GST-RGS4^{S30C}

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein. Cells were treated with P. tox (25 ng/ml for 16 hours) and membranes were prepared. The capacity of varying concentrations of adrenaline (0.1 nM – 1 mM) to stimulate high-affinity GTPase activity was then measured in the absence (black) or presence of 1 µM GST-RGS4 (red) or 1 µM GST-RGS4^{S30C} (blue) using 0.5 µM [γ^{32}P]GTP. Data shown are from quadruplicate determinations (mean ± SEM) and are representative of three individual experiments performed**.**

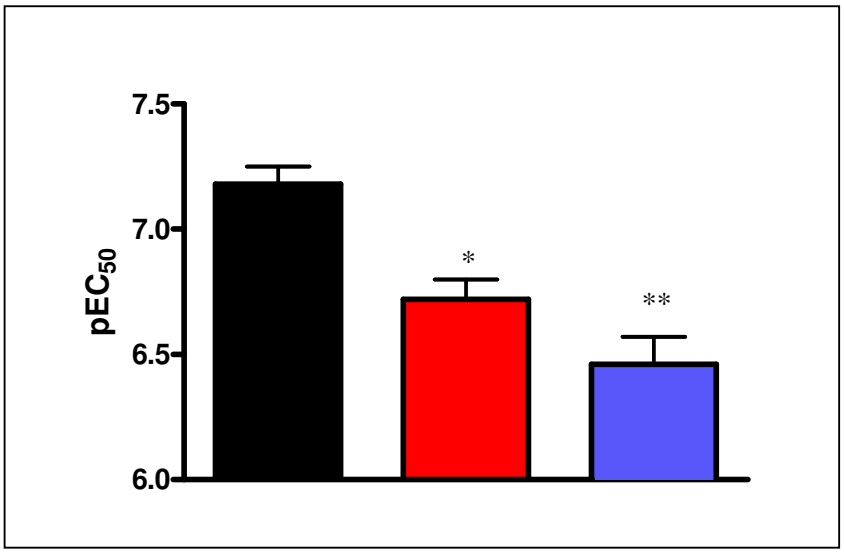


Figure 4.6 Comparison of GST-RGS4 or GST-RGS4^{S30C} on pEC_{50} values of adrenaline to stimulate the GTPase activity of the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein

The pEC_{50} results from Figure 4.5 are presented for comparison. The pEC_{50} in the absence of RGS protein (black), presence of GST-RGS4 (red) or presence of GST-RGS4^{S30C} (blue) are mean ± SEM. from three individual experiments performed. * denotes significantly different from the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein and ** denotes significantly different from the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein + 1 µM GST-RGS4 ($p<0.01$).

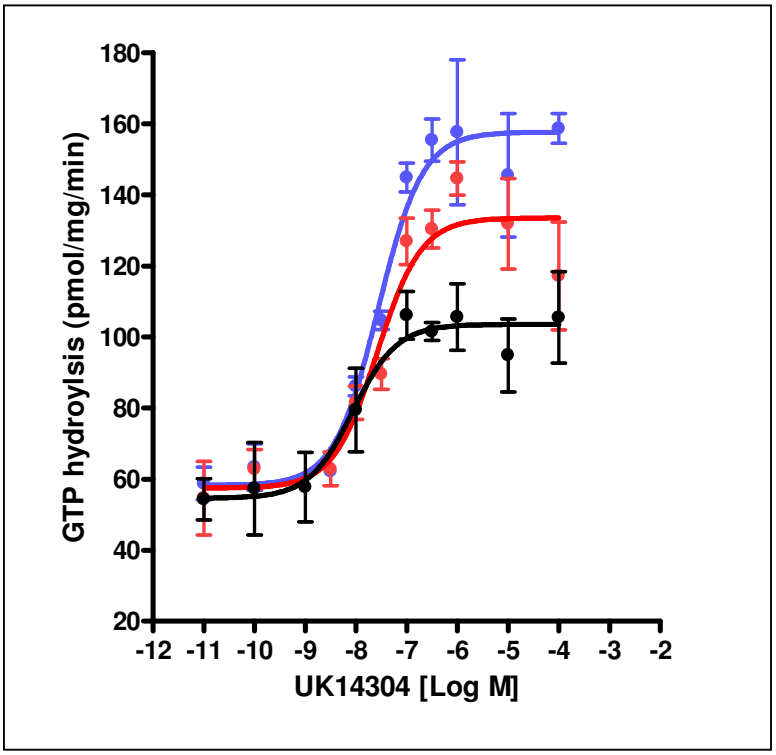


Figure 4.7 UK14304-stimulated GTPase activity of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein in the presence and absence of GST-RGS4 or GST-RGS4^{S30C}

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein. Cells were treated with P. tox (25 ng/ml for 16 hours) and membranes were prepared. The capacity of varying concentrations of UK14304 to stimulate high-affinity GTPase activity was then measured in the absence (black) and presence of 1 µM GST-RGS4 (red) or 1 µM GST-RGS4^{S30C} (blue) using 0.5 µM [γ^{32}P]GTP. Data shown are from quadruplicate determinations (mean ± SEM) and are representative of three individual experiments performed.

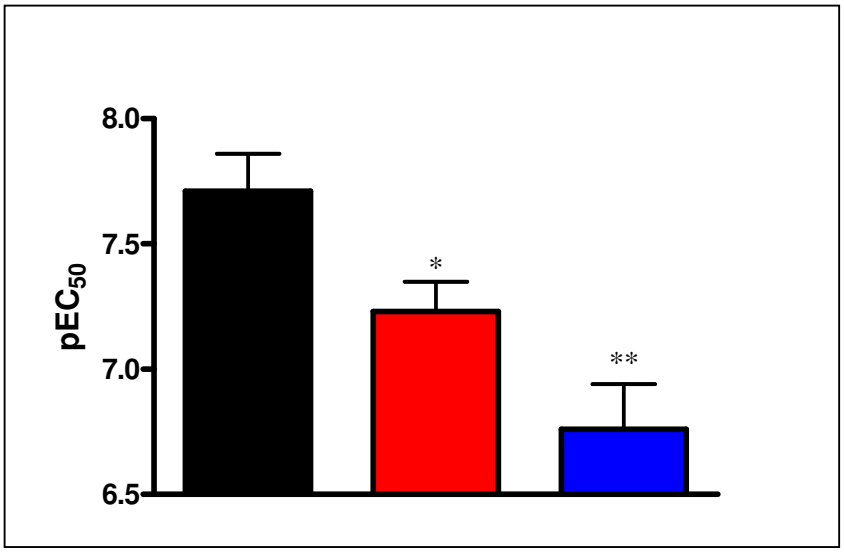


Figure 4.8 Comparison of GST-RGS4 or GST-RGS4^{S30C} on pEC_{50} values of UK14304 to stimulate the GTPase activity of the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein

The pEC_{50} results from Figure 4.7 are presented for comparison. The pEC_{50} in the absence of RGS protein (black), presence of GST-RGS4 (red) or presence of GST-RGS4^{S30C} (blue) are mean ± SEM. from three individual experiments performed. * denotes significantly different from the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein and ** denotes significantly different from the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein + 1 µM GST-RGS4 ($p<0.01$).

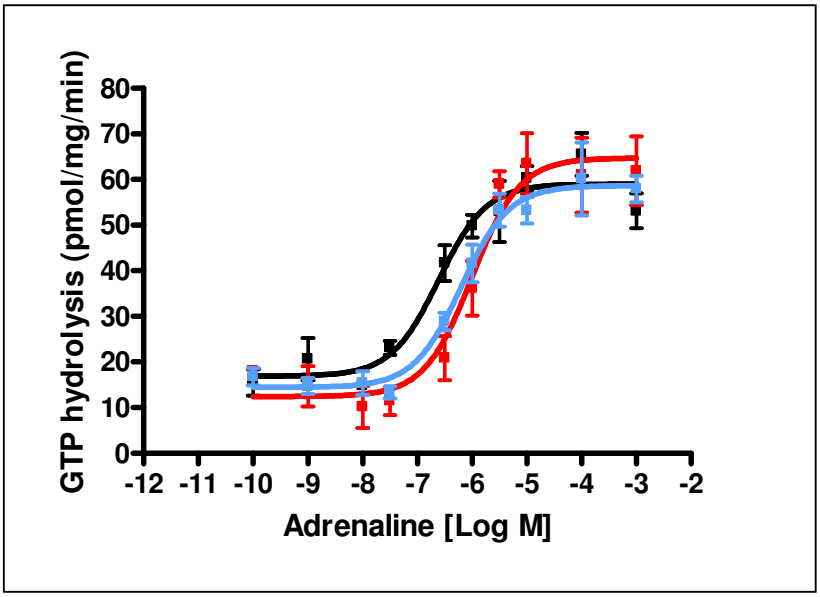


Figure 4.9 Adrenaline-stimulated GTPase activity of the α_{2A}-adrenoceptor-$G\alpha_{i1}^{C351I}$ fusion protein in the presence and absence of GST-RGS4 or GST-RGS4^{S30C}

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-$G\alpha_{i1}^{C351I}$ fusion protein. Cells were treated with P. tox (25 ng/ml for 16 hours) and membranes were prepared. The capacity of varying concentrations of adrenaline to stimulate high-affinity GTPase activity was then measured in the absence (black) or presence of 100 nM GST-RGS4 (red) or 100 nM GST-RGS4^{S30C} (blue) using 0.5 µM [γ^{32}P]GTP. Data shown are from quadruplicate determinations (mean ± SEM) and are representative of three individual experiments performed**.**

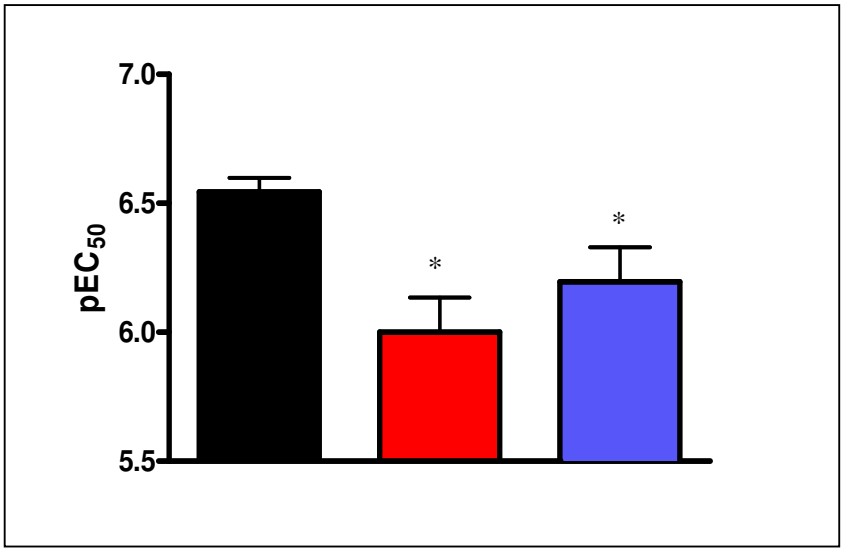


Figure 4.10 Comparison of GST-RGS4 or GST-RGS4^{S30C} on pEC_{50} values of adrenaline to stimulate the GTPase activity of the α_{2A}-adrenoceptor-Gα_{i1}^{C351I} fusion protein

The pEC_{50} results from Figure 4.9 are presented for comparison. The pEC_{50} in the absence of RGS protein (black), presence of GST-RGS4 (red) or presence of GST-RGS4^{S30C} (blue) are mean ± SEM. from three individual experiments. Results are mean ± SEM. for three individual experiments performed. * denotes significantly different from the α_{2A}-adrenoceptor-Gα_{i1}Cys^{351}Ile fusion protein ($p<0.01$).

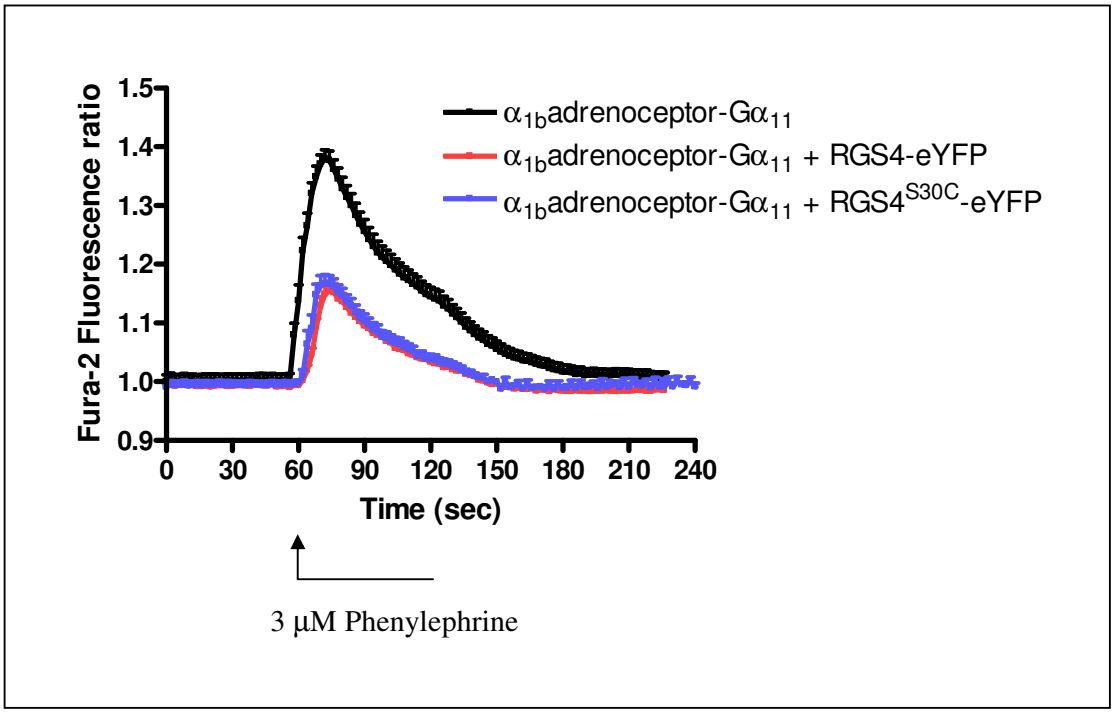


Figure 4.11 Phenylephrine-stimulated elevation of $[Ca^{2+}]_i$ in cells expressing the α_{1b}-adrenoceptor-Gα_{11} fusion protein, in the presence and absence of RGS4-eYFP RGS4^{S30C}-eYFP

HEK293T cells were transfected to express the α_{1b}-adrenoceptor-Gα_{11} fusion protein (black line), α_{1b}-adrenoceptor-Gα_{11} fusion protein + RGS4-eYFP (red line) or α_{1b}-adrenoceptor-Gα_{11} fusion protein + RGS4^{S30C}-eYFP (blue line). Cells were loaded with Fura-2 and intracellular $[Ca^{2+}]$ levels imaged before and after 3 µM phenylephrine was perfused over the cells for 60 seconds. Data represents means ± SEM. from 54 α_{1b}-adrenoceptor-Gα_{11}), 25 α_{1b}-adrenoceptor-Gα_{11} + RGS4-eYFP) and 23 α_{1b}-adrenoceptor-Gα_{11} + RGS4^{S30C}-eYFP) cells from 3 individual experiments performed.

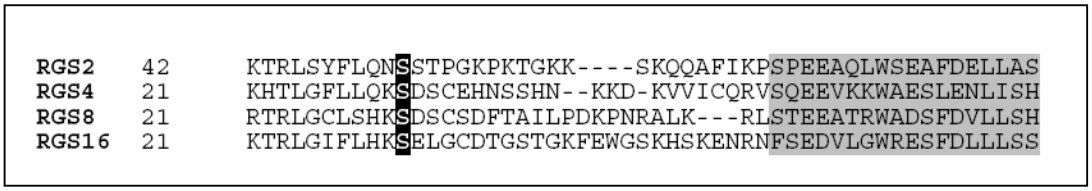


Figure 4.12 Comparison of serine 30 in the B/R4 subfamily of RGS proteins

The serine 30 residue in RGS4 is conserved amongst members of the B/R4 family. The N-terminal region of the protein sequence of four members of the B/R4 family of RGS proteins is compared. Amino acid sequences of RGS2, RGS4, RGS8 and RGS16 are denoted using single-letter code. Sequences were aligned using MultAlin version 5.4.1. with symbol comparison table blosum62, gap weight 12 and gap length weight 2 (Corpet, 1988). Gaps introduced to maximise alignment are indicated by -. The conserved serine residue is highlighted in black and the start of the predicted RGS domain is highlighted in grey.

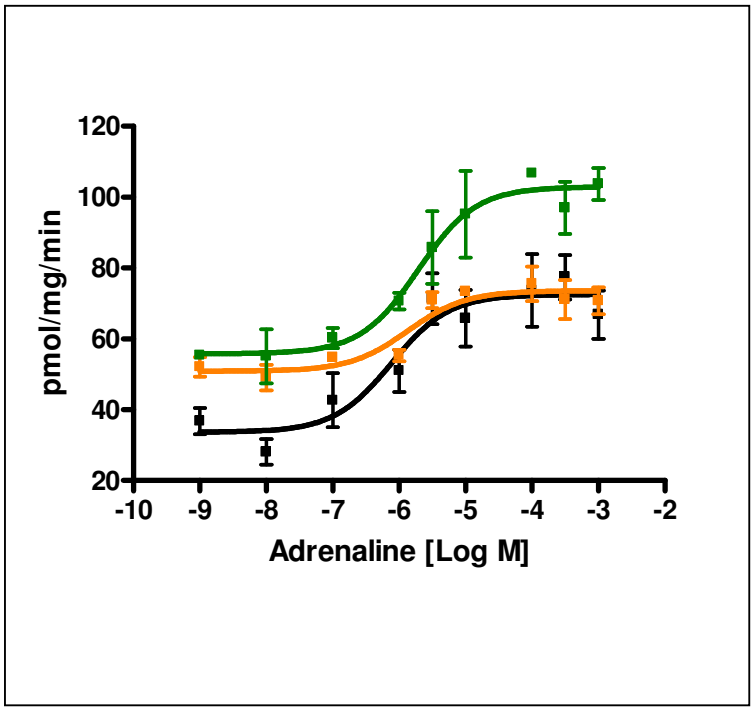


Figure 4.13 Adrenaline-stimulated GTPase activity of the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein in the presence and absence of GST-RGS16 or GST-RGS16^{S30C}

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein. Cells were treated with P. tox (25 ng/ml for 16 hours) and membranes were prepared. The capacity of varying concentrations of adrenaline to stimulate high-affinity GTPase activity was then measured in the absence (black) or presence of 100 nM GST-RGS16 (orange) or 100 nM GST-RGS16^{S30C} (green) using 0.5 µM [γ^{32}P]GTP. Data shown are from quadruplicate determinations (mean ± SEM) and are representative of three individual experiments performed.

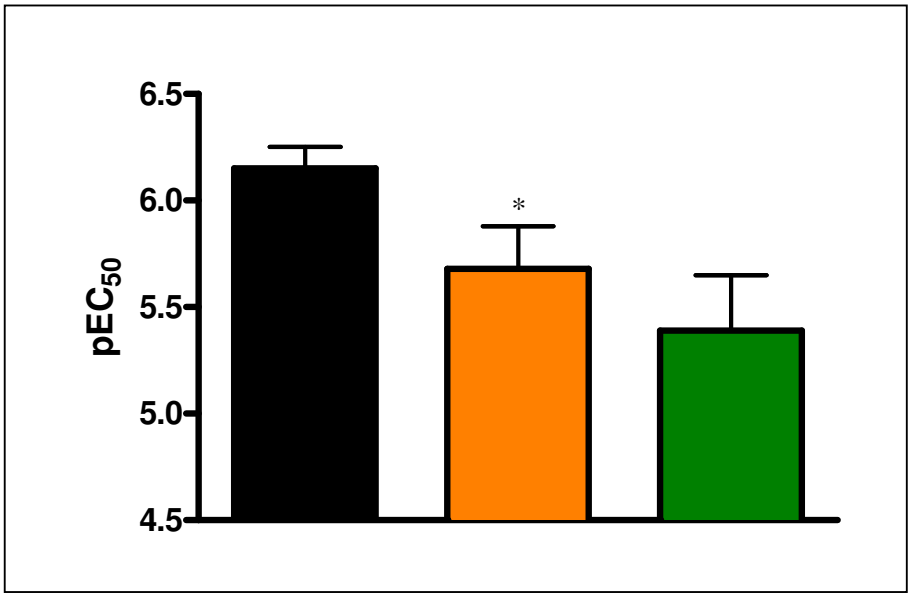


Figure 4.14 Comparison of GST-RGS16 or GST-RGS16^{S30C} on pEC_{50} values of adrenaline to stimulate the GTPase activity of the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein

The pEC_{50} results from Figure 4.13 are presented for comparison of results. The pEC_{50} in the absence (black) or presence of 100 nM GST-RGS16 (orange) or 100 nM GST-RGS4^{S30C} (green) are mean ± SEM. from three individual experiments performed. * denotes significantly different from the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein and ** denotes significantly different from the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein + 1 μM GST-RGS16 ($p<0.01$).

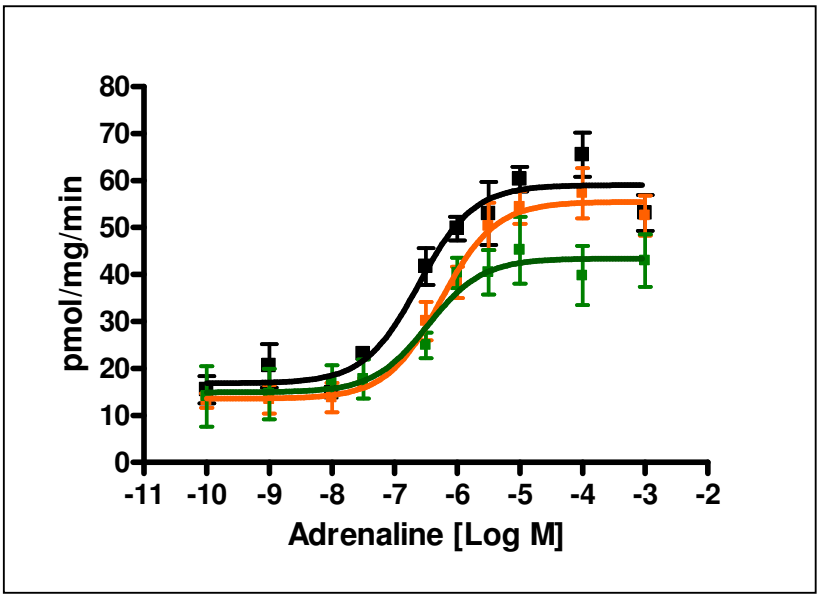


Figure 4.15 Adrenaline-stimulated GTPase activity of the α_{2A}-adrenoceptor-$G\alpha_{i1}^{C351I}$ fusion protein in the absence and presence of GST-RGS16 or GST-$RGS16^{S30C}$

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-$G\alpha_{i1}^{C351I}$ fusion protein. Cells were treated with P. tox (25 ng/ml for 16 hours) and membranes were prepared. The capacity of varying concentrations of adrenaline to stimulate high-affinity GTPase activity was then measured in the absence (black) or presence of 100 nM GST-RGS16 (orange) or 100 nM GST-$RGS16^{S30C}$ (green) using 0.5 µM [γ^{32}P]GTP. Data shown are from quadruplicate determinations (mean ± SEM) and are representative of three individual experiments performed.

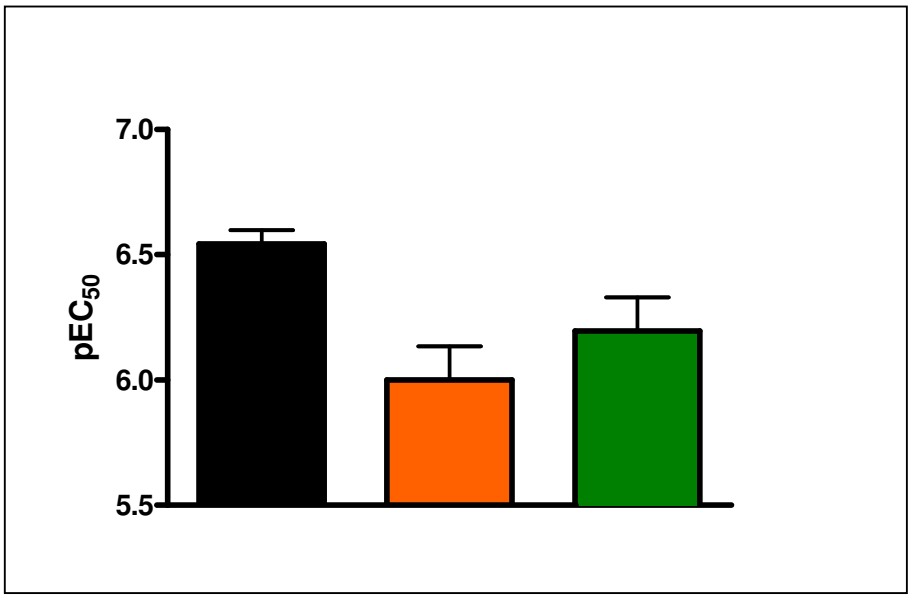


Figure 4.16 Comparison of GST-RGS16 or GST-RGS16^{S30C} on pEC_{50} values of adreanline to stimulate the GTPase activity of the α_{2A}-adrenoceptor-Gα_{i1}^{C351I} fusion protein

The pEC_{50} results from Figure 4.15 are presented for comparison of results. The pEC_{50} in the absence (balck) or presence of 100 nM GST-RGS16 (orange) or 100 nM GST-RGS16^{S30C} (green) are mean ± SEM. for three individual experiments performed.

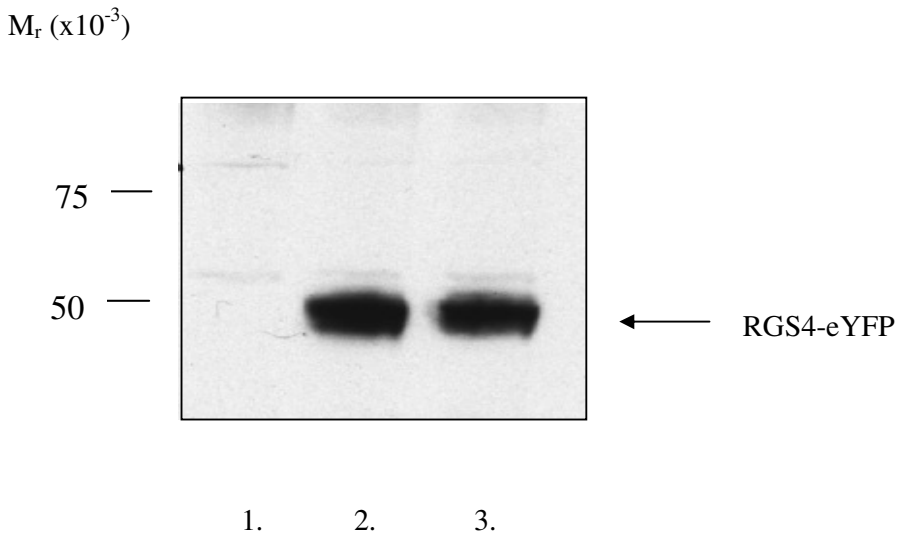
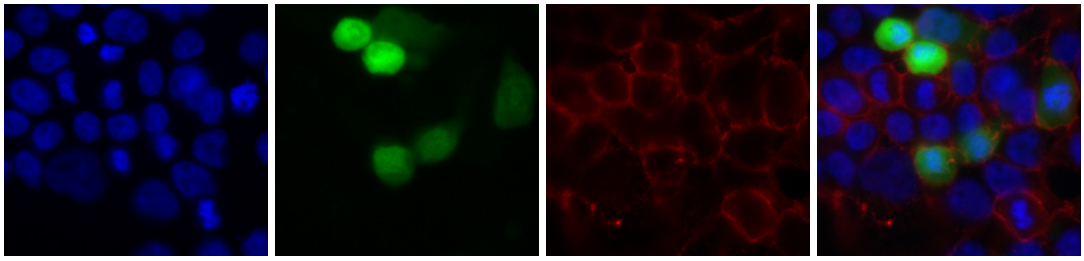


Figure 4.17 Expression and immunological detection of RGS4-eYFP and RGS4^{S30C}-eYFP

HEK293T cells were transiently transfected to express RGS4-eYFP (lane 2) or RGS4^{S30C}-eYFP (lane 3). HEK293T cells transfected with pcDNA3 were included as a control (lane 1). Cell lysates were resolved by SDS-PAGE and then immunoblotted with anti-GFP antiserum. Results shown are of a single experiment and are representative of three experiments performed.

(A) (i) (ii) (iii) (iv)



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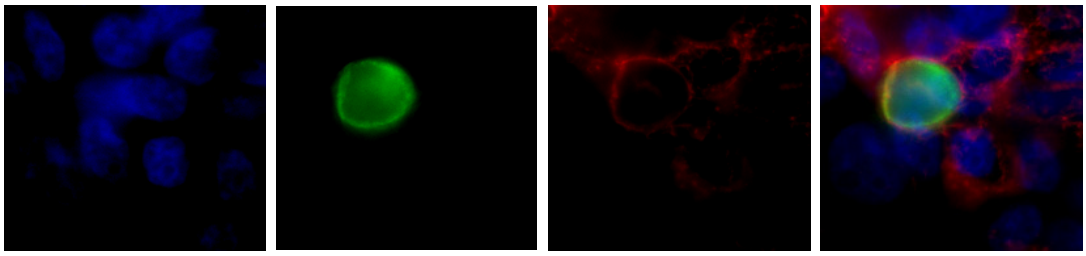

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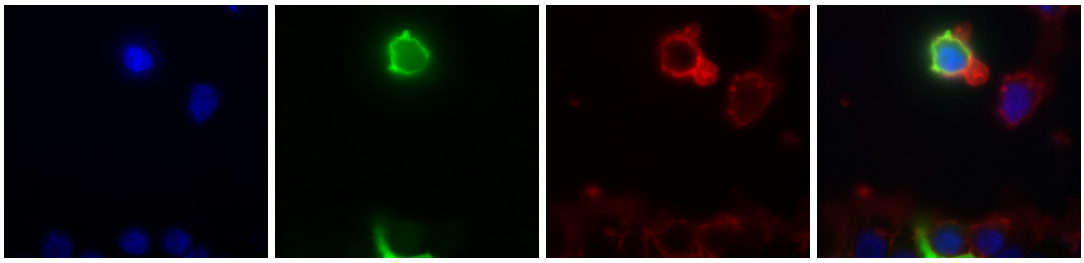

(D) (i) (ii) (iii) (iv)

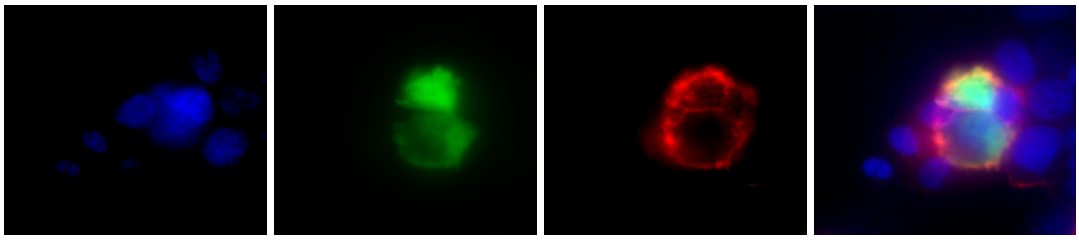

Figure 4.18 The cellular localisation of RGS4^{S30C}-eYFP

HEK293T cells grown on coverslips were transiently transfected with (A) RGS4^{S30C}-eYFP (B) RGS4^{S30C}-eYFP co-transfected with $G\alpha_{i1}$ (C) RGS4^{S30C}-eYFP co-transfected with the α_{2A}-adrenoceptor or (D) RGS4^{S30C}-eYFP co-transfected with the α_{2A}-adrenoceptor and stimulated with 100 μM UK14304 for 30 minutes. Images were generated using an inverted Nikon TE2000-E microscope equipped with a 60 x, (NA=1.4), oil-immersion Plan Fluor Apochromat lens and a cooled digital Cool Snap-HQ CCD camera. (i) Hoechst 33342 nuclei staining (blue) (ii) RGS4^{S30C}-eYFP (green) or (iii) WGA-Alexa Fluor 594 membrane staining (red) (iv) merged images. Results shown are of a single experiment and are representative of three experiments performed.

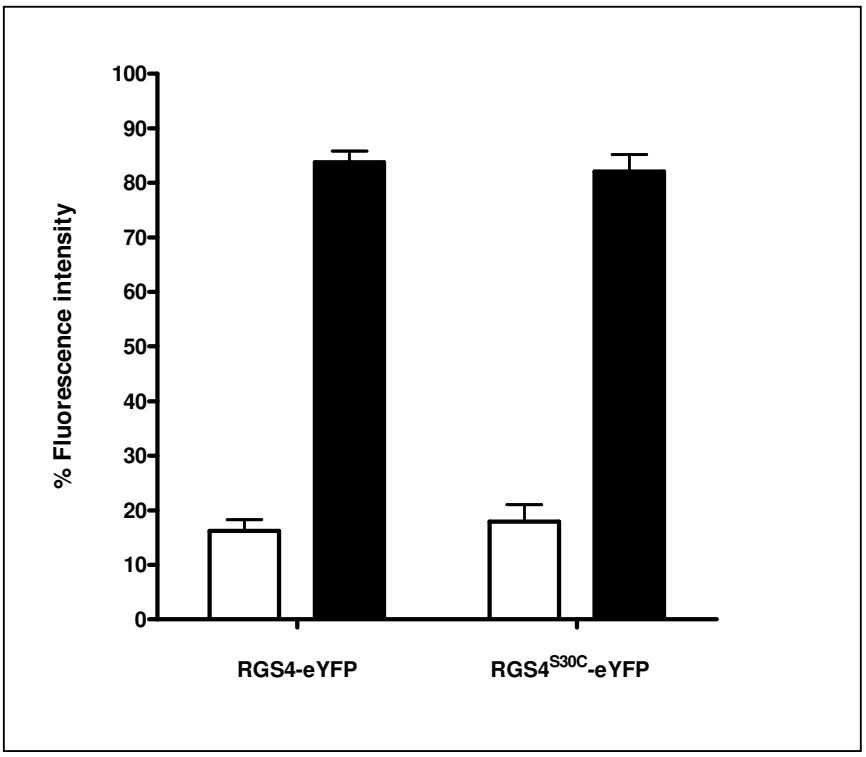

Figure 4.19 Percentage plasma membrane and cytosolic localisation of RGS4^{S30C}-eYFP fluorescence

HEK293T transfected cells were examined for RGS4-eYFP fluorescence or RGS4^{S30C}-eYFP intensity at the plasma membrane (open bars) and in the cytoplasm (closed bars). Images were deconvolved using an iterative and constrained algorithm (Chapter 2, Section 2.7.5). Surface and cytosolic masks were created and superimposed and fluorescence pixel intensity values corresponding to eYFP located at the plasma membrane and cytoplasm of the cell were quantified from each generated mask. Fluorescence was expressed as a percentage of the total fluorescence intensity using a mean of three cells ± SEM.

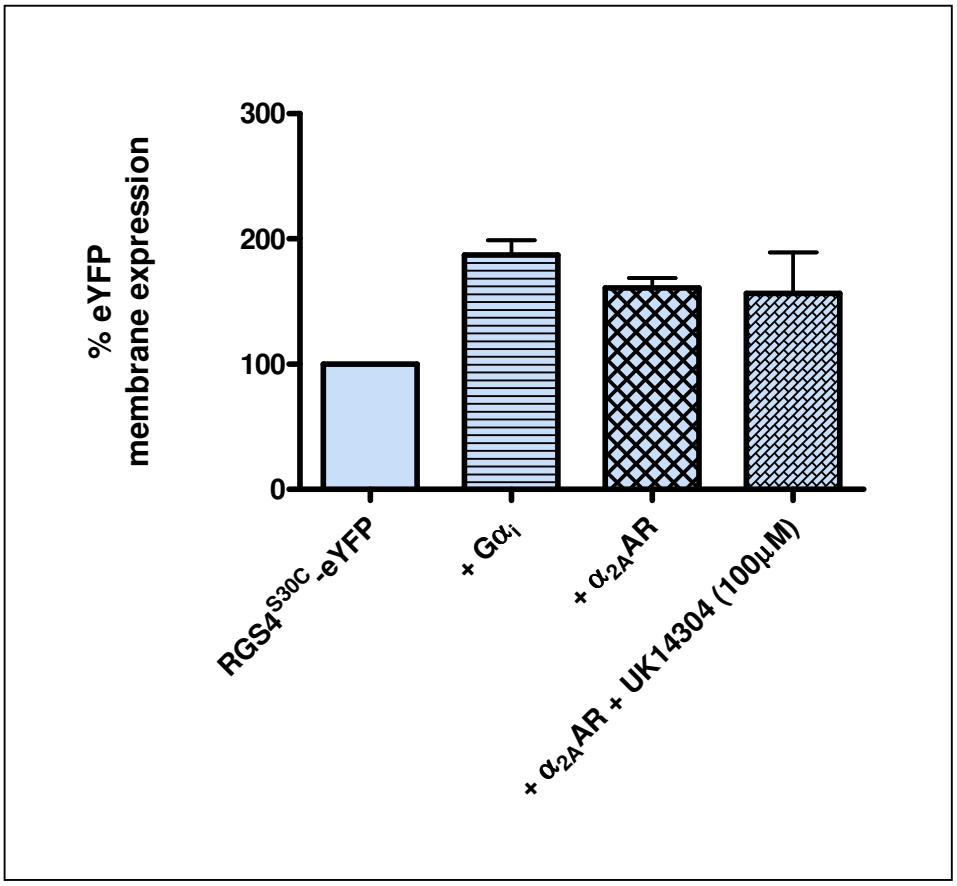

Figure 4.20 Comparison of percentage RGS4-eYFP ^{S30C} fluorescence at the plasma membrane

HEK293T cells were transiently transfected to express RGS4^{S30C}-eYFP, RGS4^{S30C}-eYFP co-transfected with Gα_{i1}, RGS4^{S30C}-eYFP co-transfected with the α_{2A}-adrenoceptor or RGS4^{S30C}-eYFP co-transfected with the α_{2A}-adrenoceptor and stimulated with 100 μM UK14304 for 30 minutes. The membrane fraction was measured for total RGS4^{S30C}-eYFP (defined as 100%) and compared to the percentage eYFP fluorescence of RGS4^{S30C}-eYFP co-expressed with other signalling proteins. Data shown are from triplicate determinations (mean ± SEM.) and are representative of three experiments performed.

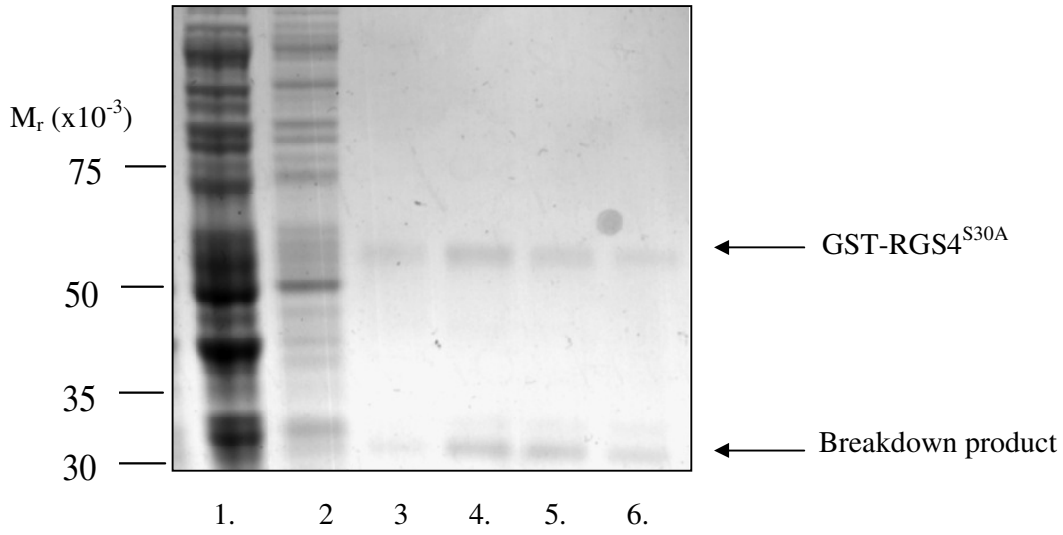

Figure 4.21 Coomassie Blue staining for purified GST-RGS4^{S30A}

BL21 bacteria were transformed with the plasmid pGEX 6P-1 encoding GST-RGS4^{S30A}. GST-fused protein was induced and purified and equal volumes of each fraction were resolved by SDS-PAGE and stained with Coomassie Blue. Induced cell lysates of BL21bacteria before addition of glutathione sepharose beads (lane 1), induced cell lysates of BL21after incubation with glutathione sepharose beads (lane 2), elution 1 (lane 3), elution 2 (lane 4), elution 3 (lane 5), elution 4 (lane 6).

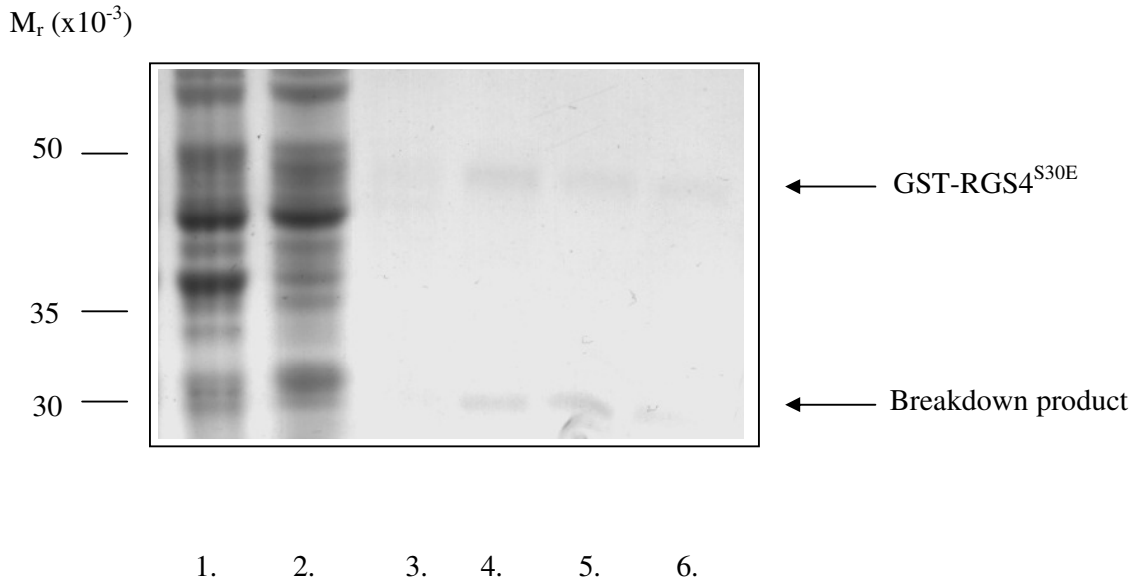

Figure 4.22 Coomassie Blue staining for purified GST-RGS4^{S30E}

BL21 bacteria were transformed with the plasmid pGEX 6P-1 encoding GST-RGS4^{S30E}. GST-fused protein was induced and purified and equal volumes of each fraction were resolved by SDS-PAGE and stained with Coomassie Blue. Induced soluble clarified extract of BL21 bacteria before addition of glutathione sepharose beads (lane 1), induced soluble clarified extract of BL21 bacteria after incubation with glutathione sepharose beads (lane 2), elution 1 (lane 3), elution 2 (lane 4), elution 3 (lane 5), elution 4 (lane 6).

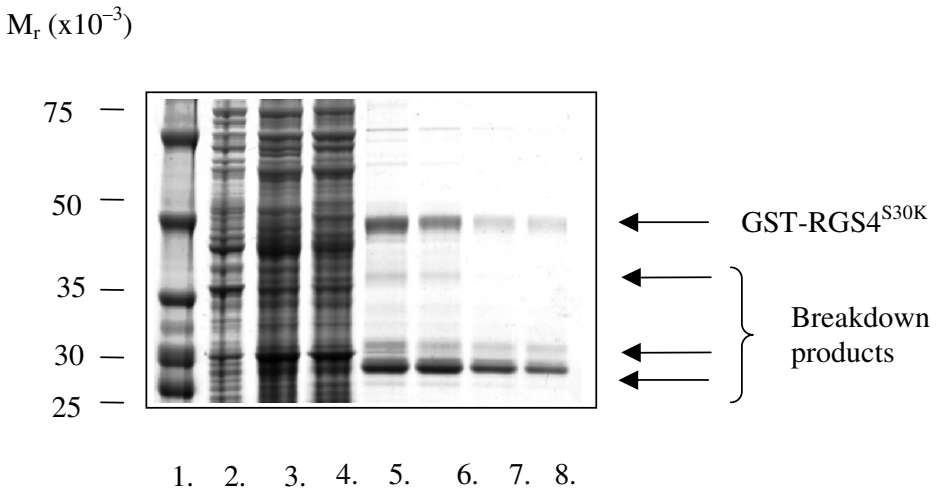

Figure 4.23 Coomassie Blue staining for purified GST-RGS4^{S30K}

BL21 bacteria were transformed with the plasmid pGEX 6P-1 encoding GST-RGS4^{S30K}. GST-fused protein was induced and purified and equal volumes of each fraction were resolved by SDS-PAGE and stained with Coomassie Blue Marker (lane 1), crude bacterial lysate (lane 2), soluble clarified extract before incubation with glutathione sepharose beads (lane 3), soluble clarified extract after incubation with glutathione sepharose beads (lane 4), elution 1 (lane 5), elution 2 (lane 6), elution 3 (lane 7), elution 4 (lane 8).

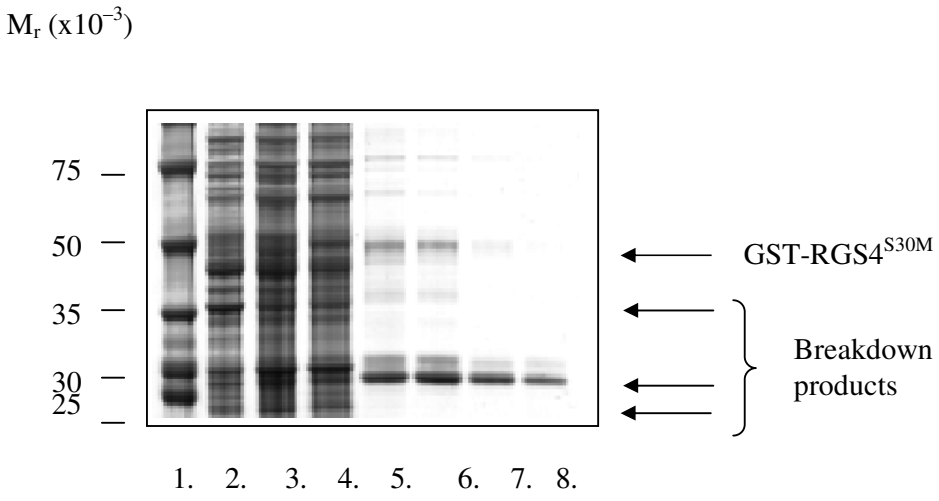

Figure 4.24 Coomassie Blue staining for purified GST-RGS4^{S30M}

BL21 bacteria were transformed with the plasmid pGEX 6P-1 encoding GST-RGS4^{S30M}. GST-fused protein was induced and purified and equal volumes of each fraction were resolved by SDS-PAGE and stained with Coomassie Blue Marker (lane 1), crude bacterial lysate (lane 2), soluble clarified extract before incubation with glutathione sepharose beads (lane 3), soluble clarified extract after incubation with glutathione sepharose beads (lane 4), elution 1 (lane 5), elution 2 (lane 6), elution 3 (lane 7), elution 4 (lane 8).

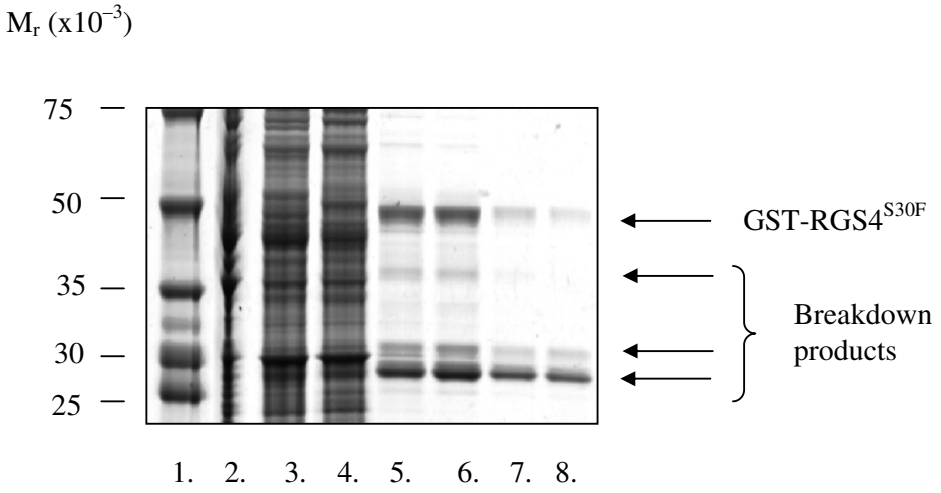

Figure 4.25 Coomassie Blue staining for purified GST-RGS4^{S30F}

BL21 bacteria were transformed with the plasmid pGEX 6P-1 encoding GST-RGS4^{S30F}. GST-fused protein was induced and purified and equal volumes of each fraction were resolved by SDS-PAGE and stained with Coomassie Blue Marker (lane 1), crude bacterial lysate (lane 2), soluble clarified extract before incubation with glutathione sepharose beads (lane 3), soluble clarified extract after incubation with glutathione sepharose beads (lane 4), elution 1 (lane 5), elution 2 (lane 6), elution 3 (lane 7), elution 4 (lane 8).

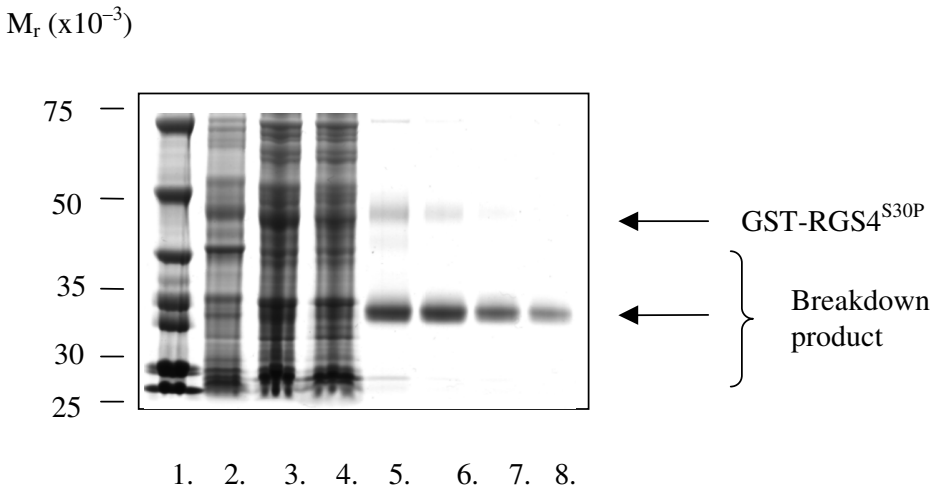

Figure 4.26 Coomassie Blue staining for purified GST-RGS4^{S30P}

BL21 bacteria were transformed with the plasmid pGEX 6P-1 encoding GST-RGS4^{S30P}. GST-fused protein was induced and purified and equal volumes of each fraction were resolved by SDS-PAGE and stained with Coomassie Blue Marker (lane 1), crude bacterial lysate (lane 2), soluble clarified extract before incubation with glutathione sepharose beads (lane 3), soluble clarified extract after incubation with glutathione sepharose beads (lane 4), elution 1 (lane 5), elution 2 (lane 6), elution 3 (lane 7), elution 4 (lane 8).

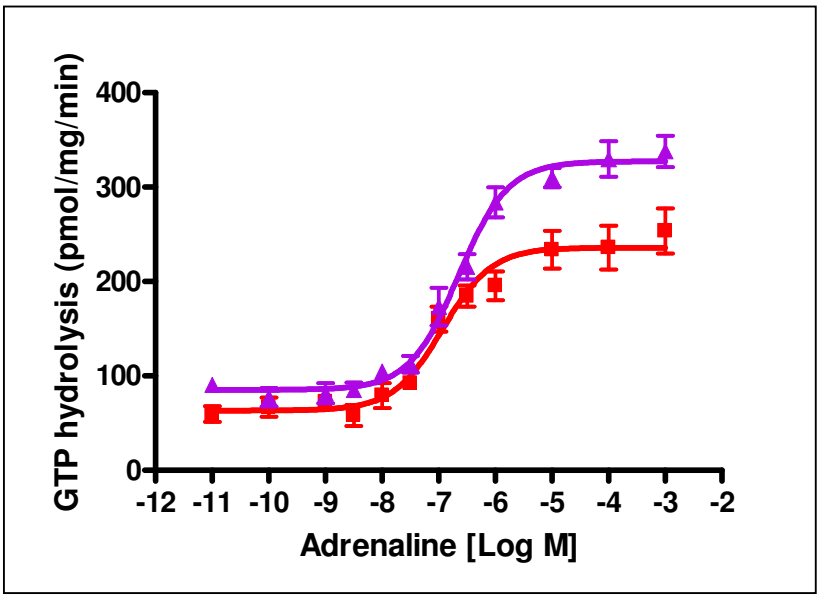

Figure 4.27 Adrenaline-stimulated GTPase activity of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein in the presence of GST-RGS4 or GST-RGS4^{S30A}

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein. Cells were treated with P. tox (25 ng/ml for 16 hours) and membranes were prepared. The capacity of varying concentrations of adrenaline to stimulate high-affinity GTPase activity was then measured in presence of 1 µM GST-RGS4 (red) or 1 µM GST-RGS4^{S30A} (purple) using 0.5 µM [γ^{32}P]GTP. Data shown are from quadruplicate determinations (mean ± SEM) and are representative of three individual experiments performed.

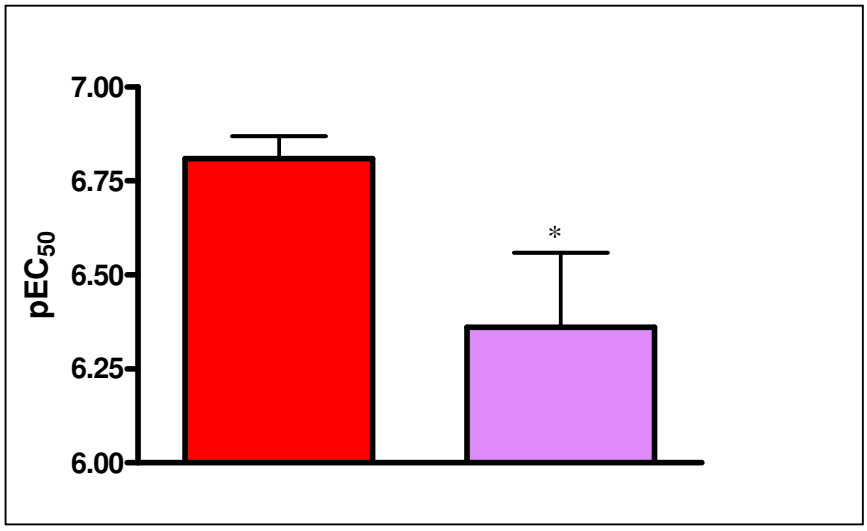

Figure 4.28 Comparison of pEC_{50} values of adrenaline to stimulate the GTPase activity of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein in the presence of GST-$RGS4^{S30A}$

The results from Figure 4.27 are presented in graphical form for comparison of results. The pEC_{50} in the presence of GST-RGS4 (red) or presence of GST-$RGS4^{S30A}$ (purple) are mean ± SEM. from three individual experiments performed. * denotes significantly different from the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein + 1 µM GST-RGS4 ($p<0.05$).

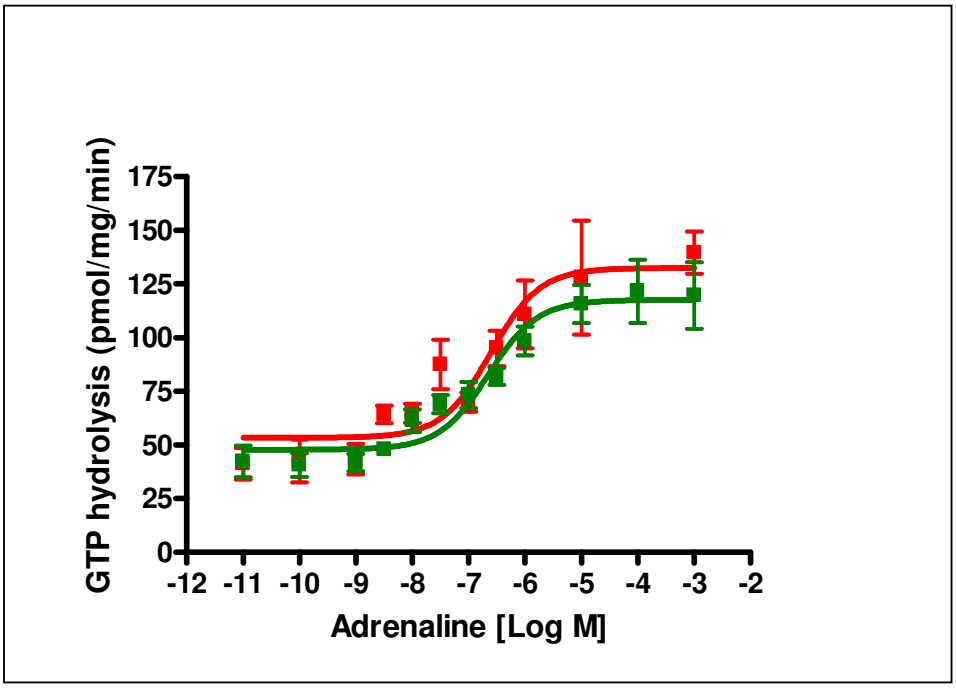

Figure 4.29 Adrenaline-stimulated GTPase activity of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein in the presence of GST-$RGS4^{S30E}$

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein. Cells were treated with P. tox (25 ng/ml for 16 hours) and membranes were prepared. The capacity of varying concentrations of adrenaline to stimulate high-affinity GTPase activity was then measured in presence of 1 µM GST-RGS4 (red) or 1 µM GST-$RGS4^{S30E}$ (green) using 0.5 µM [γ^{32}P]GTP. Data shown are from quadruplicate determinations (mean ± SEM) and are representative of three individual experiments performed.

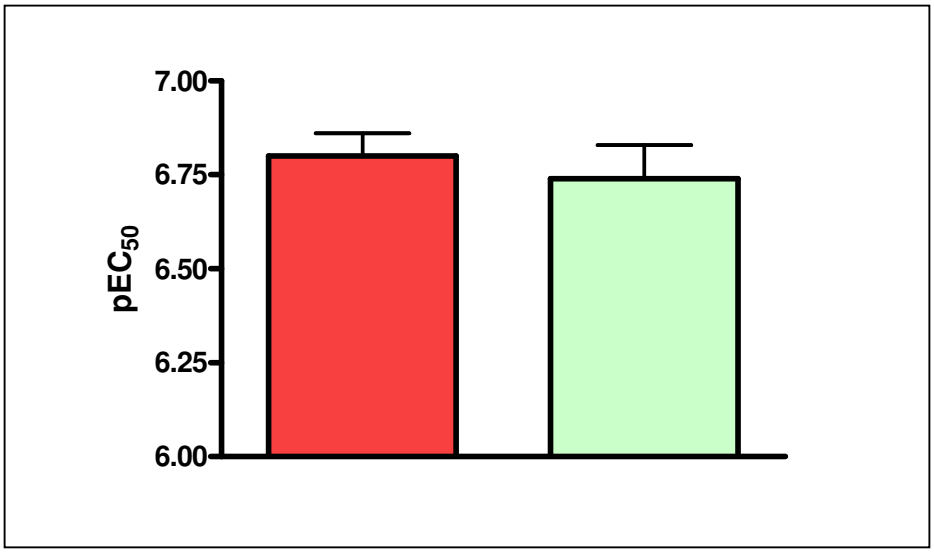

Figure 4.30 Comparison of pEC_{50} values of adrenaline to stimulate the GTPase activity of the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein in the presence of GST-RGS4^{S30E}

The results from Figure 4.29 are presented in graphical form for comparison of results. The pEC_{50} in the presence of GST-RGS4 (red) or presence of GST-RGS4^{S30E} (green) are mean ± SEM. from three individual experiments performed.

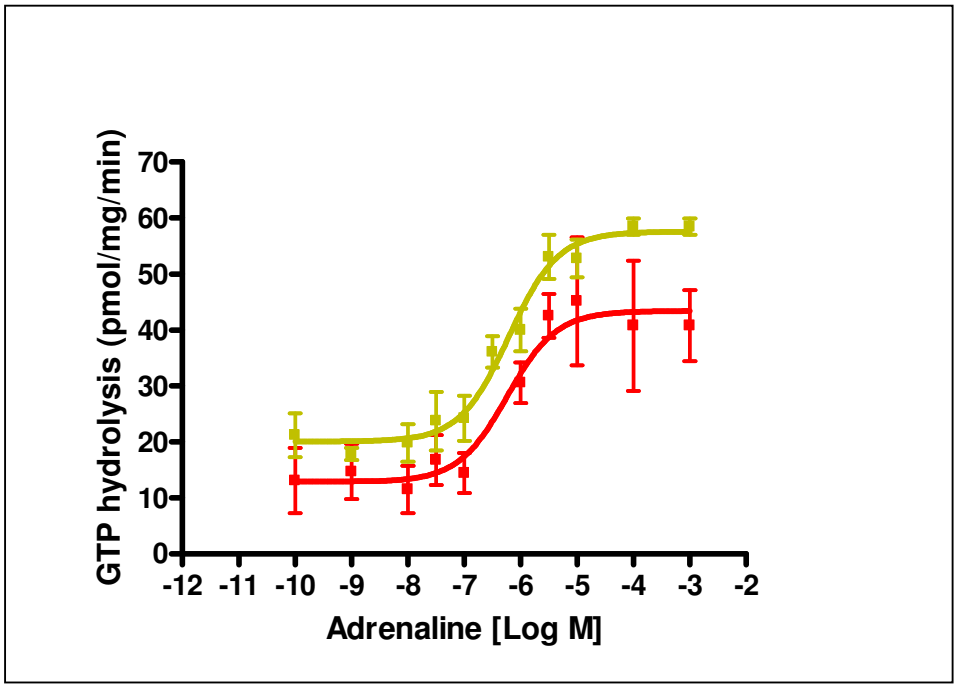

Figure 4.31 Adrenaline-stimulated GTPase activity of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein in the presence of GST-$RGS4^{S30K}$

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein. Cells were treated with P. tox (25 ng/ml for 16 hours) and membranes were prepared. The capacity of varying concentrations of adrenaline to stimulate high-affinity GTPase activity was then measured presence of 100 nM GST-RGS4 (red) or 100 nM GST-$RGS4^{S30K}$ (mustard) using 0.5 μM [γ^{32}P]GTP. Data shown are from quadruplicate determinations (mean ± SEM) and are representative of three individual experiments performed.

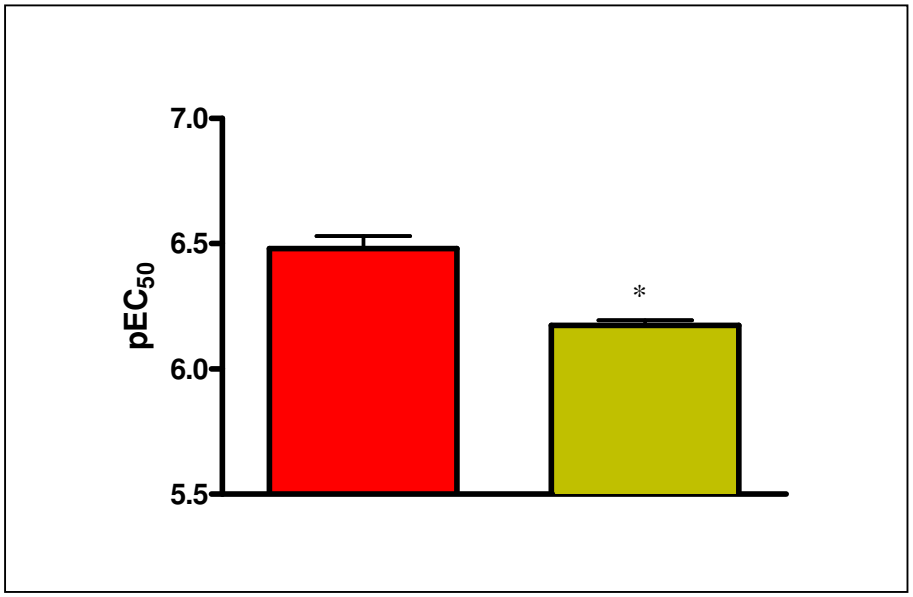

Figure 4.32 Comparison of pEC_{50} values of adrenaline to stimulate the GTPase activity of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein in the presence of GST-$RGS4^{S30K}$

The pEC_{50} results from Figure 4.31are presented for comparison of results. The pEC_{50} in the presence of 100 nM GST-RGS4 (red) or 100 nM GST-$RGS4^{S30K}$ (mustard) are mean ± SEM. from three individual experiments performed. * denotes significantly different from α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein + 100 nM GST-RGS4 ($p<0.05$).

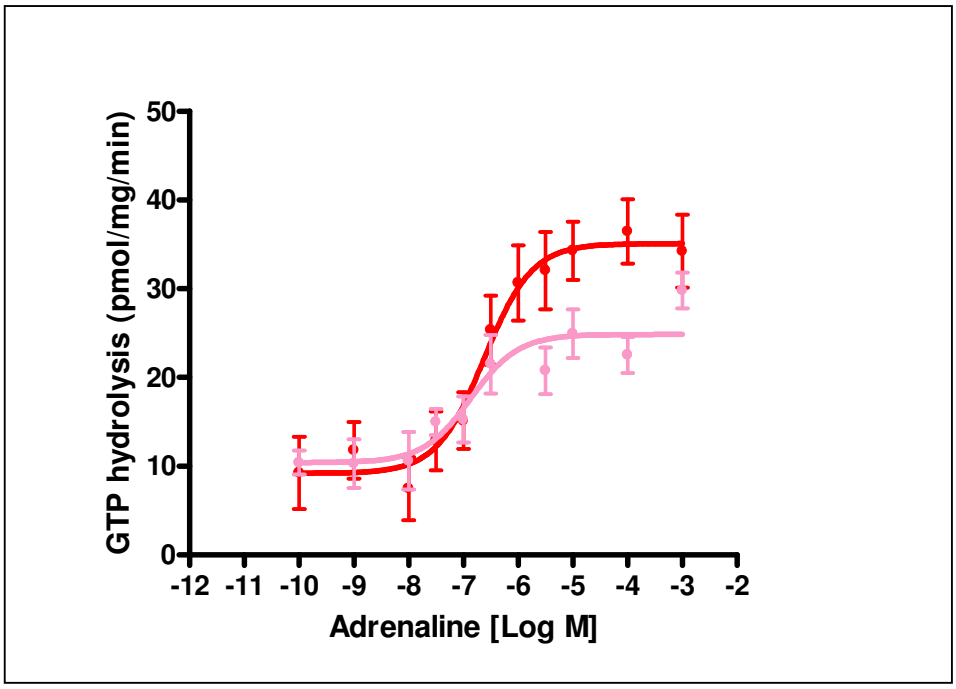

Figure 4.33 Adrenaline-stimulated GTPase activity of the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein in the presence of GST-RGS4^{S30M}

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-Gα_{o1}^{C351}I fusion protein. Cells were treated with P. tox (25 ng/ml for 16 hours) and membranes were prepared. The capacity of varying concentrations of adrenaline to stimulate high-affinity GTPase activity was then measured presence of 100 nM GST-RGS4 (red) or 100 nM GST-RGS4^{S30M} (pink) using 0.5 µM [γ^{32}P]GTP. Data shown are from quadruplicate determinations (mean ± SEM) and are representative of three individual experiments performed.

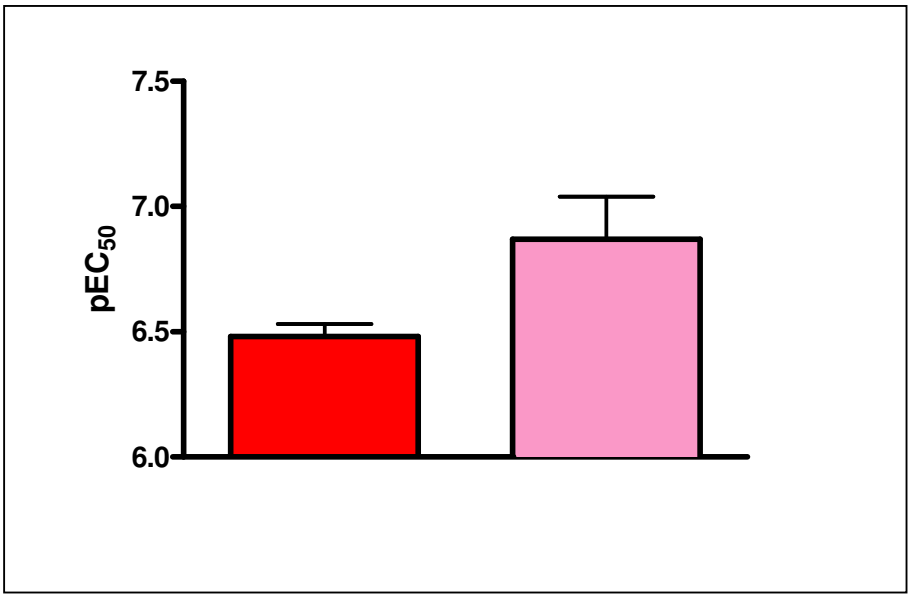

Figure 4.34 Comparison of pEC_{50} values of adrenaline to stimulate the GTPase activity of the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein in the presence of GST-RGS4^{S30M}

The pEC_{50} results from Figure 4.33 are presented for comparison of results. The pEC_{50} in the presence of 100 nM GST-RGS4 (red) or 100 nM GST-RGS4^{S30M} (pink) are mean ± SEM. from three individual experiments performed.

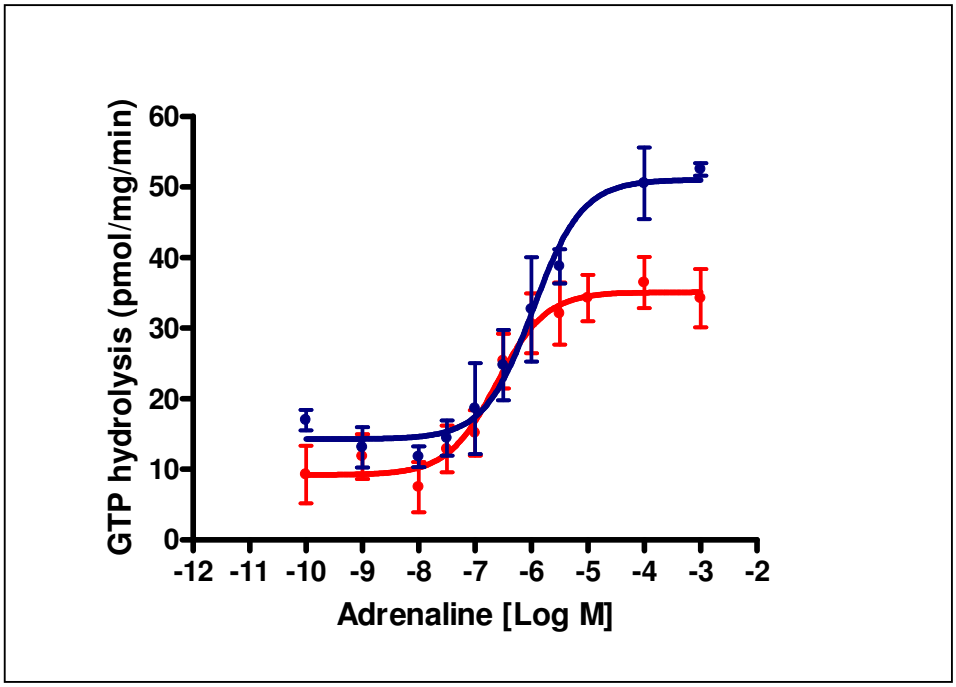

Figure 4.35 Adrenaline stimulated GTPase activity of the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein in the presence of GST-RGS4^{S30F}

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein. Cells were treated with P. tox (25 ng/ml for 16 hours) and membranes were prepared. The capacity of varying concentrations of adrenaline to stimulate high-affinity GTPase activity was then measured presence of 100 nM GST-RGS4 (red) or 100 nM GST-RGS4^{S30F} (navy) using 0.5 µM [γ^{32}P]GTP. Data shown are from quadruplicate determinations (mean ± SEM) and are representative of three individual experiments performed.

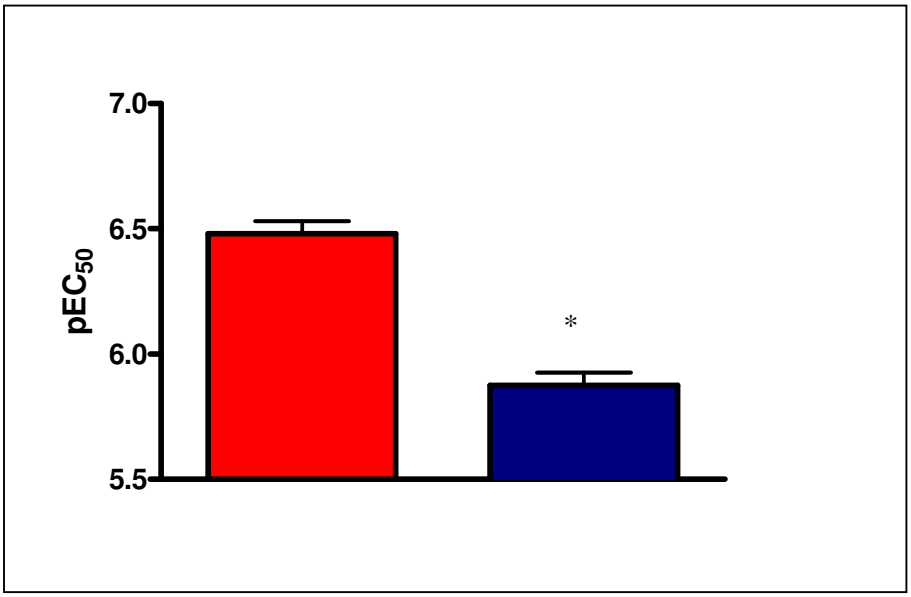

Figure 4.36 Comparison of pEC_{50} values of adrenaline to stimulate the GTPase activity of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein in the presence of GST-$RGS4^{S30F}$

The pEC_{50} results from Figure 4.35 are presented in for comparison of results. The pEC_{50} in the presence of 100 nM GST-RGS4 (red) or 100 nM GST-$RGS4^{S30F}$ (navy) are mean ± SEM. from three individual experiments performed. Where ** denotes significantly different from the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein + 100 nM GST-RGS4 ($p<0.01$)

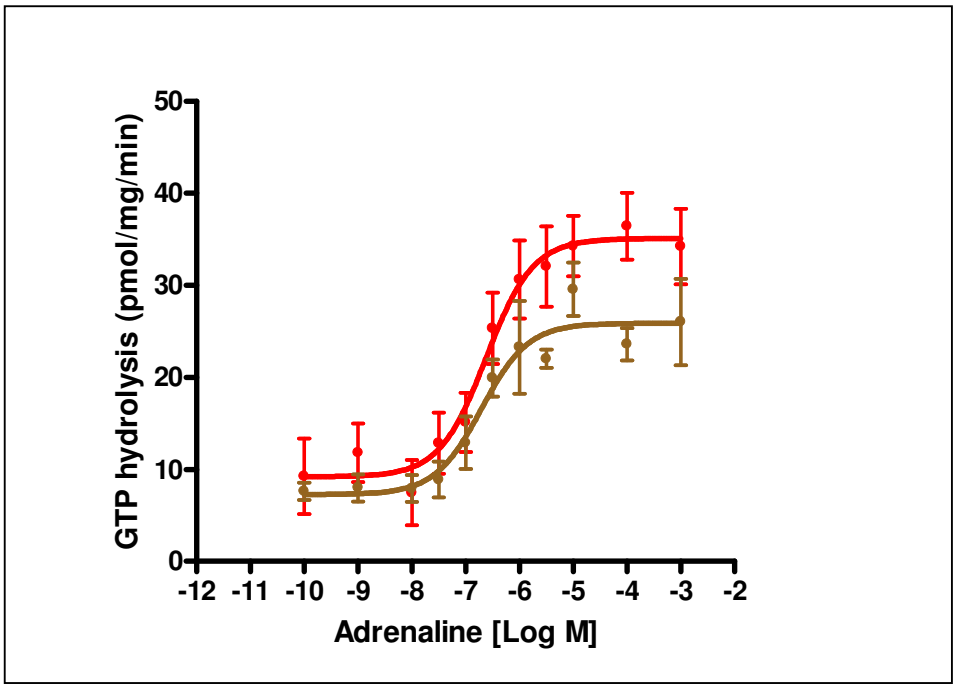

Figure 4.37 Adrenaline stimulated GTPase activity of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein in the presence of GST-$RGS4^{S30P}$

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ fusion protein. Cells were treated with P. tox (25 ng/ml for 16 hours) and membranes were prepared. The capacity of varying concentrations of adrenaline to stimulate high-affinity GTPase activity was then measured presence of 100 nM GST-RGS4 (red) or 100 nM GST-$RGS4^{S30P}$ (brown) using 0.5 µM [γ^{32}P]GTP. Data shown are from quadruplicate determinations (mean ± SEM) and are representative of three individual experiments performed.

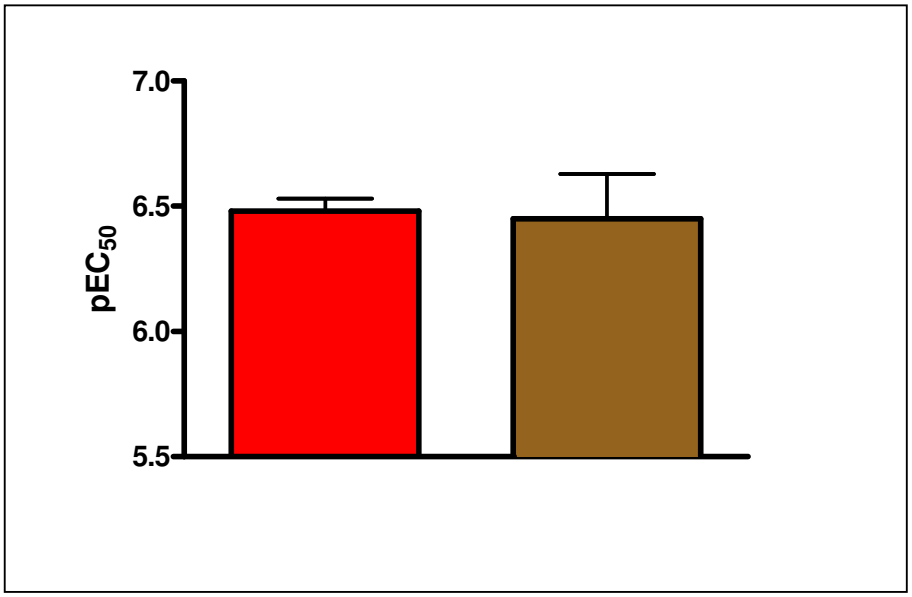

Figure 4.38 Comparison of pEC_{50} values of adrenaline to stimulate the GTPase activity of the α_{2A}-adrenoceptor-Gα_{o1}^{C351I} fusion protein in the presence of GST-RGS4^{S30P}

The pEC_{50} results from Figure 4.37 are presented for comparison of results. The pEC_{50} in the presence of 100 nM GST-RGS4 (red) or 100 nM GST-RGS4^{S30P} (brown) are mean ± SEM. from three individual experiments performed.

	pEC_{50}	
Amino acid at position 30	**Wild-Type RGS4**	**Mutant**
C	6.72 ± 0.08	6.46 ± 0.11*
A	6.80 ± 0.06	6.36 ± 0.2 **
E	6.80 ± 0.06	6.74 ± 0.09
K	6.48 ± 0.05	6.21 ± 0.04 **
M	6.48 ± 0.05	6.87 ± 0.17 **
F	6.48 ± 0.05	6.11 ± 0.24*
P	6.48 ± 0.05	6.45 ± 0.18

Table 4.1 Comparison of pEC_{50} values in RGS4 serine 30 mutants

The comparison of pEC_{50} values for high-affinity adrenaline-stimulated activation of the α_{2A}-adrenoceptor-$G\alpha_{o1}^{C351I}$ for each serine 30 mutant compared to wild-type RGS4. Data shown are the means of three independent experiments ± SEM. Statistical significance was determined using a Student's *t* test, * represents $p<0.05$ and ** represents $p<0.005$. It should be noted that results for K, M, F and P wild-type controls were produced from the same experiment.

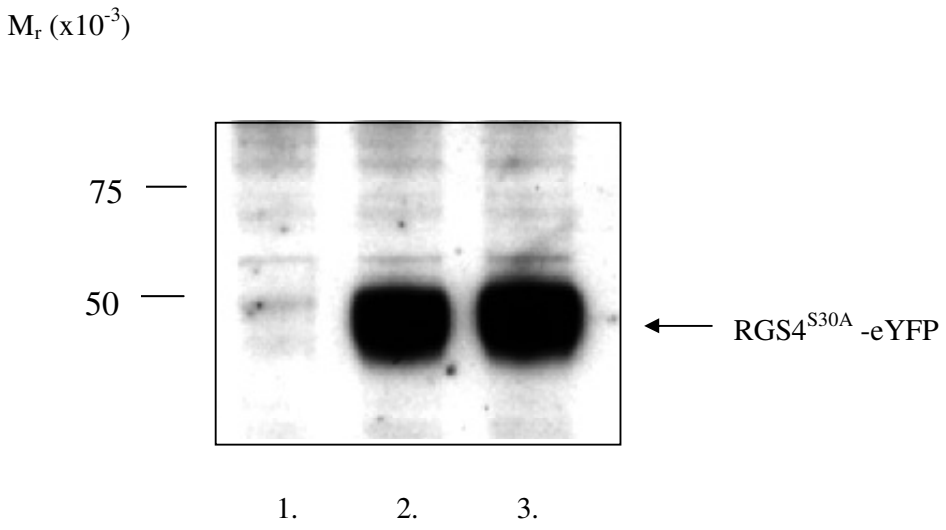

Figure 4.39 Expression and immunological detection of RGS4-eYFP and RGS4^{S30A} -eYFP

HEK293T cells were transiently transfected to express RGS4-eYFP (lane 2) or RGS4^{S30A}-eYFP (lane 3). HEK293T cells transfected with pcDNA3 were included as a control (lane 1). Cell lysates were resolved by SDS-PAGE and then immunoblotted with anti-GFP antiserum. Results shown are from a single experiment and are representative of three experiments performed.

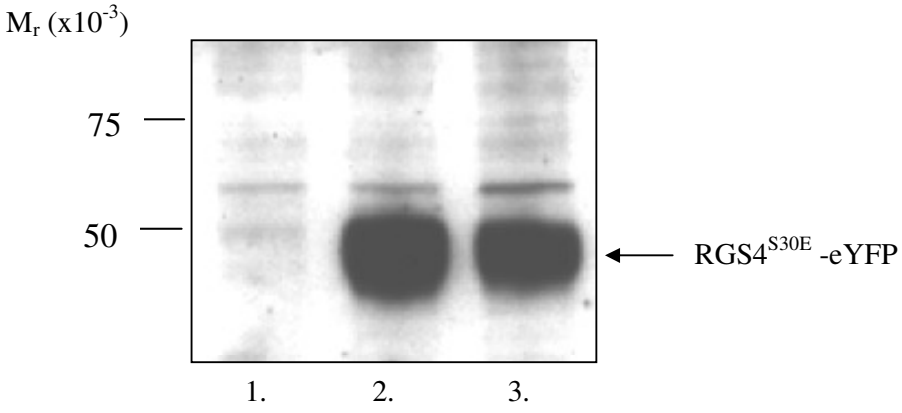

Figure 4.40 Expression and immunological detection of RGS4-eYFP and RGS4 ^{S30E} -eYFP

HEK293T cells were transiently transfected to express RGS4-eYFP (lane 2) or RGS4^{S30E}-eYFP (lane 3). HEK293T cells transfected with pcDNA3 were included as a control (lane 1). Cell lysates were resolved by SDS-PAGE and then immunoblotted with anti-GFP antiserum. Results shown are from a single experiment and are representative of three experiments performed.

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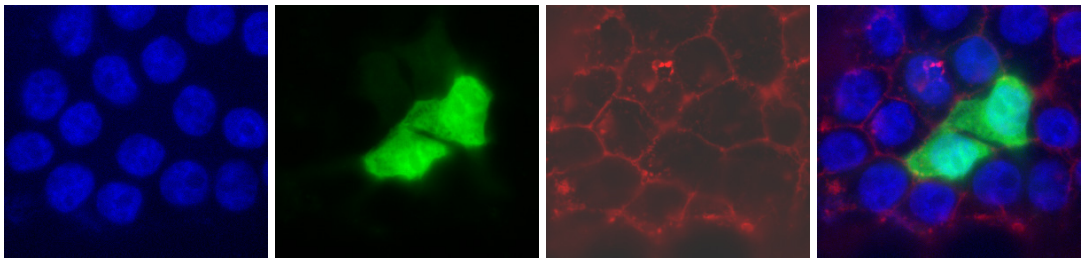

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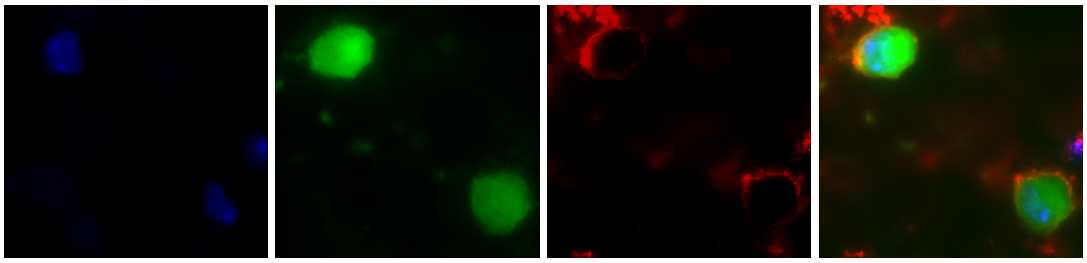

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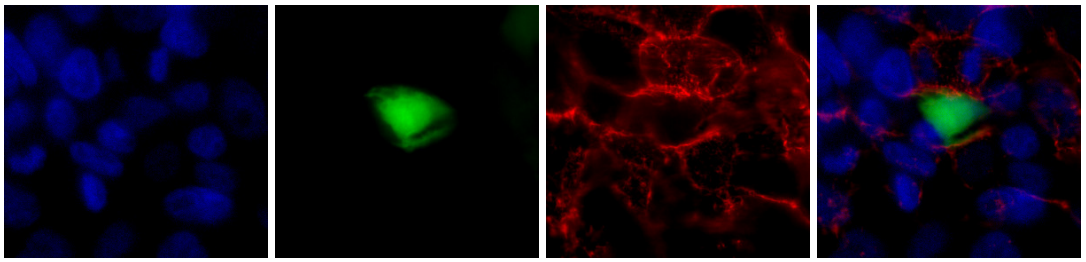

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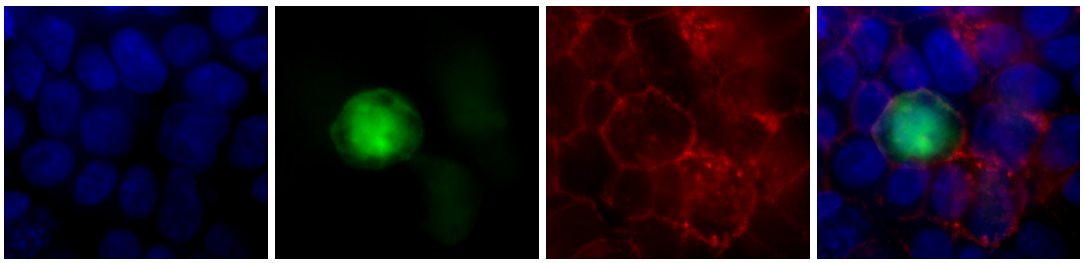
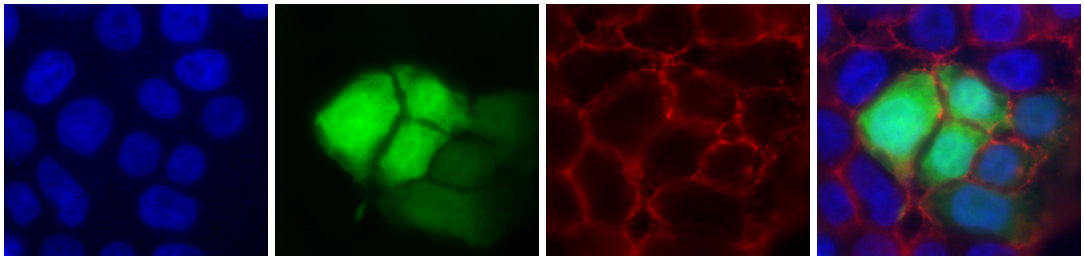


Figure 4.41 The cellular localisation of RGS4^{S30A}-eYFP

HEK293T cells grown on coverslips were transiently transfected with (A) RGS4^{S30A}-eYFP (B) RGS4^{S30A}-eYFP co-transfected with $G\alpha_{i1}$ (C) RGS4^{S30A}-eYFP co-transfected with the α_{2A}-adrenoceptor or (D) RGS4^{S30A}-eYFP co-transfected with the α_{2A}-adrenoceptor and stimulated with 100 μM UK14304 for 30 minutes. Images were generated using an inverted Nikon TE2000-E microscope equipped with a 60 x, (NA=1.4), oil-immersion Plan Fluor Apochromat lens and a cooled digital Cool Snap-HQ CCD camera. (i) Hoechst 33342 nuclei staining (blue) (ii) RGS4^{S30A}-eYFP (green) or (iii) WGA-Alexa Fluor 594 membrane staining (red) (iv) merged images. Results shown are from a single experiment and are representative of three experiments performed.

(A) (i) (ii) (iii) (iv)



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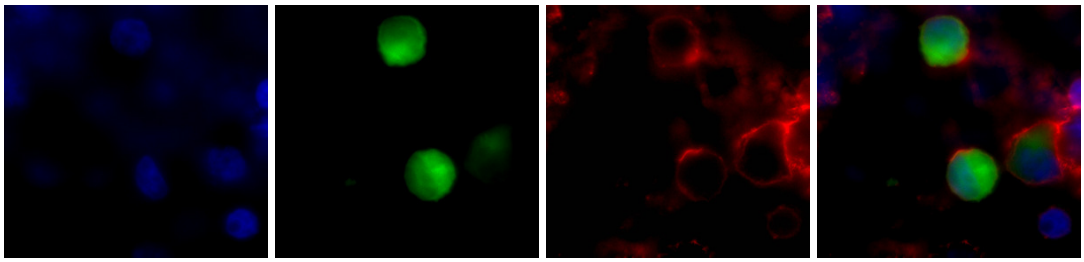

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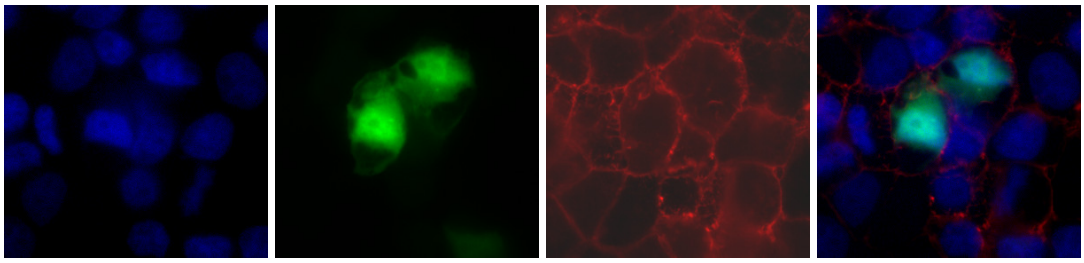

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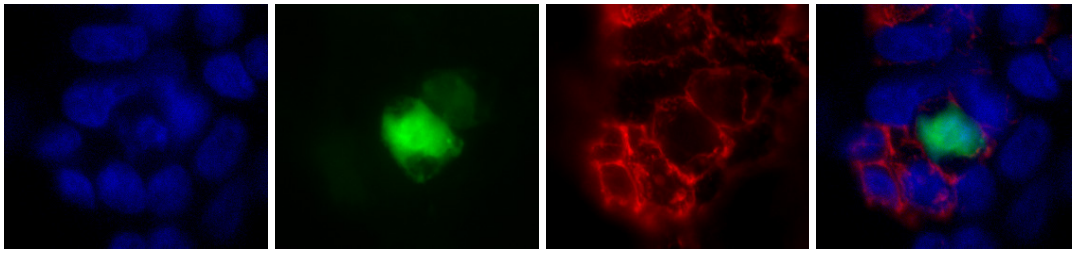

Figure 4.42 The cellular localisation of RGS4^{S30E}-eYFP

HEK293T cells grown on coverslips were transiently transfected with (A) RGS4^{S30E}-eYFP (B) RGS4^{S30E}-eYFP co-transfected with $G\alpha_{i1}$ (C) RGS4^{S30E}-eYFP co-transfected with the α_{2A}-adrenoceptor or (D) RGS4^{S30E}-eYFP co-transfected with the α_{2A}-adrenoceptor and stimulated with 100 μM UK14304 for 30 minutes. Images were generated using an inverted Nikon TE2000-E microscope equipped with a 60 x, (NA=1.4), oil-immersion Plan Fluor Apochromat lens and a cooled digital Cool Snap-HQ CCD camera. (i) Hoechst 33342 nuclei staining (blue) (ii) RGS4^{S30E}-eYFP (green) or (iii) WGA-Alexa Fluor 594 membrane staining (red) (iv) merged images. Results shown are from a single experiment and are representative of three experiments performed.

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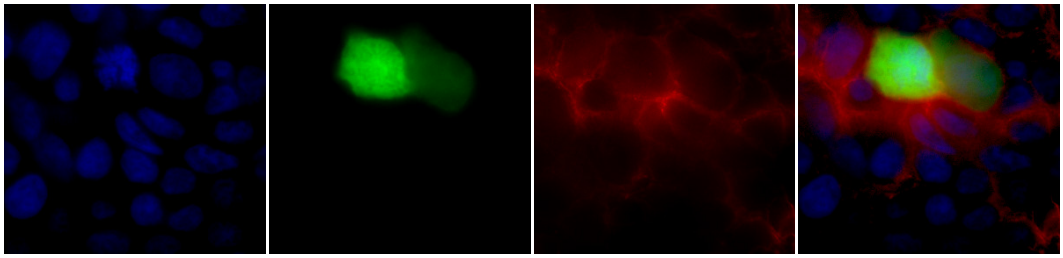

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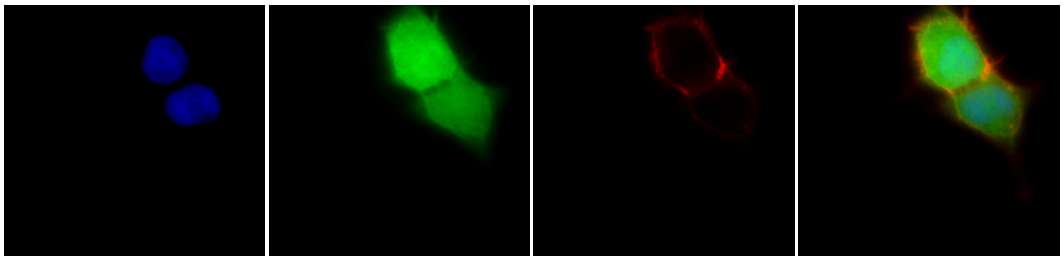

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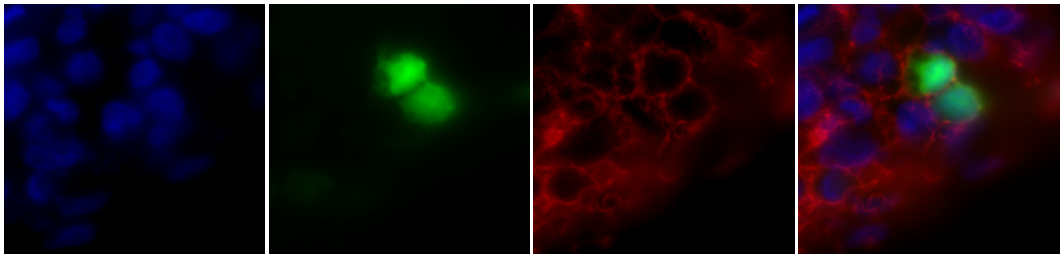

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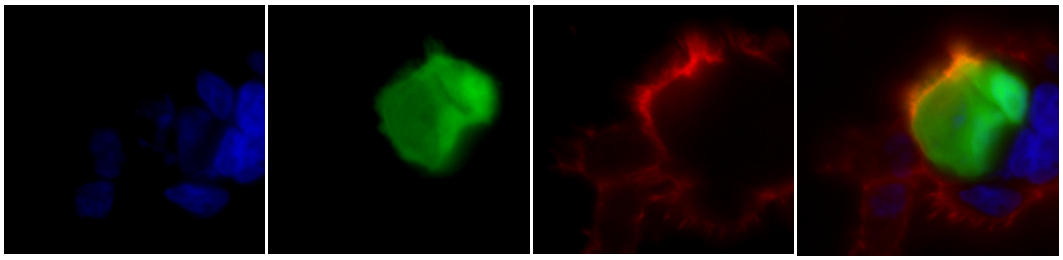

Figure 4.43 The cellular localisation of RGS4^{S30K}-eYFP

HEK293T cells grown on coverslips were transiently transfected with (A) RGS4^{S30K}-eYFP (B) RGS4^{S30K}-eYFP co-transfected with $G\alpha_{i1}$ (C) RGS4^{S30K}-eYFP co-transfected with the α_{2A}-adrenoceptor or (D) RGS4^{S30K}-eYFP co-transfected with the α_{2A}-adrenoceptor and stimulated with 100 μM UK14304 for 30 minutes. Images were generated using an inverted Nikon TE2000-E microscope equipped with a 60 x, (NA=1.4), oil-immersion Plan Fluor Apochromat lens and a cooled digital Cool Snap-HQ CCD camera. (i) Hoechst 33342 nuclei staining (blue) (ii) RGS4^{S30K}-eYFP (green) or (iii) WGA-Alexa Fluor 594 membrane staining (red) (iv) merged images. Results shown are from a single experiment and are representative of three experiments performed.

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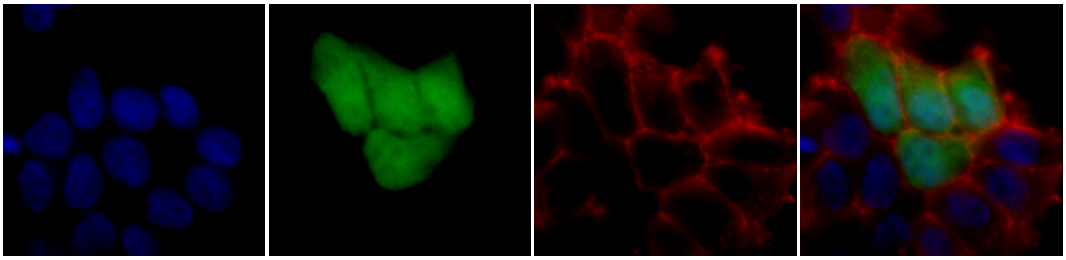

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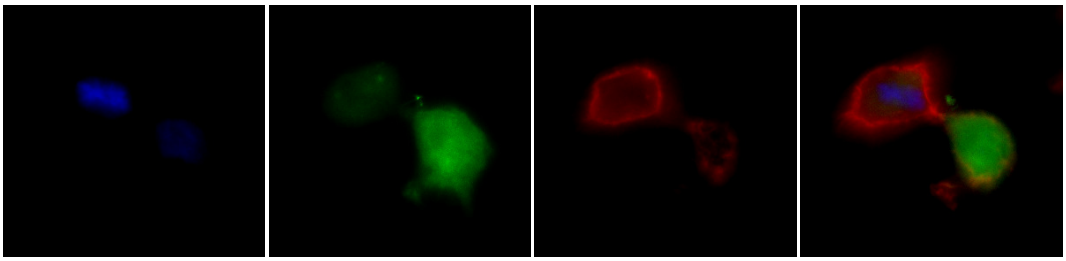

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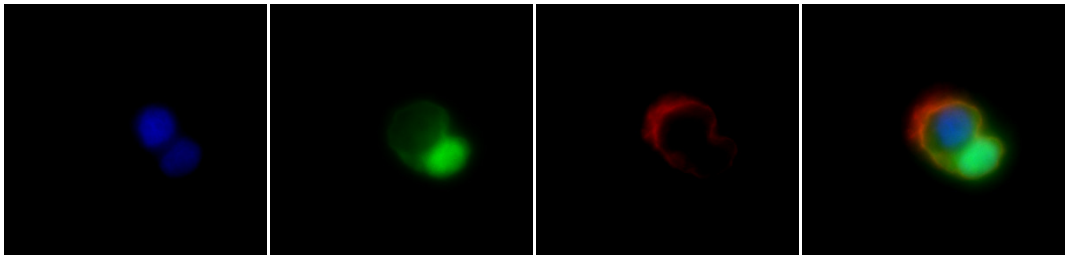

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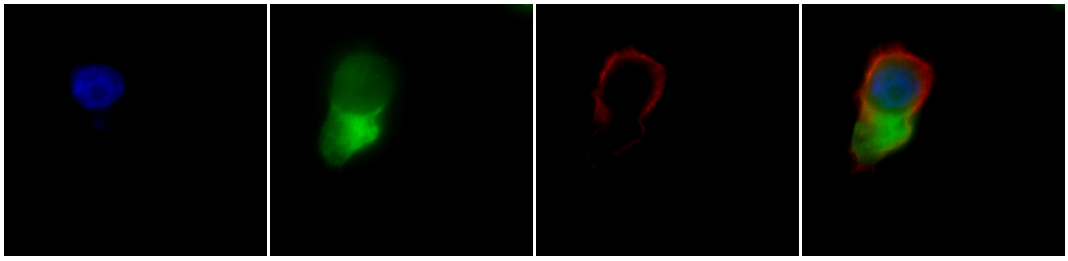
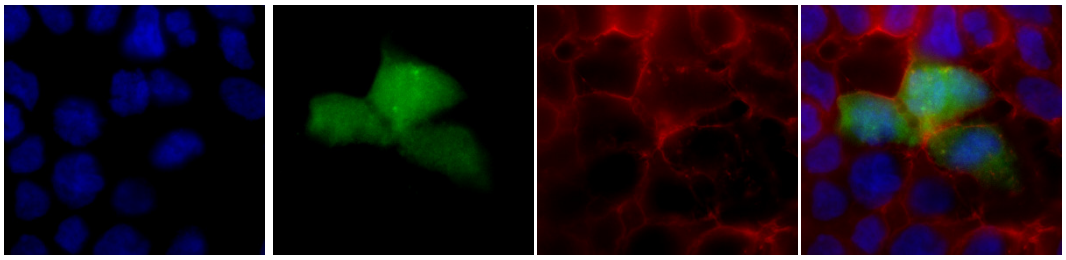


Figure 4.44 The cellular localisation of RGS4^{S30M}-eYFP

HEK293T cells grown on coverslips were transiently transfected with (A) RGS4^{S30M}-eYFP (B) RGS4^{S30M}-eYFP co-transfected with Gα_{i1} (C) RGS4^{S30M}-eYFP co-transfected with the α_{2A}-adrenoceptor or (D) RGS4^{S30M}-eYFP co-transfected with the α_{2A}-adrenoceptor and stimulated with 100 μM UK14304 for 30 minutes. Images were generated using an inverted Nikon TE2000-E microscope equipped with a 60 x, (NA=1.4), oil-immersion Plan Fluor Apochromat lens and a cooled digital Cool Snap-HQ CCD camera. (i) Hoechst 33342 nuclei staining (blue) (ii) RGS4^{S30M}-eYFP (green) or (iii) WGA-Alexa Fluor 594 membrane staining (red) (iv) merged images. Results shown are from a single experiment and are representative of three experiments performed.

(A) (i) (ii) (iii) (iv)



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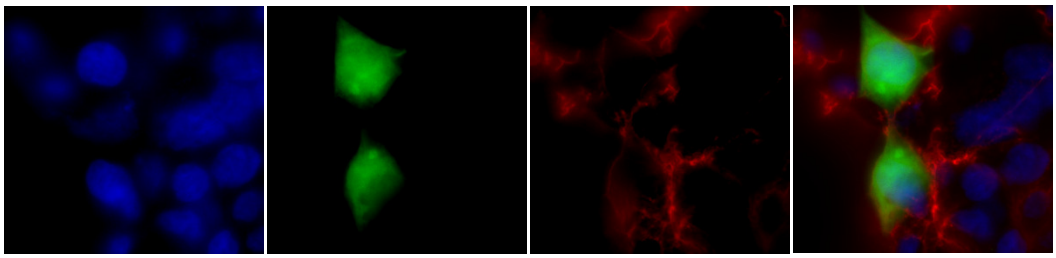

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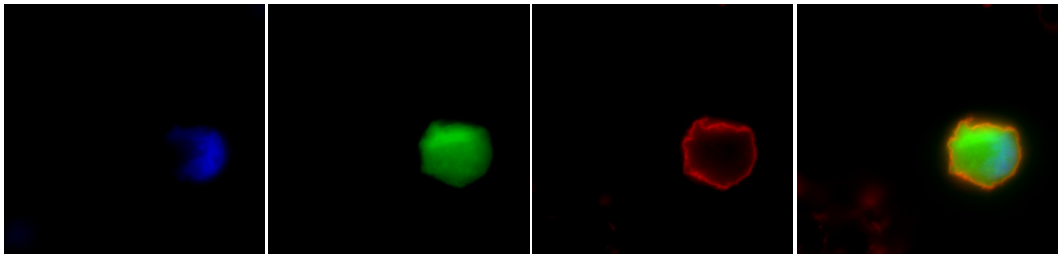

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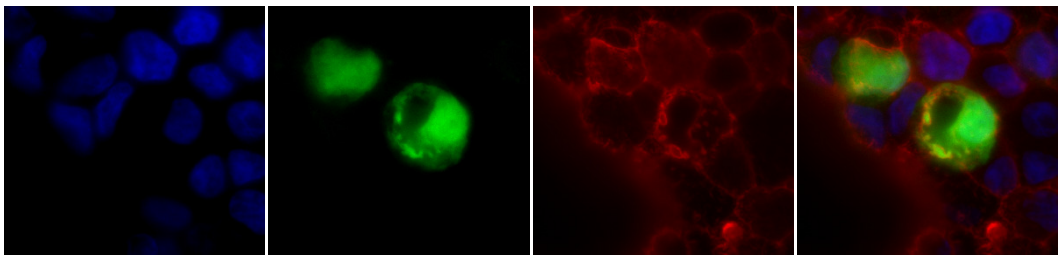
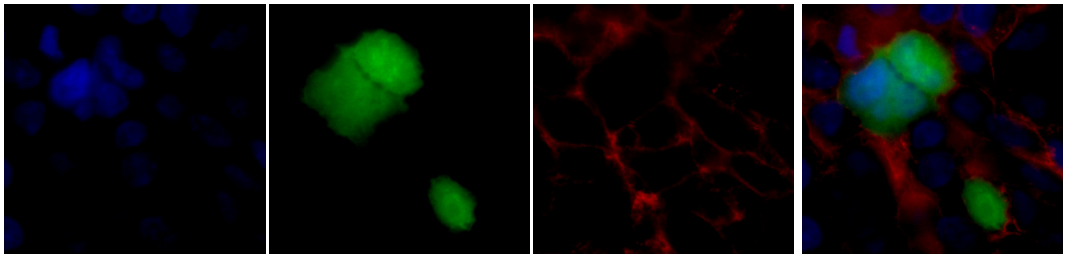


Figure 4.45 The cellular localisation of RGS4^{S30F}-eYFP

HEK293T cells grown on coverslips were transiently transfected with (A) RGS4^{S30F}-eYFP (B) RGS4^{S30F}-eYFP co-transfected with $G\alpha_{i1}$ (C) RGS4^{S30F}-eYFP co-transfected with the α_{2A}-adrenoceptor or (D) RGS4^{S30F}-eYFP co-transfected with the α_{2A}-adrenoceptor and stimulated with 100 μM UK14304 for 30 minutes. Images were generated using an inverted Nikon TE2000-E microscope equipped with a 60 x, (NA=1.4), oil-immersion Plan Fluor Apochromat lens and a cooled digital Cool Snap-HQ CCD camera. (i) Hoechst 33342 nuclei staining (blue) (ii) RGS4^{S30F}-eYFP (green) or (iii) WGA-Alexa Fluor 594 membrane staining (red) (iv) merged images. Results shown are from a single experiment and are representative of three experiments performed.

(A) (i) (ii) (iii) (iv)



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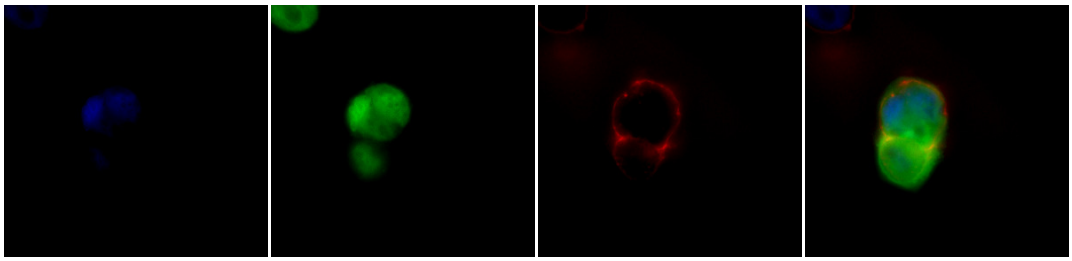

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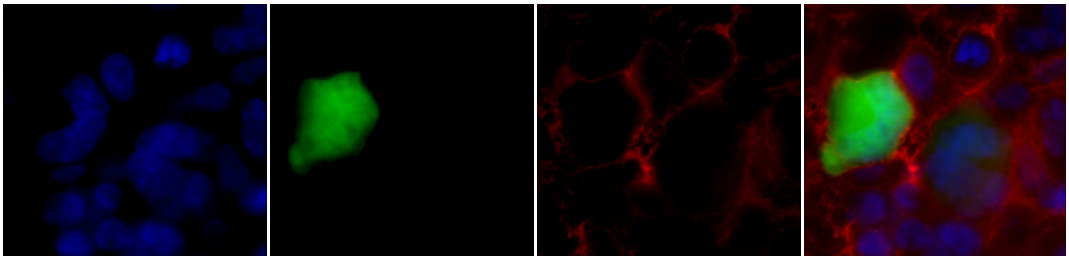

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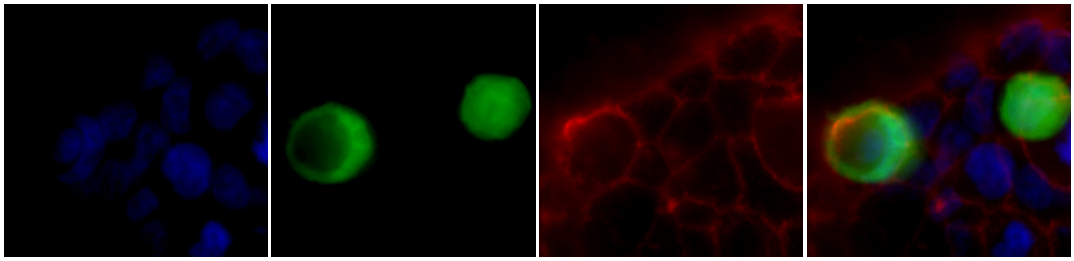

Figure 4.46 The cellular localisation of RGS4^{S30P}-eYFP

HEK293T cells grown on coverslips were transiently transfected with (A) RGS4^{S30P}-eYFP (B) RGS4^{S30P}-eYFP co-transfected with $G\alpha_{i1}$ (C) RGS4^{S30P}-eYFP co-transfected with the α_{2A}-adrenoceptor or (D) RGS4^{S30P}-eYFP co-transfected with the α_{2A}-adrenoceptor and stimulated with 100 μM UK14304 for 30 minutes. Images were generated using an inverted Nikon TE2000-E microscope equipped with a 60 x, (NA=1.4), oil-immersion Plan Fluor Apochromat lens and a cooled digital Cool Snap-HQ CCD camera. (i) Hoechst 33342 nuclei staining (blue) (ii) RGS4^{S30P}-eYFP (green) or (iii) WGA-Alexa Fluor 594 membrane staining (red) (iv) merged images. Results shown are from a single experiment and are representative of three experiments performed.

4.3 Discussion

In this study, the GOF properties of $RGS4^{S30C}$, initially identified in a yeast based screen, are confirmed in an *in vitro* high-affinity GTPase assay. $RGS4^{S30C}$ demonstrated an enhanced α_{2A}-adrenoceptor-activated increase in high-affinity GTPase activity of $G\alpha_{o1}$ compared to wild-type RGS4. This increased GTPase activity was accompanied by a concurrent decrease in the potency of the adrenoceptor agonists adrenaline and UK14304 to stimulate high-affinity GTPase activity compared to wild-type RGS4. This serine residue is conserved in other members of the B/R4 family of RGS proteins and the conversion of serine 30 to cysteine in RGS16 also demonstrated an increase in high-affinity GTPase activity and decrease in agonist potency compared to wild-type RGS16. These results suggest that the conserved serine residue in the B/R4 subfamily of RGS proteins is important for the GAP activity of these proteins. Interestingly, the only other GOF mutation identified in an RGS protein was identified in *S. cerevisiae* and was also located in the N terminus of this protein at position 20 ($SST2^{P20L}$) (Dohlman *et al.*, 1995). Serine 30 in RGS4 is positioned in the N-terminal amphipathic helix suggesting perhaps that this residue could be implicated in the ability of RGS4 to directly interact with GPCRs (Srinivasa *et al.*, 1998;Riddle *et al.*, 2005;Itoh *et al.*, 2006). The N-terminus, and in particular, residue 30 may help correctly position RGS4 at the receptor where it can optimally inactivate the G protein α subunit via the GAP activity of the RGS domain. Mutation of this residue may serve to change the orientation of RGS4 and subsequently the GAP activity of the RGS domain.

To compare the GTPase activity of the RGS proteins studied, GST-RGS proteins were expressed and purified using previously published techniques (Hoffmann *et al.*, 2001). A high-affinity GTPase assay using a fixed concentration of agonist (100 μM) and increasing concentrations of RGS4 revealed that addition of 1 μM purified RGS4, as previously used by Cavalli *et al.*, (Cavalli *et al.*, 2000) gave sufficient activity to effectively measure enhanced GTPase activity of the G protein. Purification of RGS16 and $RGS16^{S30C}$ however, was experimentally more difficult than RGS4. In this study, maximal GTPase activity of RGS4 and RGS16 are never directly compared, therefore it was possible to lower the concentration of purified RGS16 added to high-affinity GTPase assays to 100 nM. In the current studies, 10 μL of each purified RGS protein was manually added to each assay plate, perhaps future experiments could make use of an automated liquid handling device to precisely transfer a smaller volume of purified RGS protein from a

microplate to the assay plate allowing the final concentration of difficult to purify RGS proteins to be increased and perhaps ultimately enabling total assay volume to be reduced.

In addition to an increase in the maximal high-affinity GTPase activity of $G\alpha_{o1,}$ both RGS4 and RGS16 caused a simultaneous significant decrease in potency of the adrenoceptor agonist. These observed decreases in potency are in accordance with other published results for the addition of RGS proteins to high-affinity GTPase assays (Ward and Milligan, 1999;Cavalli *et al.*, 2000). Hoffmann *et al.*, compared the capacity of RGS16, RGS1 and GAIP to regulate GTP hydrolysis by $G\alpha_{o1}$ following its activation by a range of agonists at the α_{2A}-adrenoceptor (Hoffmann *et al.*, 2001). RGS16 was the most active RGS protein and concomitantly reduced the potency of adrenaline (pEC_{50} 6.0 ± 0.1). Marked differences of the three different RGS proteins to alter the efficacy and the potency of the α_{2A}-adrenoceptor-$G\alpha_{o1}$ following agonist activation provided evidence that RGS proteins must interact with receptors. If the RGS proteins were to interact only with the G proteins the agonist binding site would remain unchanged and no effect would be expected in the potency of the agonist

In this study, the potency of adrenaline to stimulate the α_{2A}-adrenoceptor high-affinity GTPase activity of $G\alpha_{o1}$ on addition of purified RGS16 (pEC_{50} 5.68 ± 0.3) was lower on the addition of RGS4 (pEC_{50} 6.72 ± 0.08). This perhaps provides yet further evidence for the selective interactions between RGS protein and GPCRs. RGS4 and RGS16 must interact with the α_{2A}-adrenoceptor, altering the conformation of the receptor. These closely related RGS proteins do not function equally and the specificity of the interaction is perhaps fundamental to the GAP activity of the RGS protein and the ability to control biological functions. Indeed, previous evidence has suggested regions of RGS proteins outwith the RGS domain are particularly important for the selective interaction with GPCRs. Interactions between RGS proteins and GPCRs are thought to be promoted through the N-terminal of the RGS protein. Co-immunoprecipitation studies have demonstrated that the N-terminus of RGS2 binds to the third intracellular loop of the M3 muscarinic receptors. This binding was also seen for another member of the B/R4 family of RGS proteins, RGS16, but not for a further member of this family, RGS1 (Bernstein *et al.*, 2004). Also of interest are previous studies in an oocyte expression system which have suggested that the N-terminal of RGS8 is responsible for its ability to inhibit signalling by either the M1 muscarinic receptors or the substance P receptors but not the M3 muscarinic receptors (Itoh *et al.*, 2006). It is therefore conceivable that perhaps residues within the N-

terminal of RGS4 and RGS16, are important for the alteration in potency of adrenaline to stimulate the α_{2A}-adrenoceptor high-affinity GTPase activity of $G\alpha_{o1}$.

High-affinity GTPase assays using the α_{2A}-adrenoceptor-$G\alpha_{i1}$ fusion protein revealed that the GTPase enhancing activity of RGS4 and RGS16 are selective for $G\alpha_{o1}$ over $G\alpha_{i1}$. Addition of purified RGS4 or RGS16 to high-affinity GTPase assays did not increase maximal GTPase activity for $G\alpha_{i1,}$ but did, however, decrease the potency for adrenaline to stimulate high-affinity GTPase activity. These conflicting results could suggest that the $G\alpha_{i1}$ protein is working at maximal capacity in this assay system, and presence of additional exogenous RGS proteins interacts with the α_{2A}-adrenoceptor but cannot further increase this maximal GTPase activity. However, it has previously been established that the B/R4 subfamily of RGS proteins show selectivity for $G\alpha_{o1}$ over $G\alpha_{i1}$ (Cavalli *et al.*, 2000;Riddle *et al.*, 2005), so perhaps, both RGS4 and RGS16 can interact with the α_{2A}-adrenoceptor but are not able to act as efficient GAP proteins for $G\alpha_{i1}$.

Previously, residues involved in RGS4-Gα interaction were identified by examining the crystal structure of the RGS domain of RGS4 complexed with $G\alpha_{i1}$ (Tesmer *et al.*, 1997). Due to the globular nature of the N-terminal of RGS4, this region could not be crystallised and residues identified in the RGS-$G\alpha_{i1}$ interaction were all within the RGS-fold of RGS4. Residues important for Gα selectivity within the N-terminus of RGS4 may have been unwittingly overlooked. Future advances in crystallisation may allow the crystallisation of the full-length of RGS4-$G\alpha_{o1}$ and reveal resides within the N-terminus important for this interaction.

In this study, addition of purified $RGS4^{S30C}$ or $RGS16^{S30C}$ to high-affinity GTPase assays did not increase maximal GTPase activity for $G\alpha_{i1}$. However, $RGS4^{S30C}$ and $RGS16^{S30C}$ decreased the potency for adrenaline to stimulate high-affinity GTPase activity compared to basal activity. This decrease in potency, unlike $G\alpha_{o1}$, was not significantly different to the wild-type RGS protein. These results suggest that, like their equivalent wild-type counterparts, these RGS proteins interact with the α_{2A}-adrenoceptor and alter the conformation of the receptor despite the Gα subunit fused to the C-terminal. RGS4 and RGS16 are also known to selectively interact with $G\alpha_{i2}$ (Cavalli *et al.*, 2000;Hoffmann *et al.*, 2001). It would now be of interest to investigate the α_{2A}-adrenoceptor-$G\alpha_{i2}$ high-affinity GTPase activity to reveal further selectivity of the B/R4 subfamily of RGS proteins using high-affinity GTPase assays.

An increase in the membrane attachment of $RGS4^{S30C}$ in HEK293T cells would orientate the mutated RGS protein in closer proximity to G proteins and therefore could be responsible for the enhanced GTPase activity of the protein. Mutation of residue 30 to cysteine may have allowed palmitoylation of this residue. Palmitoylation is the post-translational, reversible addition of palmitate to cysteine residues that can alter both subcellular localisation and GAP activity of the modified protein. Indeed, palmitolyation of cysteine residues at position 2 and 12 and 95 of RGS4 have already been reported (Tu *et al.*, 1999). It may therefore be plausible that further palmitolyation of cysteine at residue 30 of RGS4 could also occur. The cytosolic and membrane localisation of $RGS4^{S30C}$ was comparable to wild-type RGS4 in both unstimulated and stimulated cells. However, the cellular localisation of $RGS4^{S30C}$ co-expressed with G proteins was conducted using $G\alpha_{i1}$. At this point, high-affinity GTPase results in this study show selectivity for $G\alpha_{o1}$ over $G\alpha_{i1}$, and it would therefore be of more interest if $G\alpha_{o1}$ had been selected for co-expression and perhaps future localisation studies of RGS4 should take this into account.

It was also conceivable that an increase in the level of cellular expression of $RGS4^{S30C}$ compared to wild-type RGS4 was responsible for the GOF properties of the mutant. However, detection of cell lysates transiently expressing RGS4-eYFP or $RGS4^{S30C}$-eYFP with an antiserum directed against GFP showed bands of equal intensity suggesting equal levels of expression of the two proteins. However, the transient over-expression of both RGS4-eYFP and $RGS4^{S30C}$-eYFP could perhaps have concealed any differences in subcellular localisation or expression levels of the $RGS4^{S30C}$ mutant.

This transient over-expression of RGS4-eYFP or $RGS4^{S30C}$-eYFP could also have concealed differences in the inhibition of single cell $[Ca^{2+}]_i$ mobilisation from agonist activated HEK293T cells transiently expressing the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein. Addition of phenylephrine to these cells transiently expressing the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein and $RGS4^{S30C}$-eYFP did not demonstrate any further inhibition or change in the kinetics of the downstream signalling response compared to cells expressing the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein and wild-type RGS4-eYFP. The transient expression of the eYFP tagged RGS protein routinely allowed individual cells to be selected for analysis that were the most highly fluorescent and therefore cells that were unequivocally expressing the RGS4 protein. It is therefore reasonable to suggest that the inhibition of $[Ca^{2+}]_i$ release by RGS4 was near maximum capacity for the system and identification of further inhibition by an RGS4 GOF mutation would be difficult to detect.

Mutating serine 30 to a range of other amino acids established the importance of this particular residue on the effect on the α_{2A}-adrenoceptor-activated high-affinity GTPase activity. Amino acids were selected for mutational analysis by the properties of their side chains. The mutation of serine 30 to alanine, lysine, or phenylalanine resulted in a similar increase in α_{2A}-adrenoceptor-$G\alpha_{o1}$ activated high-affinity GTPase activity to $RGS4^{S30C}$. As predicted for GOF mutants, all the mutants also caused a concomitant decrease in potency of the adrenoceptor agonist adrenaline. It is interesting to note that $RGS4^{S30F}$ and $RGS4^{S30K}$ were also the most active mutants in *Sz. pombe* (Hill *et al*., personal communication). As amino acids other than cysteine also diplay GOF activity, it is therefore unlikely that palmitoylation is responsible for the increase in RGS activity. Other chemical properties shared by all GOF mutants are more likely to be responsible. Crucially, the hydroxyl group of serine at position 30 seems to be important to maintain wild-type activity of RGS4. Future experiments mutating residue 30 to tyrosine or threonine would allow further examination of this hypothesis.

Interestingly, conversion of residue 30 to glutamic acid or proline decreased the activity of RGS4 against $G\alpha_{o1}$ in high-affinity GTPase assays. Surprisingly, both of these mutants demonstrated no alteration in the potency of adrenaline to stimulate high-affinity GTPase activity. This perhaps suggests that the presence of glutamic acid or proline alters the conformation of the RGS amphiphatic helix such that the RGS now no longer interacts with α_{2A}-adrenoceptor. The side chain dimensions of the two most active mutants, phenylalanine and lysine, are comparably large. However, the amino acids cysteine and alanine at position 30 in RGS4 also demonstrated GOF properties, but the dimensions of these amino acids are not bigger than the least active, proline. It is, therefore, most likely that chemical properties of the side chains are contributing to the GTPase activity alterations. However, noticeable differences in the chemical properties between the most active mutants and least active $RGS4^{S30X}$ mutants are difficult to detect.

It has been determined that two members of the B/R4 subfamily of RGS proteins, RGS5 and RGS18 contain proline at the amino acid corresponding to residue 30 in RGS4. Since $RGS4^{S30P}$ resulted in a reduction in RGS activity, it would be interesting to compare $RGS5^{P30S}$ and $RGS18^{P30S}$ with $RGS4^{S30P}$ and further elucidate the role of proline in the orientation and interaction of Gα subunits with RGS proteins.

Despite serine 30 being outwith the predicted RGS domain in RGS4 and RGS16, mutation of this residue can directly affect its ability to enhance GTPase activity. This discovery

may have many future therapeutic implications as alterations in RGS signalling play a role in many disease states. An RGS GOF mutation could be used directly to decrease a signalling pathway or current small molecule inhibitors (Ingi *et al.*, 1998) could be tested to examine their ability to inhibit the enhanced RGS activity of these mutants. The specificity of both RGS4 and RGS16 for $G\alpha_{o1}$ is an important aspect of future therapeutics. This selectivity allows novel inhibitors to be designed to act on selective $G\alpha_{o1}$ signalling pathways of interest.

This study describes the GOF properties of $RGS4^{S30C}$ in a α_{2A}-adrenoceptor high-affinity GTPase assay. Mutation of a single amino acid in the N-terminus of this protein to enhance the RGS activity was transposed to another B/R4 family member, RGS16. The demonstrated Gα selectivity of both wild-type and mutant proteins has important future therapeutic implications for many signalling pathways regulated by RGS proteins.

5 Chapter 5

5.1 Introduction

RGS proteins increase the intrinsic rate of GTP hydrolysis on Gα subunits. It has been suggested that RGS proteins increase this hydrolysis by at least 40-fold by stabilising the transition state of the GTPase complex (Watson *et al.*, 1996;Druey and Kehrl, 1997). The highly conserved nine-helix bundle of the RGS domain is predicted to directly contact the Gα subunit at three switch regions. These switch regions undergo a conformational change during the GTPase cycle and specific amino acids within the RGS domain have been identified that, through non-covalent interactions, contribute to stabilising this conformational change.

Biochemical studies have shown that RGS proteins have little or no affinity for Gα-GDP complexes, and therefore do not alter the rate of GDP release. Instead, RGS proteins bind to the GTP-bound forms of Gα subunits and stimulate GTP hydrolysis. Interestingly, RGS proteins had higher affinity for $G\alpha_{i1}$ bound to GDP and AlF_4^-, a complex proposed to mimic the transition state of the GTPase reaction, indicating that RGS proteins act by stabilising the transition state (Watson *et al.*, 1996;Berman *et al.*, 1996b). The Ras family are also weak GTPases and data from p120GAP binding to, and accelerating the GTP hydrolysis of $p21^{ras}$, provided evidence that p120GAP introduced residues into the Ras active site that directly participate in the catalysis of GTP hydrolysis (Gideon *et al.*, 1992;Mittal *et al.*, 1996;Scheffzek *et al.*, 1996). Crystallisation of RGS4 complexed with $G\alpha_{i1}$ bound to GDP, AlF_4^- and Mg^{2+}, showed only the RGS domain of RGS4 visible in the crystal. However, due to the highly conserved nature of the RGS domain, the data from the crystallisation revealed features important for all members of the RGS family (Tesmer *et al.*, 1997). In contrast to the Ras family proteins, RGS proteins show no structural similarity to p120GAP, and appear not to contribute catalytical residues to the stimulation of GTP hydrolysis.

Mutagenesis of residues identified from the crystallisation complex has revealed residues essential for normal function of RGS4. In particular, $asparagine^{128}$ has been suggested as a key amino acid for the GAP action of RGS4. It has been demonstrated that $RGS4^{N128A}$ and $RGS4^{N88S,N128A}$ have impaired GAP activity against the α_{2A}-adrenoceptor-$G\alpha_{o1}$ fusion protein (Bahia *et al.*, 2003). Interestingly, it was also shown that $RGS4^{N88S}$ was both able

(Cavalli *et al.*, 2000) and unable (Bahia *et al.*, 2003) to act as an effective GAP for $G\alpha_{o1}$. The aim of this chapter was to investigate these RGS4 mutants using western blotting, microscopy and agonist-activated $[Ca^{2+}]_i$ mobilisation. By adding eYFP to the C-terminal of $RGS4^{N88S}$, $RGS4^{N128A}$ and $RGS4^{N88S,N128A}$, these techniques were used to examine the GAP activity of these RGS4 mutants against $G\alpha_{11}$.

5.2 Results

Mutations previously introduced into RGS4 to explore the functionality of the protein as a GAP, were C-terminally tagged with eYFP. This fluorescent tag was introduced to RGS4 in which asparagine residues 88 and 128 were mutated to serine and alanine respectively. In addition, eYFP was also introduced to the C-terminal of $RGS4^{N88S}$ and $RGS4^{N128A}$ to investigate the importance of each single mutation. Figure 5.1 shows the structure of these amino acids for comparison.

5.2.1 Expression of aspargine RGS4 mutants

To investigate the effect of mutating residues 88 and 128 in RGS4 on the expression level of the protein, RGS4-eYFP and $RGS4^{N88S,N128A}$-eYFP were individually transiently expressed in HEK293T cells and cell lysates were separated by SDS-PAGE (Figure 5.2). HEK293T cells transfected with pcDNA3 were included as a control (lane 1). Proteins were detected by western blotting using an anti-GFP antiserum that recognises eYFP. Bands corresponding to RGS4-eYFP (lane 2) and $RGS4^{N88S,N128A}$-eYFP (lane3) at ~50kDa were of comparable intensity indicating equal expression of the two proteins. No immunoreactivity was observed at ~50kDa in the control sample. However, immunoreactivity of a non-specific protein in all lanes at ~70kDa demonstrates equal loading of all three protein samples.

This protocol was repeated to investigate the expression level of $RGS4^{N88S}$-eYFP. Bands of equal intensity corresponding to RGS4-eYFP and $RGS4^{N88S}$-eYFP were observed suggesting equivalent expression of the two proteins by HEK293T cells (Figure 5.3). As observed previously, no immunoreactivity at ~50kDa, the predicted molecular weight for an eYFP-tagged RGS4, was observed in lane 1 in which cells were transfected only with pcDNA3. Again, a non-specific band at ~70kDa was observed in all three samples, suggesting identical total protein concentrations were present.

This protocol was again repeated to compare the expression levels of RGS4^{N128A}-eYFP to wild-type RGS4-eYFP. Cell lysates of transfected HEK293T cells separated by SDS-PAGE and immunoblotted by an anti-GFP antiserum demonstrated equal expression of both the wild-type and the mutant RGS4 protein (Figure 5.4).

5.2.2 Functional activity of asparagine RGS4 mutants

As previously described, following expression of the α_{1b}-adrenoceptor-Gα_{11} fusion protein, agonist activation causes an elevation in $[Ca^{2+}]_i$ (Chapter 5, Section 1.22) (Stevens *et al.*, 2001). In HEK293T cells, Gα_{11} activates PLCβ which in turn hydrolyses PIP2 and produces IP3 and DAG, leading to activation of downstream effector molecules and a rise in $[Ca^{2+}]_i$. The amplification of this signal is limited by the return of the Gα subunit to the inactive GDP-bound state by GTPase activity. Therefore, increasing the rate of GTP hydrolysis by the GAP action of RGS proteins should further limit this amplification. Expression of RGS4 with a fluorescent tag attached allows the visual identification of cells positively transfected with RGS4. Expression of RGS4-eYFP negatively regulates $[Ca^{2+}]_i$ mobilisation by agonist-stimulated α_{1b}-adrenoceptor-Gα_{11} fusion protein (Figure 5.5).

The co-expression of the α_{1b}-adrenoceptor-Gα_{11} fusion protein with an RGS mutant that has impaired GAP activity would be anticipated to limit the effect on $[Ca^{2+}]_i$ mobilisation. Indeed, co-expression of RGS4^{N88S}-eYFP, RGS4^{N128A}-eYFP and RGS4^{N88S,N128A}-eYFP with the α_{1b}-adrenoceptor-Gα_{11} fusion protein had no effect on agonist-mediated elevation of $[Ca^{2+}]_i$ (Figure 5.6).

The consistency of these results was demonstrated by repeating this protocol using the thyrotropin-releasing hormone receptor (TRHR-1)-Gα_{11} fusion protein. Co-expression of this receptor-G protein fusion with RGS4-eYFP also demonstrated reduced amplification of $[Ca^{2+}]_i$ (Figure 5.7). However, co-expression of RGS4^{N88S}-eYFP, RGS4^{N128A}-eYFP and RGS4^{N88S,N128A}-eYFP with the TRHR-1-Gα_{11} fusion protein did not affect the maximal $[Ca^{2+}]_i$ mobilisation, suggesting that all three RGS4 mutants have ablated GAP activity with no additive effect following expression of the double mutant (Figure 5.8).

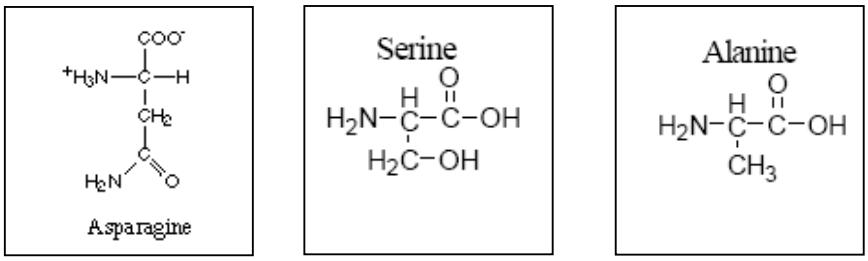

Figure 5.1 Amino acids selected for substitution of asparagine 88 and 128 in RGS4

Asparagine residues in RGS4 were selected for site directed mutagenesis. Asparagine at position at 88 was mutated to serine and asparagine at position 128 was mutated to alanine to produce three constructs, RGS4^{N88S}-eYFP, RGS4^{N128A}-eYFP and RGS4^{N88S,N128A}-eYFP.

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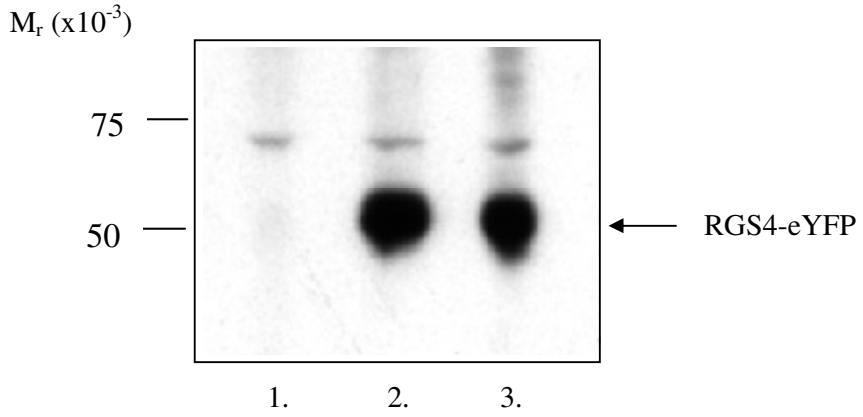


Figure 5.2 Expression and immunological detection of RGS4-eYFP and RGS4^{N88S,N128A}-eYFP

HEK293T cells were transiently transfected to express RGS4-eYFP (lane 2) or RGS4^{N88S,N128A}-eYFP (lane 3). HEK293T cells transfected with pcDNA3 were included as a control (lane 1). Cell lysates were resolved by SDS-PAGE and then immunoblotted with anti-GFP antiserum. Results shown are of a single experiment and are representative of three experiments performed.

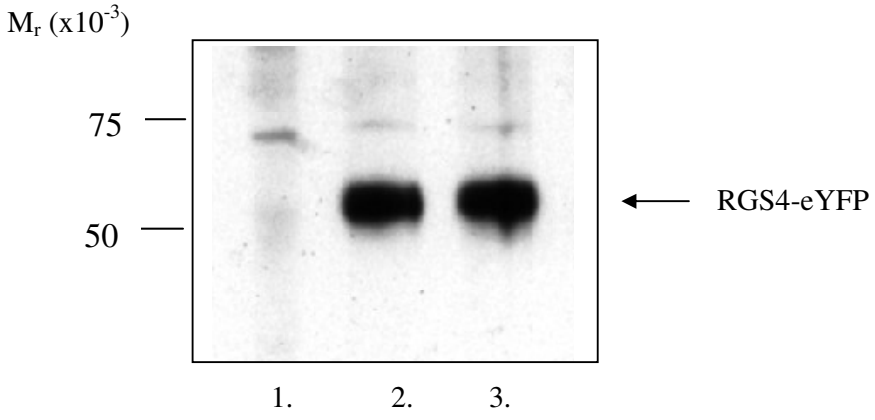


Figure 5.3 Expression and immunological detection of RGS4-eYFP and RGS4^{N88S}-eYFP

HEK293T cells were transiently transfected to express RGS4-eYFP (lane 2) or RGS4^{N88S}-eYFP (lane 3). HEK293T cells transfected with pcDNA3 were included as a control (lane 1). Cell lysates were resolved by SDS-PAGE and then immunoblotted with anti-GFP antiserum. Results shown are of a single experiment and are representative of three experiments performed.

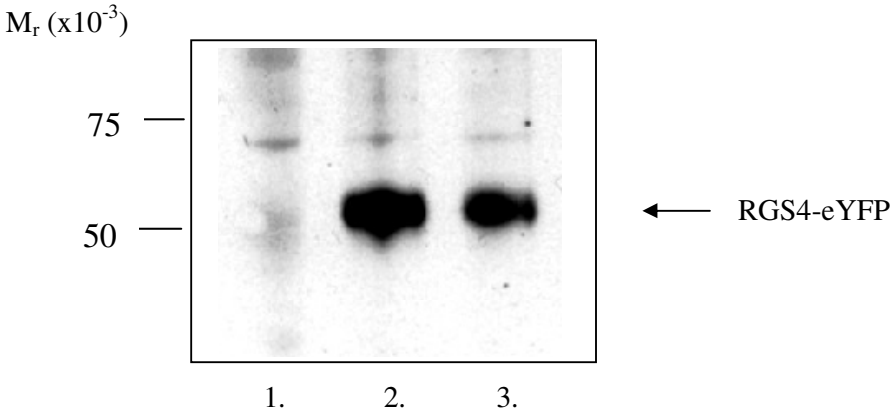


Figure 5.4 Expression and immunological detection of RGS4-eYFP and RGS4^{N128A}-eYFP

HEK293T cells were transiently transfected to express RGS4-eYFP (lane 2) or RGS4^{N128A}-eYFP (lane 3). HEK293T cells transfected with pcDNA3 were included as a control (lane 1). Cell lysates were resolved by SDS-PAGE and then immunoblotted with anti-GFP antiserum. Results shown are of a single experiment and are representative of three experiments performed.

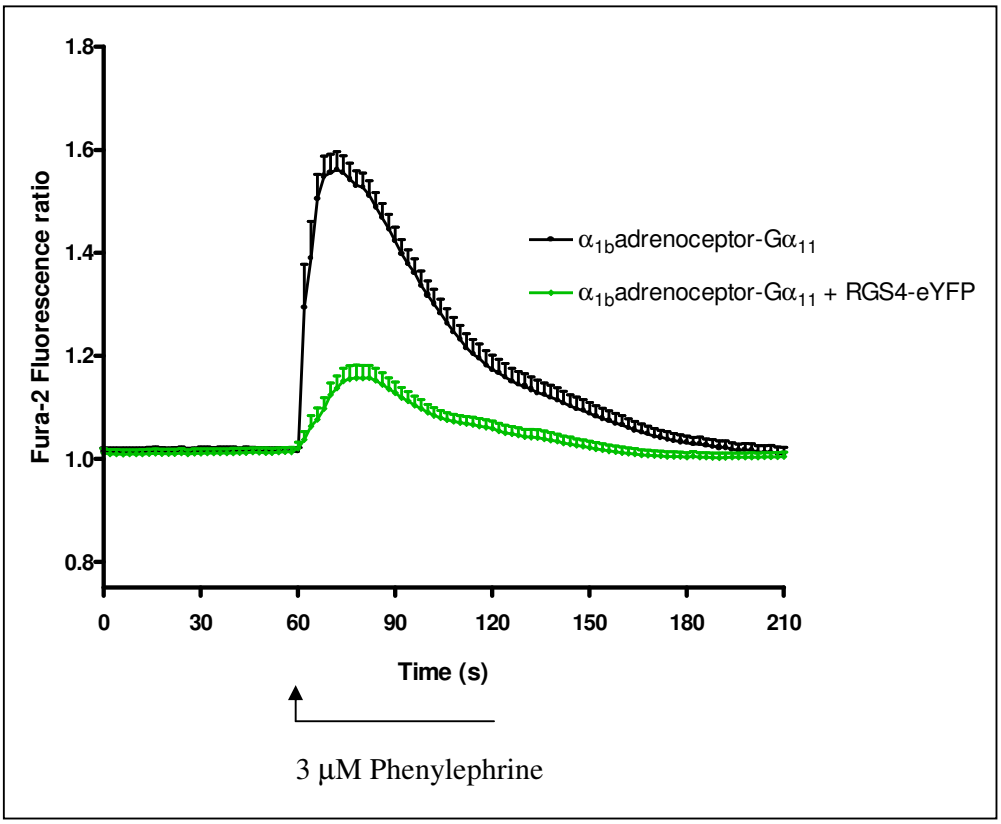


Figure 5.5 RGS4-eYFP reduces phenylephrine stimulation of $[Ca^{2+}]_i$ via the α_{1b}-adrenoceptor-Gα_{11} fusion protein

HEK293T cells were transfected to express the α_{1b}-adrenoceptor-Gα_{11} fusion protein (black line) or the α_{1b}-adrenoceptor-Gα_{11} fusion protein and RGS4-eYFP (green line). Cells were loaded with Fura-2/AM and $[Ca^{2+}]_i$ levels imaged before and after 3 μM phenylephrine was perfused over the cells for 60 seconds. Data represents means ± SEM. from 15 (α_{1b}-adrenoceptor-Gα_{11}), and 17 (α_{1b}-adrenoceptor-Gα_{11} and RGS4-eYFP) cells from 3 individual experiments performed.

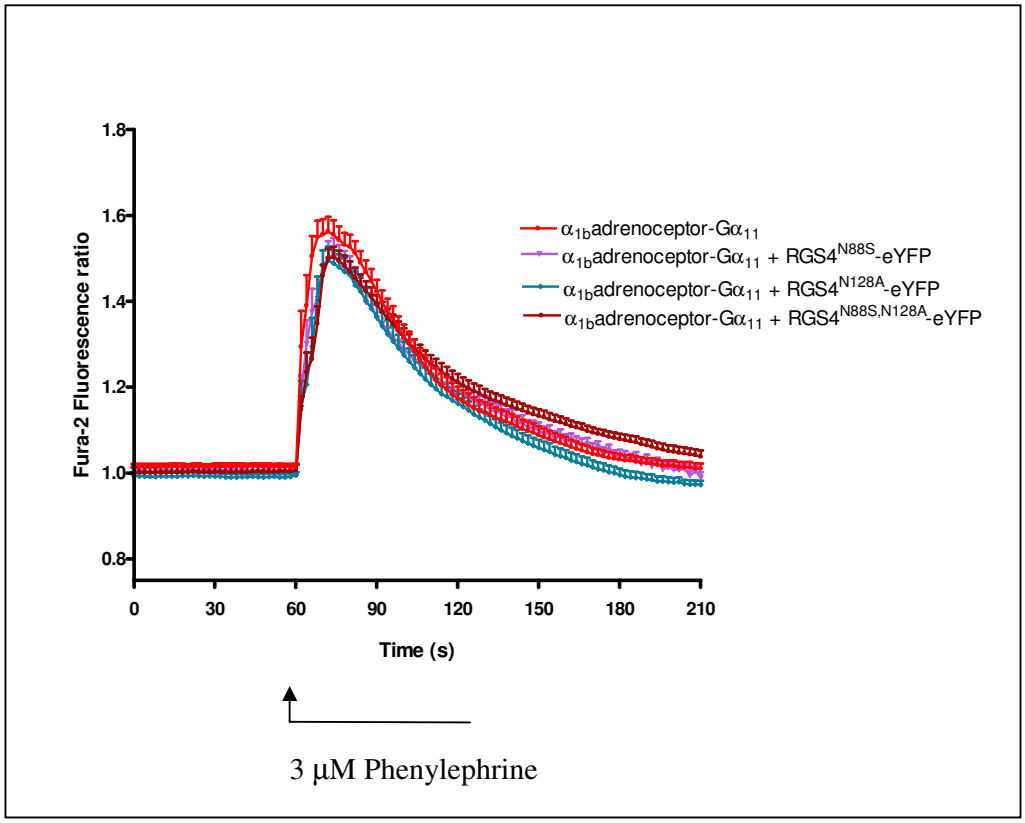


Figure 5.6 Mutation of asparagine 88 or 128 in RGS4 eliminates GAP activity against phenylephrine-stimulation of $[Ca^{2+}]_i$ via the α_{1b}-adrenoceptor-Gα_{11} fusion protein

HEK293T cells were transfected to express the α_{1b}-adrenoceptor-Gα_{11} fusion protein (black line), the α_{1b}-adrenoceptor-Gα_{11} fusion protein and RGS4^{N88S}-eYFP (pink), the α_{1b}-adrenoceptor-Gα_{11} fusion protein and RGS4^{N128A}-eYFP (orange), or the α_{1b}-adrenoceptor-Gα_{11} fusion protein and RGS4^{N88S,N128A}-eYFP (brown). Cells were loaded with Fura-2/AM and $[Ca^{2+}]_i$ levels imaged before and after 3 µM phenylephrine was perfused over the cells for 60 seconds. Data represents means ± SEM. from 15 (α_{1b}-adrenoceptor-Gα_{11}), 27 (α_{1b}-adrenoceptor-Gα_{11} and RGS4^{N88S}-eYFP), 9 (α_{1b}-adrenoceptor-Gα_{11} and RGS4^{N128A}-eYFP), and 20 (α_{1b}-adrenoceptor-Gα_{11} and RGS4^{N88S,N128A}-eYFP) cells from 3 individual experiments performed.

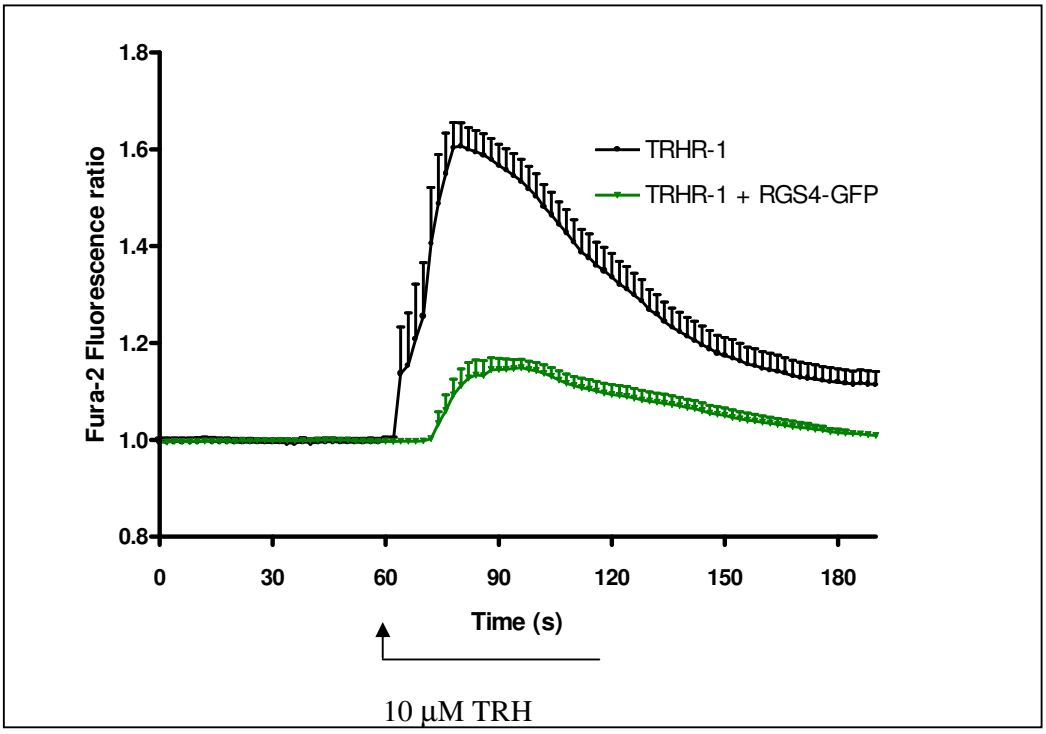


Figure 5.7 RGS4-GFP^2 reduces TRH stimulation of $[Ca^{2+}]_i$ via theTRHR–1-Gα_{11} fusion protein

HEK293T cells were transfected to express the TRHR-1-Gα_{11} fusion protein (black line) or the TRHR-1 and RGS4-GFP^2 (green line). Cells were loaded with Fura-2/AM and $[Ca^{2+}]_i$ levels imaged before and after 10 μM thyrotropin-releasing hormone (TRH) was perfused over the cells for 60 seconds. Data represents means ± SEM. from 12 (TRHR-1) and 9 (TRHR-1and RGS4-GFP^2) cells from 3 individual experiments performed.

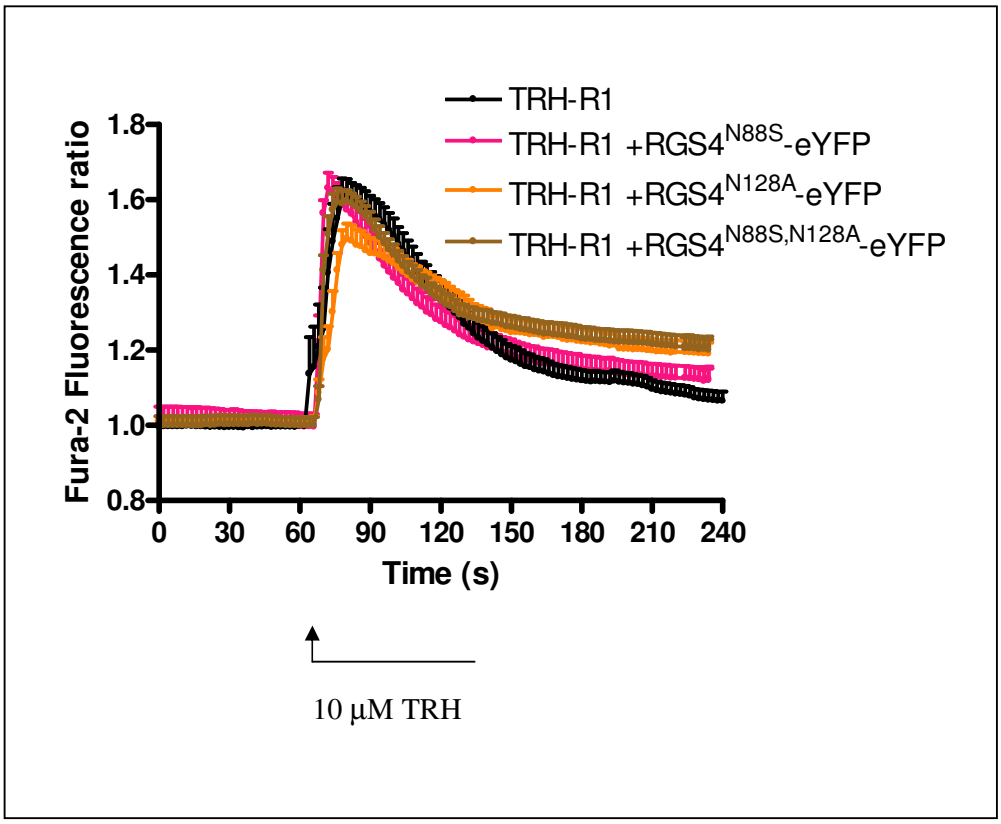


Figure 5.8 Mutation of asparagine 88 or 128 in RGS4 elimanes GAP activity against TRH stimulation of $[Ca^{2+}]_i$ via the TRHR–1-Gα_{11} fusion protein

HEK293T cells were transfected to express the TRHR-1 (black line), the TRHR-1 and RGS4^{N88S}-eYFP (pink line), the TRHR-1 and RGS4^{N128A}-eYFP (orange line), or the TRHR-1 and RGS4^{N88S,N128A}-eYFP (brown) line. Cells were loaded with Fura-2/AM and $[Ca^{2+}]_i$ levels imaged before and after 10 µM TRH was perfused over the cells for 60 seconds. Data represents means ± SEM. from 12 (TRHR-1), 13 (TRHR-1and RGS4^{N88S}-eYFP), 17 (TRHR-1and RGS4^{N128S}-eYFP), 15 (TRHR-1and RGS4^{N88S,N128A}-eYFP) cells from 3 individual experiments performed.

5.3 Discussion

Mutational analysis of RGS proteins previously identified specific amino acids important for the GAP activity of these proteins. Changing specific single amino acids at the $G\alpha_{i1}$-RGS4 binding interface can result in impaired GAP activity of RGS4. The structure of RGS4-$G\alpha_{i1}$ complexed in the transition state suggests that RGS4 could stimulate GTP hydrolysis by contributing to the overall stability of the transition state complex.

The importance of asparagine^{88} and asparagine^{128} to the GAP activity of RGS4 has previously been reported and was further investigated in this study. Mutation of both residue 88 and residue 128 to serine and alanine respectively, resulted in ablated GAP activity RGS4 towards $G\alpha_{11}$, a previously unknown detail. In addition, the importance of each individual mutation was also studied. Both RGS4^{N88S} and RGS4^{N128A} demonstrated ablated GAP activity, showing there was no additive effect of the double mutant. These RGS4 mutants were selected for use in this study due to their previous successful application to show impaired GAP activity. However, no previous study has demonstrated the use of these RGS4 mutants towards $G\alpha_{11}$.

Introduction of a mutation can often cause an alteration in expression of the protein. However, no differences in protein size or expression levels were observed among the aspragine RGS4 mutant proteins. This suggests that the impaired GAP activity of the RGS4 mutants was caused by disruption of the binding interface between the Gα subunit and RGS4.

Structural data shows that the three switch regions of $G\alpha_{i1}$ interact with the most highly conserved regions of RGS4 (Tesmer *et al.*, 1997). Threonine^{182} of switch region I interacts exclusively with seven highly conserved residues of RGS4, including asparagine^{88}. It could therefore be anticipated that when asparagine^{88} was mutated to serine, the loss of an amide and a carbonyl group would disrupt the interaction between switch region I and RGS4, causing impaired GAP activity. A previous study has investigated the mutation of asparagine^{88} to alanine (Srinivasa *et al.*, 1998). The alanine substitution also demonstrated diminished GAP activity of RGS4 and was proposed to disrupt the binding pocket in RGS4 in which theronine^{182} of the G protein binds. In this way, serine at position 88 in RGS4 would also cause a change in the amino acid side chains contributing to the binding pocket, and a loss of hydrogen bonding to the Gα subunit. Further evidence has also suggested that asparagine^{88} interacts co-operatively with the neighbouring amino acid,

glutamic acid[77]. A double mutant in which both these amino acids were mutated to alanine had a much stronger defect in GAP activity than either single mutation (Srinivasa *et al.*, 1998). However, a study using a RGS4^{N88S} fused in-frame to the α-adrenoceptor demonstrated that this fusion protein was both able to stimulate and then deactivate $G\alpha_{o1}$ (Bahia *et al.*, 2003). In the present study, asparagine[88] was functionally important in the regulation of $[Ca^{2+}]_i$ amplification. RGS4^{N88S} demonstrated a loss of GAP activity by the inability to inhibit signalling by activated $G\alpha_{11}$ in HEK293T cells. It could therefore be reasoned that aspargine[88] of RGS4 also interacts with the equivalent theronine in $G\alpha_{11}$, $G\alpha_{11}{}^{T186}$.

Also in switch region I, lysine[180] of $G\alpha_{i1}$ forms extensive van der Waals contacts with asparagine[128] of RGS4. Although a transient, weak electrical attraction of one atom for another, a number of van der Waals forces can provide an important component of protein structure. The force arises from the transient attraction between the nucleus of one atom and the electron cloud of a neighbouring atom. When asparagine is mutated to alanine, the loss of a carbonyl group and a hydroxyl group could be anticipated to affect the spatial distance between the molecules. The attraction between atoms can only operate over a short distance and if two atoms move closer together then severe repulsion between the two nuclei of the atoms can occur. If the atoms move further apart the weak attractions will be broken. The presence of alanine at position 128 could therefore disrupt this bonding and reduce the contact of RGS4 and $G\alpha_{i1}$, weakening the ability of RGS4 to stabilise the transition state of the GTPase cycle.

Switch region I and II of $G\alpha_{i1}$ also interact with asparagine[128] in RGS4 (Tesmer *et al.*, 1997). The importance of asparagine[128] to the function of RGS4 is highlighted by the almost complete conservation of this residue within the RGS family. The only natural substitution is for serine in some RGS proteins such as GAIP (Vries *et al.*, 1995;Berman *et al.*, 1996b;Popov *et al.*, 1997;Posner *et al.*, 1999). Natochin *et al.*, mutated aspargine[128] in RGS4 to serine to and demonstrated that serine proves to be the best substitution for asparagine in regards to preserving the GAP activity of the protein (Natochin *et al.*, 1998). Asparagine[128] is the only RGS4 residue that projects into the active site of $G\alpha_{i1}$ and together with its location at the binding interface and interaction with three $G\alpha_{i1}$ residues at the active site (glutamine[204], glutamic acid[207] and lysine[180]) further underline the importance of asparagine[128] to the GAP activity of RGS4.

Previous results from mutating asparagine^{128} in RGS4 to several other amino acids have indicated that some GAP activity of RGS4 can be retained (Natochin *et al.*, 1998). However, all results using RGS4^{N128A} suggested that the alanine mutant had low GAP activity (Natochin *et al.*, 1998;Srinivasa *et al.*, 1998;Posner *et al.*, 1999;Bahia *et al.*, 2003). Although now disputed (Srinivasa *et al.*, 1998), asparagine^{128} has previously been suggested to be critical in orientating and polarising a hydrolytic water molecule in the transition state of GTP hydrolysis (Posner *et al.*, 1999). Replacing asparagine^{128} with the hydrophobic amino acid alanine would prevent any interaction with a water molecule and the RGS protein would be unable to stabilise conformational changes in the GTPase cycle.

This study demonstrated that RGS4^{N88S,N128A} displayed loss of GAP activity towards $G\alpha_{11}$. Both RGS4^{N88S} and RGS4^{N128A} also displayed no functional GAP activity towards $G\alpha_{11}$ with no apparent change in GAP activity following expression of a single asparagine mutation. Following transfection, single cells were selected that were fluorescent, a marker for cells positively transfected with RGS4-eYFP. In a field of view, cells invariably show a range of fluorescence expression and to allow reliable comparison between mutants, only the brightest fluorescent cells were selected. It is possible, therefore, that in using agonist-activated $[Ca^{2+}]_i$ amplification to measure GAP activity, small differences in GAP activity between mutants may have been concealed. It would perhaps been hypothesised that RGS4^{N88S} and RGS4^{N128A} could have retained some GAP activity, compared to the double mutant. As previously mentioned, structural and functional data suggest that RGS4 stimulates GTP hydrolysis by stabilising the transition state conformation. The additive effect of disrupting two areas of the binding interface between RGS4 and $G\alpha_{11}$ would be anticipated to further impair the GAP activity of RGS4. However, these single amino acid mutations seem able to totally disrupt the GAP activity of the protein, perhaps being sufficient to achieve a global conformational change of the complex.

Finally, the identity of Gα subtype may play a role in determining the GAP activity of RGS proteins. The loss of GAP activity of the asparagine RGS4 mutants in this study is consistent with other studies, when the Gα substrate was $G\alpha_o$ and $G\alpha_{i1}$ (Druey and Kehrl, 1997;Bahia *et al.*, 2003). The amino acids of Gα subunit proposed to be involved in binding to residues 88 and 128 of RGS4 are highly conserved, although Posner *et al.*, demonstrated RGS4^{N128F} differentially decreased the GAP activity towards $G\alpha_{i1}$ compared to $G\alpha_q$. This mutation was suppressed by mutating the residue in $G\alpha_i$ to that found in $G\alpha_q$ (^{K180P}) (Posner *et al.*, 1999). The identity of the Gα subunit may alter the function of

asparagine[128] and perhaps comparing the agonist-activated $[Ca^{2+}]_i$ mobilisation by the α_{1b}-adrenoceptor fused to $G\alpha_q$ would have been informative.

The lifetime of the RGS-Gα complexes determines the GAP activity of RGS proteins for plasma membrane-associated heterotrimeric G proteins and determines the signal strength and regulates activity of effectors. Certainly, RGS4 may also interact with the receptor and the Gβγ subunit to further stabilise the signalling complex but the total loss of all GAP activity in such mutant RGS proteins could prove useful in future drug discovery. Small molecules which inhibit RGS-Gα interaction have been proposed as novel drugs which could be used to treat numerous disease states. As such, the model of these mutant aspargine resides in RGS4 could be crucial to novel drug development.

6 Chapter 6

6.1 Introduction

Genetic and biochemical studies have demonstrated that RGS proteins interact directly with Gα subunits (Berman *et al.*, 1996b). This physical association allows RGS proteins to increase the rate of hydrolysis of GTP to GDP on the Gα subunit and negatively regulate signalling. Multiple RGS proteins are known to interact with each Gα subtype and establishing the contribution of endogenous RGS proteins to signal transduction *in vivo* was initially thought to require construction of specific knockout mutants. However, the identification and characterisation of a Gα subunit mutant that specifically disrupts the interaction with RGS proteins provided a new approach to study the endogenous function of RGS proteins (DiBello *et al.*, 1998). A single amino acid change in the Gα subunit produced a G protein insensitive to RGS action.

This point mutation in the Gα subunit was originally identified in *S. cervisiae* in a screen for yeast strains that were supersensitive to pheromone. One such strain had a single glycine to serine mutation in the yeast Gα subunit, Gpa1, which escaped regulation by the RGS protein, Sst2. This glycine residue was shown to be conserved in the mammalian Gα subunits and subsequent studies have shown that the corresponding mutation in $G\alpha_q$, $G\alpha_{i1}$ and $G\alpha_o$ also produces RGS-insensitivity. These mutant Gα subunits have become important tools for studying the endogenous role of RGS proteins. Indeed, with multiple RGS proteins being able to interact with each Gα subtype, inactivating every one of these endogenous RGS proteins would be difficult. Instead, the use of a single mutant Gα subunit can be used to ascertain the combined role of endogenous RGS proteins on the function of a particular Gα subunit.

Transient over-expression of RGS proteins, or more usually, epitope-tagged RGS proteins, produces an unnatural system and could lead to invalid conclusions. However, most previous studies have used transient over-expression to determine RGS function and overcome the low natural abundance of endogenous RGS proteins. As an alternative, transiently expressing a Gα subunit insensitive to RGS proteins could elucidate endogenous RGS function. This study determines if the previously identified RGS-insensitive mutation can be effectively transferred into $G\alpha_{11.}$ $G\alpha_q$ and $G\alpha_{11}$ share 98% homology and examination of mammalian $G\alpha_q^{G188S}$ in cells co-transfected with the $5HT_{2C}$

receptor has previously revealed that, compared to wild-type mammalian $G\alpha_q$, the response of $G\alpha_q^{G188S}$ was not inhibited by RGS7 (DiBello *et al.*, 1998). However, it has now been determined that some RGS proteins can discriminate between the two highly related $G\alpha_q$ family subunits (Ladds *et al.*, 2007) and the aim of this study was to investigate signal transduction of a potentially RGS-insensitive $G\alpha_{11}$ ($G\alpha_{11}^{G188S}$).

Agonist-activation of receptors linked to the $G\alpha_q$ subfamily of G proteins produce amplification of the second messenger, calcium. The intracellular Ca^{2+} response produced in single cells was used to investigate the role of $G\alpha_{11}^{G188S}$. Thus, the use of mouse embryonic fibroblast cells (EF88) derived from a combined $G\alpha_q$ and $G\alpha_{11}$ knockout mouse was crucial in this study. The $G\alpha_{11}^{G188S}$ protein was fused in-frame to the C-terminal of the α_{1b}-adrenoceptor. Following agonist occupation, the rise in $[Ca^{2+}]_i$ mediated by a wild-type α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein (Stevens *et al.*, 2001) was compared to the rise in $[Ca^{2+}]_i$ mediated by the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein in EF88 cells. The generality of agonist-activated $[Ca^{2+}]_i$ mediated by $G\alpha_{11}^{G188S}$ was demonstrated by transferring the ^{G188S} mutation to the $G\alpha_{11}$ protein fused in-frame to the C-terminal of the TRHR-1. The level of $[Ca^{2+}]_i$ produced upon activation of the TRHR-1-$G\alpha_{11}^{G188S}$ fusion protein was then compared to the wild-type TRHR-1-$G\alpha_{11}$ fusion protein.

Biochemical studies have previously suggested that the RGS-insensitive Gα subunits display a reduced affinity for RGS4 (Lan *et al.*, 1998). G proteins, unable to be regulated by RGS proteins, have also been reported conflictingly to be both able (Druey *et al.*, 1998) and unable (Roy *et al.*, 2003) to translocate RGS4 to the plasma membrane. Both the α_{1b}-adrenoceptor and the TRHR-1-G protein fusion proteins were used to study the subcellular localisation of RGS4-eYFP in receptor-activated HEK293T cells.

The [^{35}S]GTPγS binding assay was used to measure the level of G protein activation of $G\alpha_{11}^{G188S}$. The binding of the non-hydrolysable analogue [^{35}S]GTPγS to the Gα subunit of the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein was compared to the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein. The only reported effect of the RGS-insensitive mutation is to prevent RGS action on Gα. However, using these methods, additional novel roles for the RGS insensitive mutation, ^{G188S} in $G\alpha_{11}$ became apparent.

6.2 Results

6.2.1 Translocation of RGS4 in the presence $G\alpha_{11}^{G188S}$

The intracellular translocation of RGS4 was previously examined in this study by transfection of HEK293T cells with C-terminally eYFP-tagged RGS4 (Chapter 3, Section 3.2.3). In agreement with previous studies, the vast majority of transiently transfected RGS4-eYFP in HEK293T cells was localised within the cytoplasm (Druey *et al.*, 1998;Chatterjee and Fisher, 2000). It was also demonstrated that over-expressing agonist activated receptor promoted the translocation RGS4-eYFP from the cytoplasm to the plasma membrane in HEK293T cells (Chapter 3, Section 3.2.5).

In order to investigate the subcellular localisation of RGS4 in the presence of an agonist-activated receptor fused to an RGS-insensitive G protein, HEK293T cells were transiently co-transfected with either (A) the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein and RGS4-eYFP or (B) the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein and RGS4-eYFP (Figure 6.1). Using microscopy, the localisation of RGS4-eYFP in fixed cell samples was determined. As before, the blue colour (i) represents Hoechst 33342 DNA staining that was used to identify the nuclei in these cells. The green colour (ii) represents eYFP fluorescence from expressed RGS4-eYFP and the red colour (iii) represents WGA-Alexa Fluor 594 used to label the plasma membrane. To identify any RGS4-eYFP localised at the plasma membrane, an overlay image is also shown (iv). In these overlay images yellow colour represents any RGS4-eYFP that is co-localised with WGA-Alexa Fluor 594 at the plasma membrane. Figure 6.1 demonstrates that some RGS4-eYFP in HEK293T cells transiently co-transfected with the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein and stimulated with 100 μM phenylephrine, was located at the plasma membrane. In contrast, RGS4-eYFP in HEK293T cells transiently co-transfected with the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein and stimulated with 100 μM phenylephrine, was predominantly localised within the cytoplasm. Agonist activation of the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein therefore did not promote detectable translocation of RGS4-eYFP in HEK293T cells.

The subcellular localisation and translocation pattern of RGS4-eYFP co-expressed with $G\alpha_{11}^{G188S}$ was also confirmed by determining the percentage fluorescence of RGS4-eYFP in the plasma membrane of these co-transfected HEK293T cells (Figure 6.2). The percentage of RGS4-eYFP fluorescence in the plasma membrane of cells co-transfected with the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein (defined as 100%) was directly compared to

the percentage of RGS4-eYFP fluorescence in the plasma membrane of these cells stimulated with 100 μM phenylephrine. In these cells, agonist-stimulation translocated RGS4-eYFP to the plasma membrane (159 ± 2%). In contrast, agonist-stimulation did not translocate RGS4-eYFP to the plasma membrane when cells were co-transfected with the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein (100 ± 1%). This further confirmed that the predicted RGS-insensitive $G\alpha_{11}$ mutation must indeed directly influence the interaction of the Gα subunit and RGS4.

A similar pattern of RGS4-eYFP translocation was demonstrated when HEK293T cells were transiently co-transfected with the TRHR-1-$G\alpha_{11}$ fusion protein and challenged with the TRHR-1 selective agonist TRH (Figure 6.3). Microscopy revealed that RGS4-eYFP was still predominantly localised in the cytoplasm in cells co-transfected with the TRHR-1-$G\alpha_{11}^{G188S}$ fusion protein and stimulated with TRH (Figure 6.3(B)). In contrast, the presence of the agonist-stimulated TRHR-1-$G\alpha_{11}$ fusion protein showed co-localisation of RGS4-eYFP and the plasma membrane marker WGA-Alexa Fluor 594 (Figure 6.3 (A)). As before, the percentage of RGS4-eYFP fluorescence in the plasma membrane in cells co-transfected with the TRHR-1-$G\alpha_{11}$ fusion protein or the TRHR-1-$G\alpha_{11}^{G188S}$ fusion protein was also examined (Figure 6.4). The TRHR-1-$G\alpha_{11}^{G188S}$ fusion protein did not translocate RGS4-eYFP to the plasma membrane. The direct effect of the predicted RGS-insensitive $G\alpha_{11}$ mutation on the interaction to RGS4 is therefore not restricted to the action of a single receptor.

6.2.2 [^{35}S]GTPγS binding to the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein

In order to investigate the expression levels of the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein, membranes transiently expressing the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion proteins, were subjected to radioligand binding. Specific binding of the α_{1b}-adrenoceptor antagonist [^{3}H]prasozin was determined by subtracting the level of binding observed in the presence of the non-specific antagonist phentolamine (100 μM) (Figure 6.5). Levels of expression (B_{max}) of the receptor-G protein fusion proteins varied between individual transfections, but importantly the affinity (K_d) of the α_{1b}-adrenoceptor-$G\alpha_{11}$ (0.54 ± 0.01 nM) and of the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ (0.49 ± 0.01 nM) for [^{3}H]prasozin was not affected by the ^{G188S} mutation in $G\alpha_{11}$.

From these binding studies, HEK293T cell membranes with equivalent amounts of each transfected fusion protein were added to [^{35}S]GTPγS binding assays. A maximally effective concentration of phenylephrine increased the level of bound [^{35}S]GTPγS to the α_{1b}-adrenoceptor-Gα_{11} fusion protein. However, the specific effectiveness of phenylephrine to stimulate [^{35}S]GTPγS binding to the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion protein was significantly reduced by 90.2 ± 0.85 % ($p<0.01$) (Figure 6.6). Employing a range of concentrations of phenylephrine revealed that this difference could be attributed to the efficacy of the G protein to bind [^{35}S]GTPγS, as the reduction in the ability of phenylephrine to stimulate [^{35}S]GTPγS binding to the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion protein was not accompanied by a reduction in potency of the agonist (Figure 6.7). The pEC_{50} of phenylephrine for the α_{1b}-adrenoceptor-Gα_{11} fusion protein (pEC_{50} 6.14 ± 0.09) and the α_{1b}-adrenoceptor-Gα_{11}^{G188S} (pEC_{50} 6.35 ± 0.06) remained unchanged ($p>0.05$) (Figure 6.7 (B)).

Binding of [^{35}S]GTPγS to the α_{1b}-adrenoceptor-Gα_{11} fusion protein was determined by immunoprecipitation with an antiserum (CQ) which identifies the C-terminal decapeptide of Gα_{11}. To ensure that the reduction in the effectiveness of phenylephrine to stimulate [^{35}S]GTPγS binding on the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion protein was not due to the antiserum CQ being unable to recognise the mutated G protein, membranes expressing equal levels of receptor-Gα protein fusion, used in the [^{35}S]GTPγS binding assay, were treated with N-Glycosidase F, separated by SDS-PAGE and detected with the antiserum, CQ (Figure 6.8). Previously, western blot analyses of the α_{1b}-adrenoceptor-Gα_{11} fusion demonstrated distinct doublets (at approximately 90 kDa and 110 kDa) that probably reflected differential glycosylation of the receptor (Liu *et al.*, 2002). Pre-treatment of membranes with N-Glycosidase F released all common classes of N-glycans from the protein backbone and produced a single comparable band for each fusion protein. Bands corresponding to α_{1b}-adrenoceptor-Gα_{11} (lane 1) and α_{1b}-adrenoceptor-Gα_{11}^{G188S} (lane 2) were of comparable intensity, indicating similar reactivity of the two fusion proteins to the antiserum CQ. Decreased reactivity to the antiserum CQ of the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion protein is therefore not responsible for the reduction in the effectiveness of phenylephrine to stimulate [^{35}S]GTPγS binding.

6.2.3 Intracellular Ca^{2+} mobilisation following expression of the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein

To investigate the effect of $G\alpha_{11}^{G188S}$ on downstream signalling, the ability of the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein and the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein to elevate $[Ca^{2+}]_i$ in response to phenylephrine was compared. To investigate the effect of the mutated $G\alpha_{11}$ subunit it was imperative to use a cell line with no endogenous wild-type $G\alpha_{11}$. Presence of any wild-type $G\alpha_{11}$ would produce a response that could not be exclusively attributed to the presence of a mutation. To verify the constitutive knockout of endogenous $G\alpha_q$ and $G\alpha_{11}$ in EF88 cells, cell lysates of EF88 cells were separated by SDS-PAGE and probed with the antiserum CQ (Figure 6.9 (lane3)). As a positive control, EF88 cells transfected with $G\alpha_{11}$ (lane1) or $G\alpha_{11}^{G188S}$ (lane2) were included. Bands corresponding to $G\alpha_{11}$ at ~37 kDa in lane 1 and lane 2, but not in lane 3 confirmed that no endogenous $G\alpha_q/G\alpha_{11}$ are expressed in EF88 cells and that the transfection of cells was effective. This immunoblot also further confirms the reactivity of the RGS-insensitive Gα subunit with the antiserum CQ.

Thus, the EF88 cells used in this study do not express endogenous $G\alpha_q/G\alpha_{11}$ and, when introduced transiently, both the $G\alpha_{11}$ and the $G\alpha_{11}^{G188S}$ proteins were equally expressed by these cells. The effect of the ^{G188S} mutation in $G\alpha_{11}$ on the activity of downstream signalling could now be investigated. Expression of RGS4 with GFP^2 fused to the C terminal, allowed the identification of positively transfected fibroblasts. Following co-transfection of the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein and RGS4-GFP^2, there was a decrease in the maximal agonist-mediated elevation of $[Ca^{2+}]_i$ compared to expression of the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein alone (Figure 6.10). The α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein was transfected as before and compared to EF88 cells co-transfected with the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein (Figure 6.11). Surprisingly, expression of the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein almost eliminated the agonist-mediated elevation of $[Ca^{2+}]_i$. Co-expression of RGS4-GFP^2 did not alter the $[Ca^{2+}]_i$ mobilisation in these cells.

The $[Ca^{2+}]_i$ response in EF88 cells transiently transfected with the TRHR-1-$G\alpha_{11}^{G188S}$ fusion protein and stimulated with TRH (10 μM) also demonstrated virtually no $[Ca^{2+}]_i$ mobilisation compared to the agonist activated wild-type TRHR-1-$G\alpha_{11}$ fusion protein (Figure 6.12). The reduction in $[Ca^{2+}]_i$ elevation of the ^{G188S} mutation in $G\alpha_{11}$ is not restricted to the action of the α_{1b}-adrenoceptor.

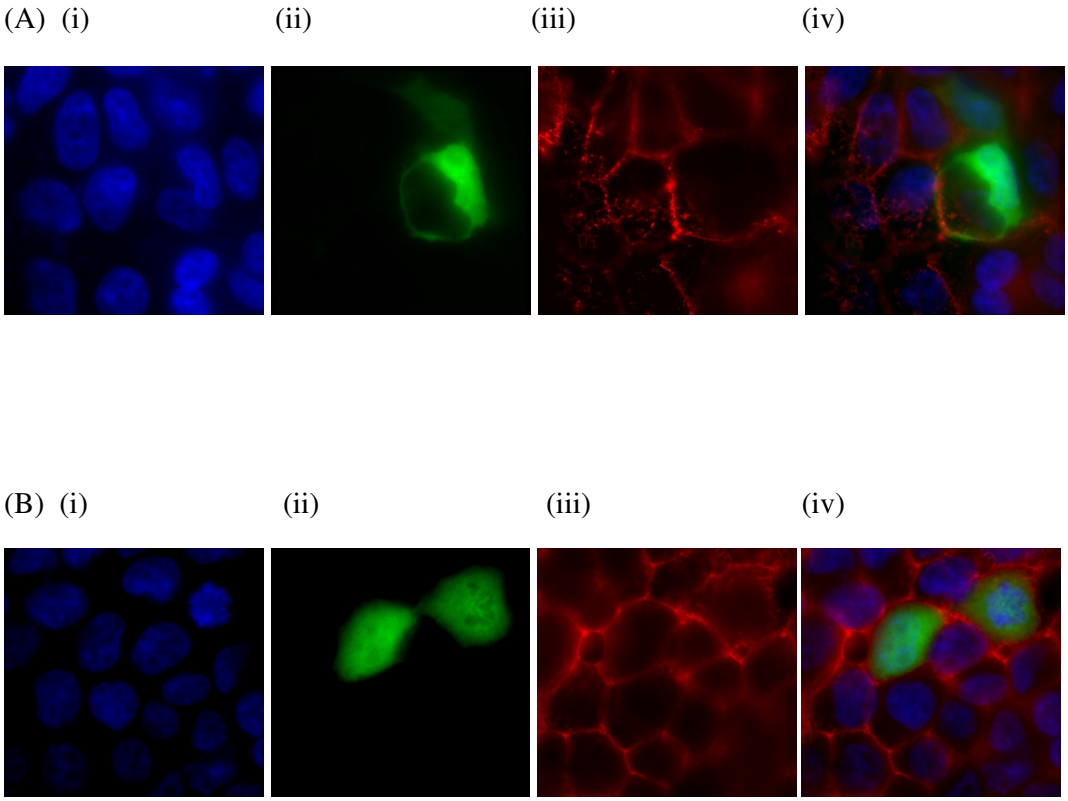


Figure 6.1 The cellular localisation of RGS4-eYFP co-expressed with the α_{1b}-adrenoceptor-Gα_{11} fusion protein or the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion protein and stimulated with phenylephrine

HEK293T cells grown on coverslips were transiently co-transfected with (A) RGS4-eYFP and the α_{1b}-adrenoceptor-Gα_{11} and stimulated with 100 µM phenylephrine for 30 minutes or (B) RGS4-eYFP and α_{1b}-adrenoceptor-Gα_{11}^{G188S} and stimulated with 100 µM phenylephrine for 30 minutes. Images were generated using an inverted Nikon TE2000-E microscope equipped with a 60 x, (NA=1.4), oil-immersion Plan Fluor Apochromat lens and a cooled digital Cool Snap-HQ CCD camera. (i) Hoechst 33342 nuclei staining (blue) (ii) RGS4-eYFP (green) or (iii) WGA-Alexa Fluor 594 membrane staining (red) (iv) merged images. Results shown are of a single experiment and are representative of three individual experiments performed.

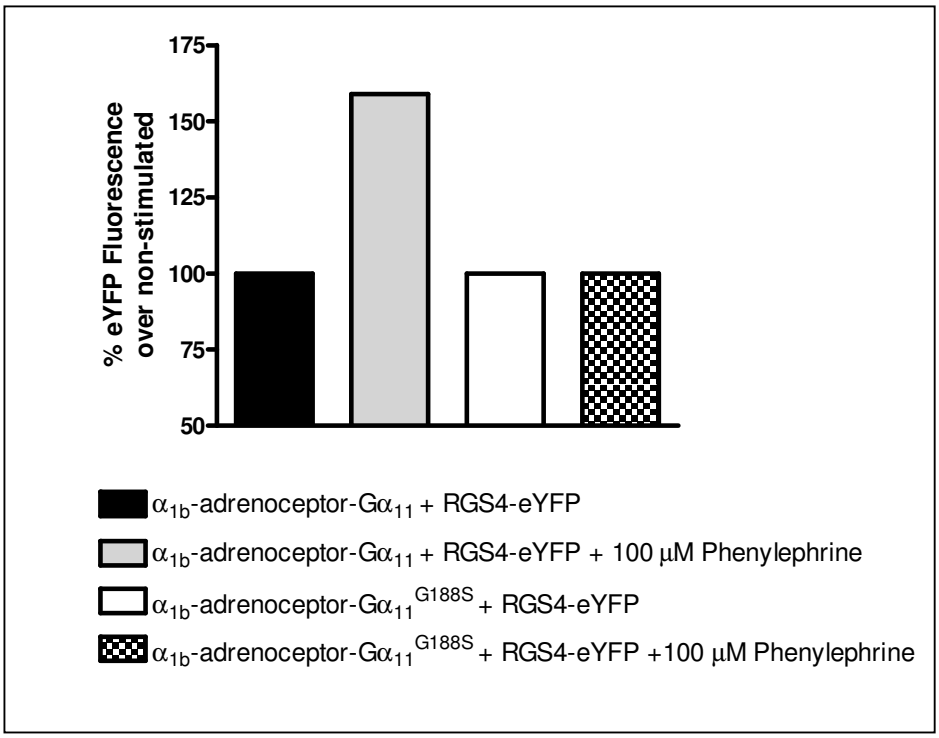


Figure 6.2 Comparison of percentage RGS4-eYFP fluorescence at the plasma membrane when co-expressed with the α_{1b}-adrenoceptor-Gα_{11} fusion protein or the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion protein

HEK293T cells were transiently transfected to express RGS4-eYFP and co-transfected with the α_{1b}-adrenoceptor-Gα_{11} fusion protein or the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion proteins and stimulated with 100 μM phenylephrine for 30 minutes. The membrane fraction of cells co-expressing the fusion proteins was measured for total RGS4-eYFP fluorescence (defined as 100%) and compared to cells not stimulated with phenylephrine. Data shown are from triplicate determinations (mean ± SEM.) and are representative of three experiments performed.

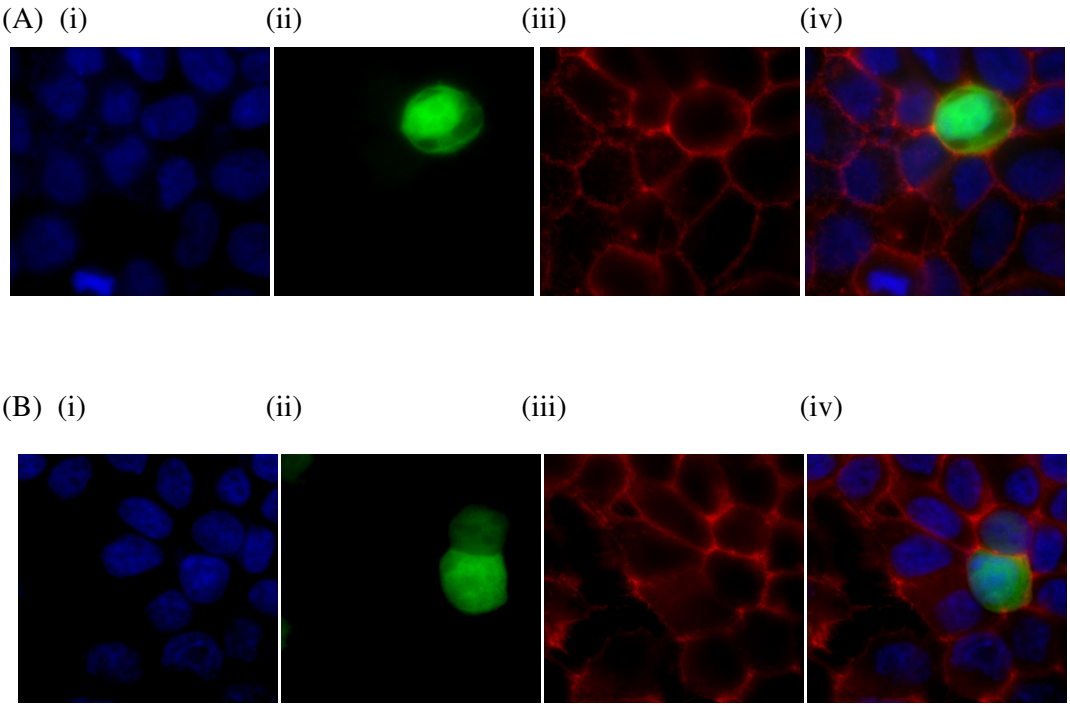


Figure 6.3 The cellular localisation of RGS4-eYFP co-expressed with the TRHR-1-Gα_{11} fusion protein or the TRHR-1-Gα_{11}^{G188S} fusion protein and stimulated with TRH

HEK293T cells grown on coverslips were transiently co-transfected with (A) RGS4-eYFP and the TRHR-1-Gα_{11} fusion protein and stimulated with 10 µM TRH for 30 minutes or (B) RGS4-eYFP and the TRHR-1-Gα_{11}^{G188S} fusion protein and stimulated with 10 µM TRH 30 minutes. Images were generated using an inverted Nikon TE2000-E microscope equipped with a 60 x, (NA=1.4), oil-immersion Plan Fluor Apochromat lens and a cooled digital Cool Snap-HQ CCD camera. (i) Hoechst 33342 nuclei staining (blue) (ii) RGS4-eYFP (green) or (iii) WGA-Alexa Fluor 594 membrane staining (red) (iv) merged images. Results shown are of a single experiment and are representative of three experiments performed.

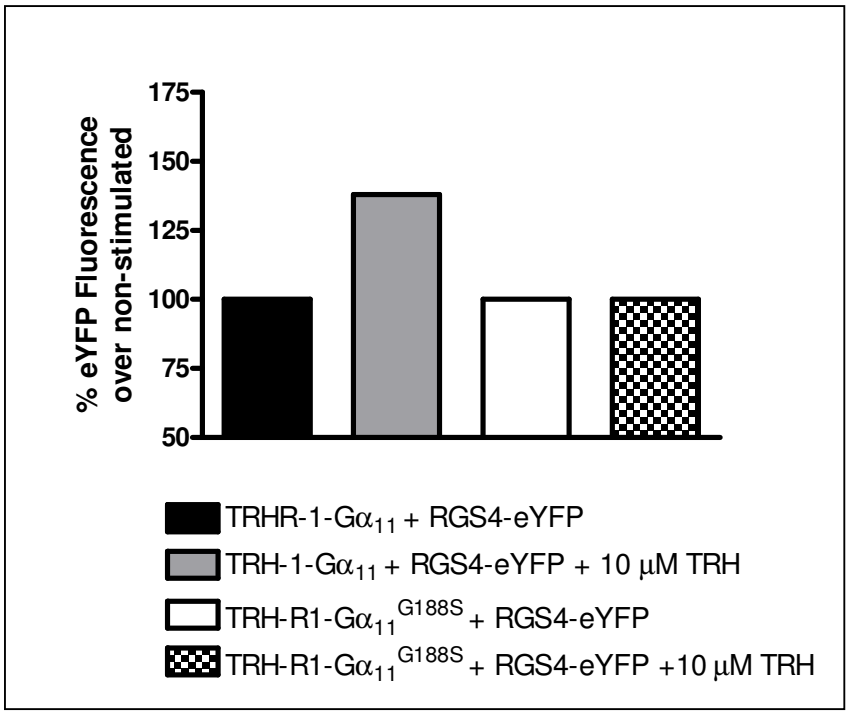
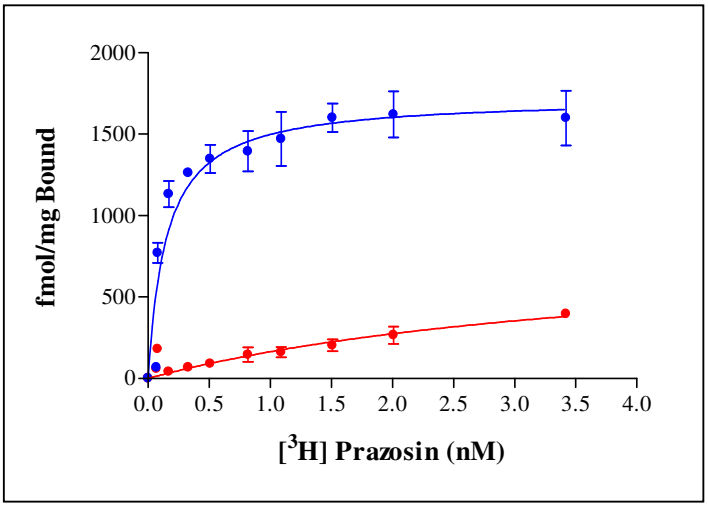


Figure 6.4 Comparison of percentage RGS4-eYFP fluorescence at the plasma membrane when co-expressed with the TRHR-1-Gα_{11} fusion protein or the TRHR-1-Gα_{11}^{G188S} fusion protein

HEK293T cells were transiently transfected to express RGS4-eYFP and co-transfected with the TRHR-1 -Gα_{11} fusion protein or the TRHR-1-Gα_{11}^{G188S} fusion protein and stimulated with 10 μM TRH for 30 minutes. The membrane fraction of cells co-expressing the fusion protein was measured for RGS4-eYFP fluorescence (defined as 100%) and compared to cells not stimulated with TRH. Data shown are from triplicate determinations (mean ± SEM, very small and therefore cannot be seen.) and are representative of three experiments performed.

(A)



(B)

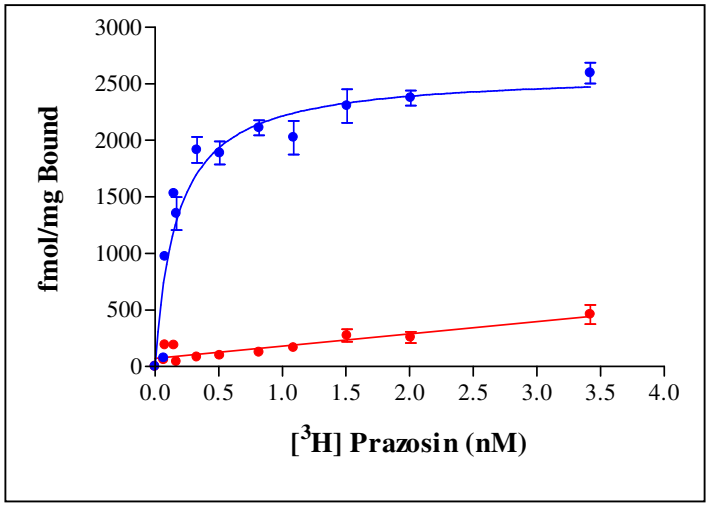


Figure 6.5 Binding of [^{3}H]prasozin to the α_{1b}-adrenoceptor-Gα_{11} fusion protein or the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion protein

HEK293T cells were transfected to express (A) the α_{1b}-adrenoceptor-Gα_{11} fusion protein or (B) the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion protein. 2 µg of each membrane preparation was used to measure the binding (blue) of [^{3}H]prasozin (0-3.4 nM). Non-specific binding (red) was determined in the presence of phentolamine (100 µM). Data shown are from triplicate determinations (mean ± SEM.) and are representative of three individual experiments performed.

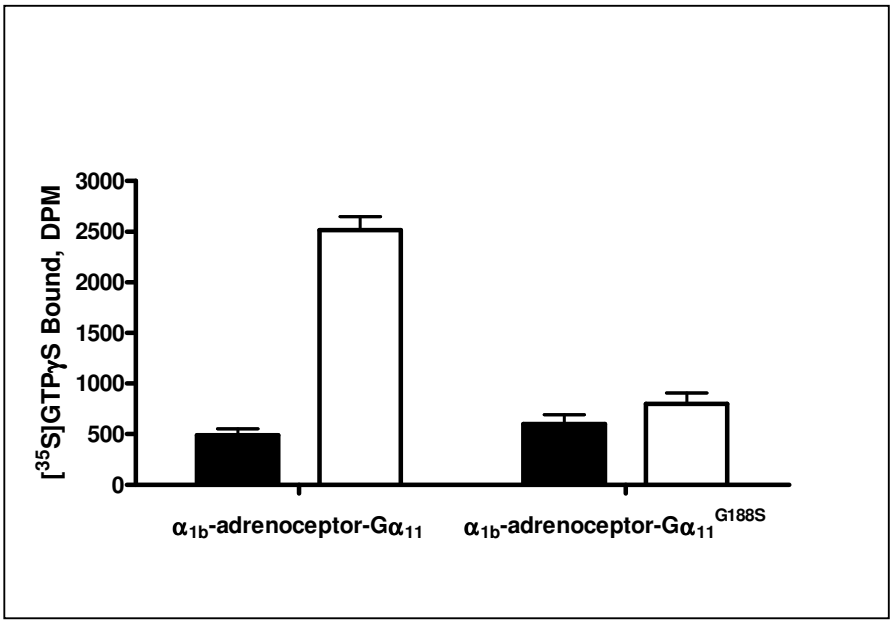
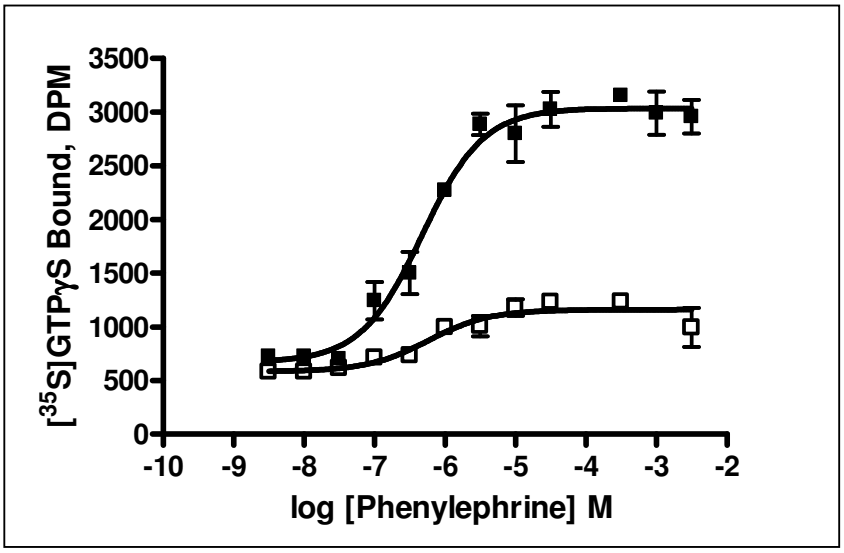


Figure 6.6 Maximally effective concentration of phenylephrine-stimulated [^{35}S]GTPγS binding to the α_{1b}-adrenoceptor-Gα_{11} fusion protein or the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion protein

Membranes expressing 90 fmol of the α_{1b}-adrenoceptor-Gα_{11} fusion protein (left) or the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion protein (right) were added to [^{35}S]GTPγS binding assays in the absence (filled bars) or presence of a single concentration of phenylephrine (100 μM) (open bars). Prior to scintillation counting samples were subsequently immunoprecipitated with an anti-G$\alpha_{q/11}$ antiserum CQ. Data shown are from triplicate determinations (mean ± SEM.) and are representative of three individual experiments performed.

(A)


[35S]GTPγS Bound, DPM
3500
3000
2500
2000
1500
1000
500
0
-10
-9
-8
-7
-6
-5
-4
-3
-2
log [Phenylephrine] M

(B)

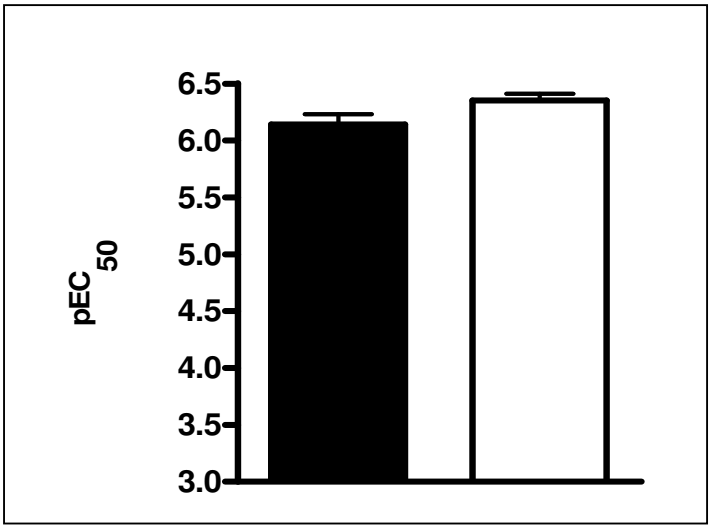

pEC50
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0

Figure 6.7 A range of concentrations of phenylephrine-stimulated [^{35}S]GTPγS binding to the α_{1b}-adrenoceptor-Gα_{11} fusion protein or the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion protein

(A) Membranes expressing 90 fmol of the α_{1b}-adrenoceptor-Gα_{11} fusion protein (filled symbols) or the α_{1b}-adrenoceptor- Gα_{11}^{G188S} fusion protein (open symbols) were added to [^{35}S]GTPγS binding assays in the presence of a range of concentrations of phenylephrine (3 nM – 3 mM). Samples were subsequently immunoprecipitated with an anti-G$\alpha_{q/11}$ antiserum and counted. Data shown are from triplicate determinations (mean ± SEM.) and are representative of three individual experiments performed.

(B) The potency of phenylephrine to stimulate [^{35}S]GTPγS binding in the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion protein compared to the wild-type fusion protein. pEC_{50} of phenylephrine for the α_{1b}-adrenoceptor- Gα_{11} fusion protein (pEC_{50} 6.14 ± 0.09) and the α_{1b}-adrenoceptor-Gα_{11}^{G188S} (pEC_{50} 6.35 ± 0.06) remains unchanged ($p>0.05$). Data shown are mean ± SEM of three individual experiments performed.

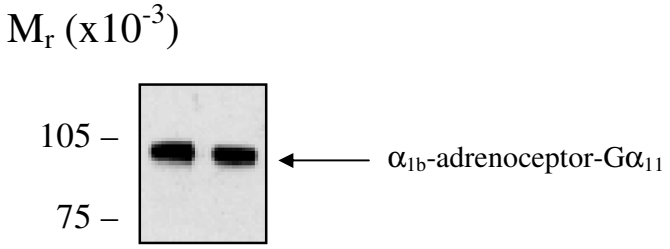


Figure 6.8 Expression and immunological detection of the α_{1b}-adrenoceptor-Gα_{11} fusion protein and the α_{1b}-adrenoceptor-Gα_{11}^{G188S} fusion protein

HEK293T cells were transfected to transiently express the α_{1b}-adrenoceptor-Gα_{11} fusion protein (lane 1) or the α_{1b}-adrenoceptor- Gα_{11}^{G188S} fusion protein (lane 2). Cell membranes containing 90 fmol of fusion protein, as calculated from [^{3}H]prazosin binding studies, were treated with N-glycosidase F and resolved by SDS-PAGE and then immunoblotted with an anti-G$\alpha_{q/11}$ antiserum CQ. Results shown are of a single experiment and are representative of three individual experiments performed.

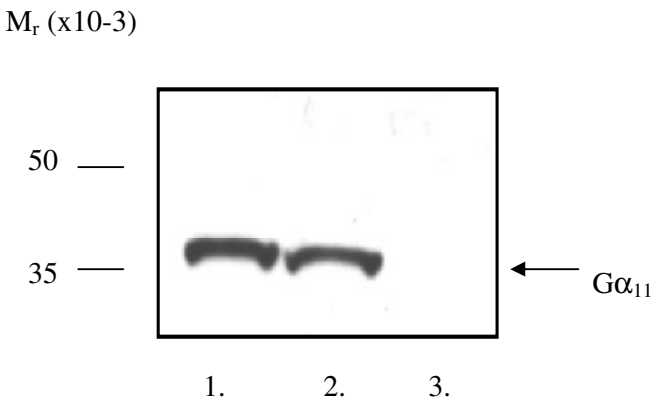


Figure 6.9 Expression and immunological detection of Gα$_{11}$ and Gα$_{11}^{G188S}$

EF88 cells were transfected, using the Amaxa nucleofection kit, to transiently express Gα$_{11}$ (lane 1) or Gα$_{11}^{G188S}$ (lane 2). EF88 cells transfected with pcDNA3 were included as a control (lane 3). Cell lysates were resolved by SDS-PAGE and then immunoblotted with the anti-Gα$_{q/11}$ antiserum CQ. Results shown are of a single experiment and are representative of three individual experiments performed.

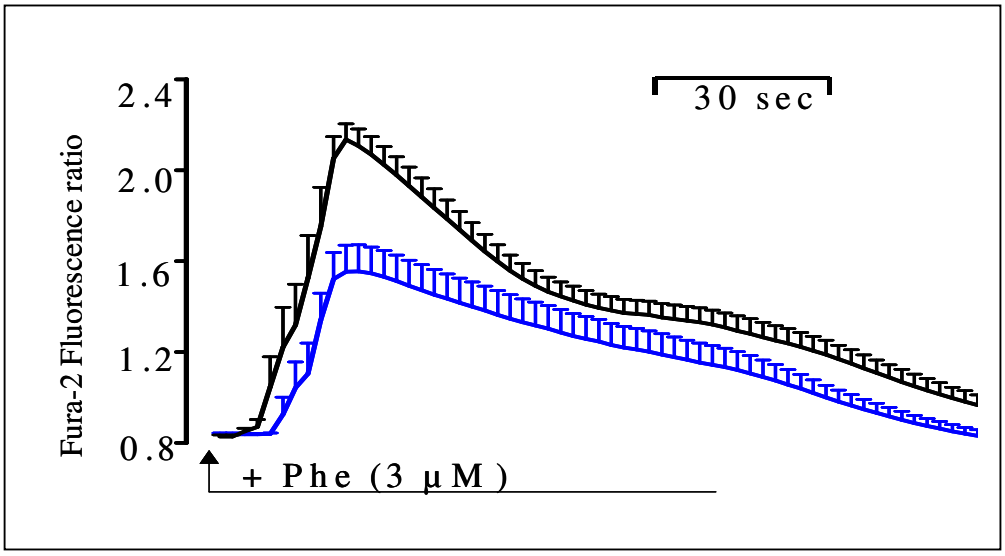


Figure 6.10 RGS4-GFP^2 reduces phenylephrine-stimulated $[Ca^{2+}]_i$ from the α_{1b}-adrenoceptor-Gα_{11} fusion protein

EF88 cells were transfected to express the α_{1b}-adrenoceptor-Gα_{11} fusion protein (black) or the α_{1b}-adrenoceptor-Gα_{11} fusion protein and RGS4-GFP (blue). Cells were loaded with Fura-2/AM and $[Ca^{2+}]_i$ levels imaged before and after 3 µM phenylephrine was perfused over the cells for 60 seconds. Data represents means ± SEM. from 13 (α_{1b}-adrenoceptor-Gα_{11}) and 11 (α_{1b}-adrenoceptor-Gα_{11} and RGS4-GFP^2) cells from 3 individual experiments.

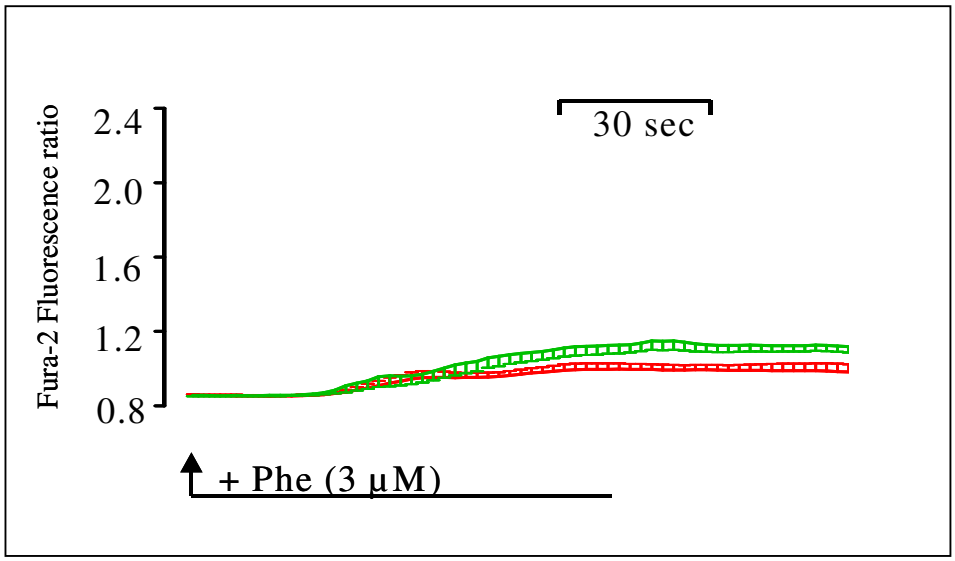


Figure 6.11 Mutation of glycine 188 of $G\alpha_{11}$ prevents phenylephrine-stimulated $[Ca^{2+}]_i$ from the α_{1b}-adrenoceptor fusion protein

EF88 cells were nucleofected to express the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein (green) or the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein and RGS4-GFP (red). Cells were loaded with Fura-2/AM and $[Ca^{2+}]_i$ levels imaged before and after 3 µM phenylephrine was perfused over the cells for 60 seconds. Data represents means ± SEM. from 17 (α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$) and 8 (α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ and RGS4-GFP) cells from 3 individual experiments.

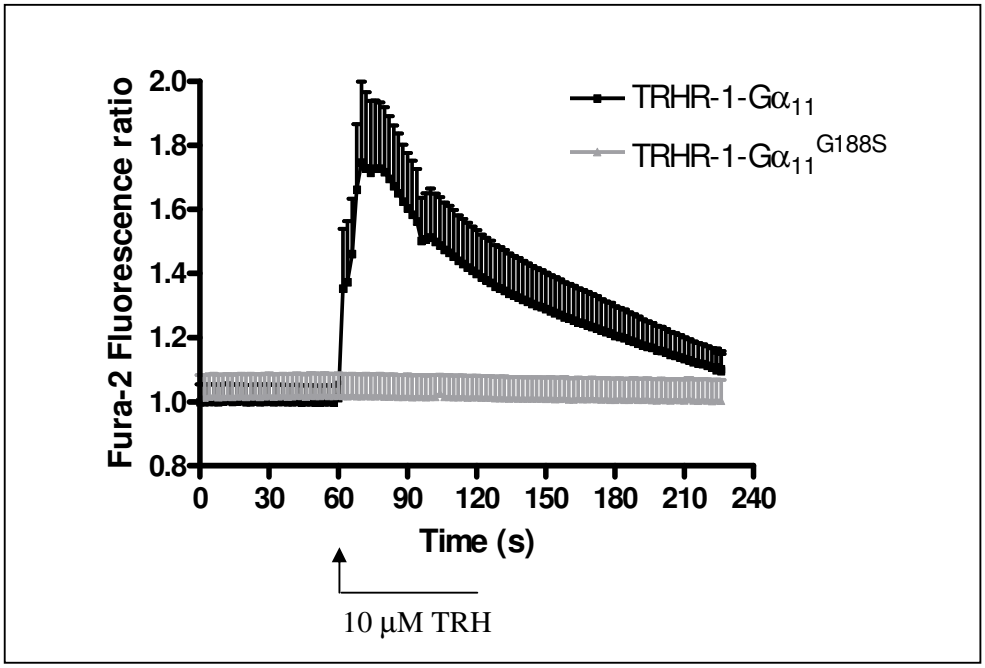
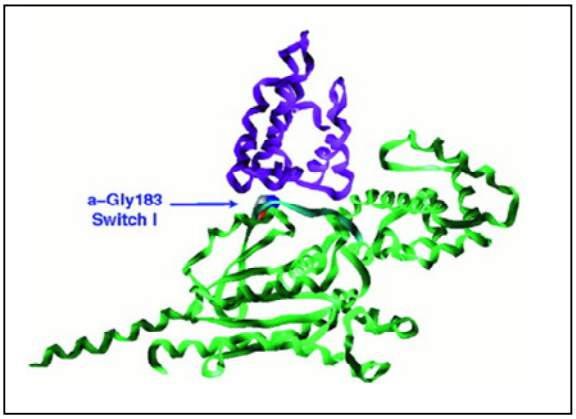


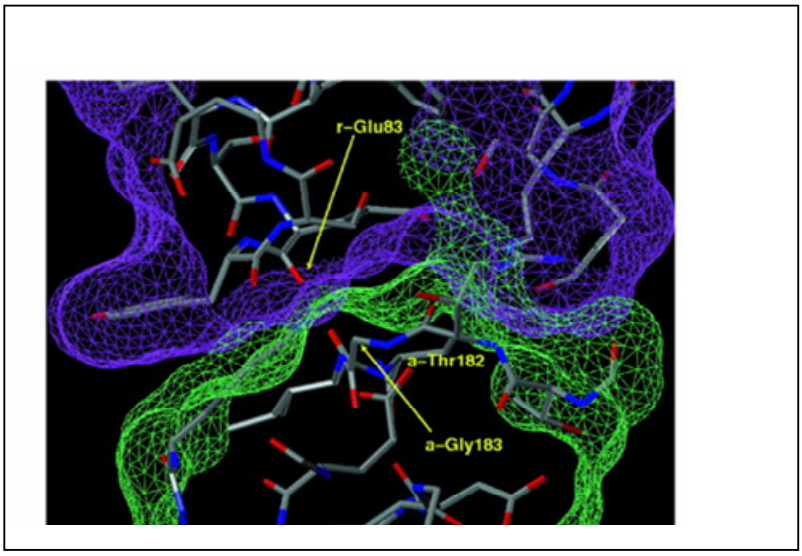
Figure 6.12 Mutation of glycine 188 of $G\alpha_{11}$ prevents TRH stimulated $[Ca^{2+}]_i$ from the TRHR- $G\alpha_{11}$ fusion protein

EF88 cells were transfected to transiently express the TRHR-1-$G\alpha_{11}$ fusion protein (black) or the TRHR-1-$G\alpha_{11}^{G188S}$ fusion protein (grey). Cells were loaded with Fura-2/AM and $[Ca^{2+}]_i$ levels imaged before and after 10 μM TRH was perfused over the cells for 60 seconds. Green fluorescent protein was co-expressed as a marker of positively transfected cells. Data represents means ± SEM. from 19 (TRHR-1-$G\alpha_{11}^{G188S}$) and 15 (TRHR-1-$G\alpha_{11}^{G188S}$) cells from 3 individual experiments.

(A)


a–Gly183
Switch I

(B)


r–Glu83
a–Thr182
a–Gly183

(C)

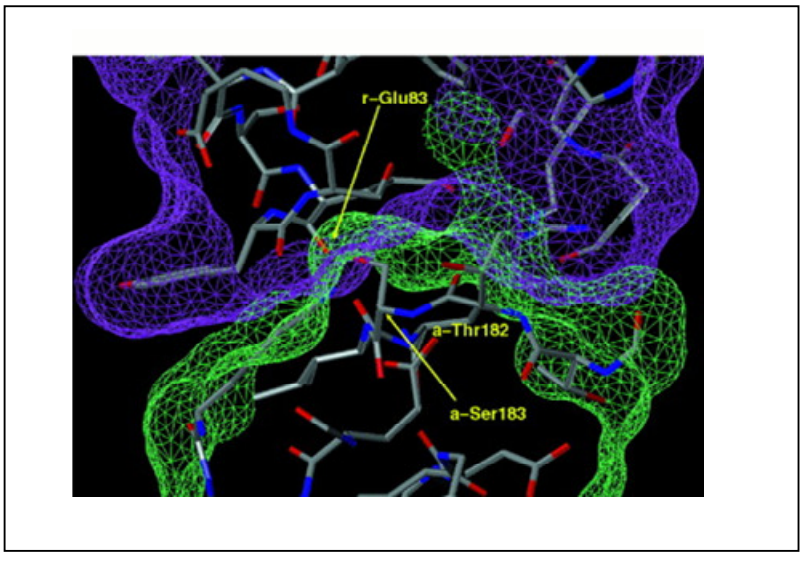

r–Glu83
a–Thr182
a–Ser183

Figure 6.13 Structure of $G\alpha_{i1}$-RGS4 complex and modelling of the glycine to serine mutation in $G\alpha_{i1}$

(A) The ribbon structure of $G\alpha_{i1}$-RGS4. Gα is shown in green, and RGS4 is shown in blue. The position of $G\alpha_{i1}^{G183}$ (corresponds to $G\alpha_{11}^{G188}$) is indicated by the space filled atoms. (B) The Connolly diagram of the switch region I of wild-type $G\alpha_{i1}$-RGS4 complex. Residues in $G\alpha_{i1}$ (glycine 183, theronine 182) and RGS4 (glutamic acid 83) are indicated. (C) Switch region I with $G\alpha_{i1}^{G183S}$ substitution. In this area, the hydroxyl group of serine is less than 1 Å from the backbone of the carbonyl of the glutamic acid 83 of RGS4 (taken from DiBello *et al.*, 1998).

6.3 Discussion

The specific functions of endogenous RGS proteins have, in the past, been poorly defined. However, a single point mutation in Gα subunits, has been suggested to render the mutant Gα protein insensitive to RGS proteins, without a change in GDP release, GTPγS binding or intrinsic GTP hydrolysis (Berman *et al.*, 2004). The structure of RGS4 complexed with $G\alpha_{i1}$, revealed the residue producing RGS-insensitivity (^{G183S} in $G\alpha_{i1}$) indicated steric and electrostatic interactions between this residue and RGS4 (Figure 6.13) (Tesmer *et al.*, 1997;DiBello *et al.*, 1998). This mutation was transferred into $G\alpha_{11}$ and investigated in the present study. Results from RGS-insensitive and –sensitive $G\alpha_{11}$ subunits fused to GPCRs demonstrated that this mutation in $G\alpha_{11}$ must also affect other components of signal transduction.

Unlike the constitutively active Gα subunit ($G\alpha_{i1}^{Q204L}$, Chapter 3 Section 3.2.4), the effects of RGS-insensitive mutations are still under control of receptors to enhance the overall efficacy of the G protein cycle in a more physiological pattern. The physiological roles of some RGS proteins (including RGS1, RGS2, RGS4 and RGS9) have been investigated using RGS knockouts. For example, the engineering of $Rgs2^{-/-}$ mutant mice allowed the endogenous role of RGS2 in developmental, behavioural and physiological tests to be explored. Symptoms recorded included only subtle behavioural and immunological differences, although subsequently, these mutant mice were found to be severely hypertensive (Heximer *et al*., 2003). The RGS4 knockout was also initially reported to have only subtle differences. Mutant mice displayed lower weight and poorer sensory motor coordination. Surprisingly, more serious defects were never displayed. There was no alteration in neuronal differentiation or opioid signalling as mutant mice had normal tolerance to pain compared to wild-type (Grillet *et al.*, 2005). Perhaps future studies should compare these knockout mice with knockdown animal models. Compensation during development may take place in knockout animals and these future experiments may reveal differences so far undiscovered.

Perhaps standard knockout or knockdown strategies targeted towards a single RGS would also underestimate the overall function of RGS proteins. The use of the RGS-insensitive Gα subunits could therefore determine the full contribution of RGS proteins mediated by a particular Gα subunit. Most of what is known about endogenous RGS proteins has been learned through the use of RGS-insensitive mutants of $G\alpha_{i/o}$ (Chen and Lambert, 2000;Jeong and Ikeda, 2000). In this way, endogenous RGS proteins have been shown to

have a negative effect on signalling and show that the GAP activity of RGS proteins provides a control that regulates potency and maximal response of agonist-activated signalling. Few studies have applied this strategy to $G\alpha_{q/11}$-mediated signalling, as the GTPase activity of $G\alpha_{q/11}$ proteins can be accelerated not only by RGS proteins but also by the effector molecule PLCβ. RGS proteins could negatively regulate $G\alpha_{q/11}$ signals by serving as effector antagonists, competing with PLCβ for binding to active G-proteins (Hepler *et al.*, 1997). Thus, it is not clear to what extent RGS proteins are essential for terminating transient $G\alpha_{q/11}$-mediated signals and it is important to consider the possibility that the RGS-insensitive mutation introduced to $G\alpha_{q/11}$ may have caused an altered kinetic response of PLCβ.

In the present work, the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein was used as a starting point to explore the effects of the presumed RGS-insensitive mutation on $G\alpha_{11}$ on cell signalling in response to agonist (Stevens *et al.*, 2001;Fu *et al.*, 2004). It has previously been demonstrated that the α_{1b}-adrenoreceptor-$G\alpha_{11}$ fusion protein is able to bind [^{35}S]GTPγS in response to phenylephrine (Carrillo *et al.*, 2002). Mutations frequently alter the expression levels of a polypeptide, and therefore the use of receptor-G protein fusion constructs allowed the expression levels of not only the receptor but also of the Gα subunit to be determined. Expression of each fusion construct was determined by [^{3}H]prasozin assays to allow the same amount of each construct to be added to [^{35}S]GTPγS binding studies. Both fusion proteins bound the [^{3}H]prazosin with the same high-affinity.

To isolate and enrich the [^{35}S]GTPγS-bound α_{1b}-adrenoreceptor-$G\alpha_{11}$ fusion proteins, after incubating [^{35}S]GTPγS with the membrane fraction of transfected cells, $G\alpha_{11}$ in the reactions was solubilised by detergents and immunoprecipitated using an anti-$G\alpha_{q/11}$ antiserum, CQ and counting the radioactivity. In concert with [^{35}S]GTPγS binding studies, membrane preparations were also separated by SDS-PAGE and immunoblotted with the anti-$G\alpha_{q/11}$ antiserum to confirm that equivalent number of expressed receptor-G-protein fusion proteins were present in the assay, and also, despite the ^{G188S} mutation, that the anti-$G\alpha_{q/11}$ antiserum was equally effective in identifying each fusion protein

It was therefore of considerable interest to note that the α_{1b}-adrenoceptor-$G\alpha_{11}{}^{G188S}$ fusion protein displayed reduced ability to bind to [^{35}S]GTPγS in response to the α_1-adrenoceptor agonist, phenylephrine. GTPγS is a poorly-hydrolysed analogue of GTP, and therefore any alteration in [^{35}S]GTPγS binding is independent of GAP activity of RGS proteins on the Gα subunit. The RGS-insensitive mutant in this assay was anticipated to have no effect on

[^{35}S]GTPγS binding compared to the wild-type fusion protein. It therefore appears that the ^{G188S} mutation in $G\alpha_{11}$ reduces the ability of [^{35}S]GTPγS to bind to $G\alpha_{11}$. However, some previous biochemical studies have suggested that the equivalent RGS-insensitive mutation preserves the kinetics of GTPγS binding in $G\alpha_o$ and $G\alpha_i$ (Lan *et al.*, 1998). The structural data for GTPγS binding to $G\alpha_{11}$ is not available but differences in GTPγS binding between $G\alpha_q$ and $G\alpha_{i/o}$ may be understandable due to the differences in their primary structure. Therefore, it is reasonable to conclude that the ^{G188S} mutation in $G\alpha_{11}$ could reduce the ability of [^{35}S]GTPγS to bind to $G\alpha_{11}$.

Boutet-Robinet *et al*., (Boutet-Robinet *et al.*, 2003) investigated the role of endogenous RGS proteins on dopaminergic D_{2S} receptor signalling in Chinese hamster ovary cells using a RGS- and P.tox-insenstive $G\alpha_o$ protein. Dopamine-mediated [^{35}S]GTPγS binding in these cells was attenuated by more that 60% compared to cells co-expressing the D_{2S} receptor and the wild-type P.tox-insensitive $G\alpha_o$ protein. It was reasoned that if Lan *et al*., (Lan *et al.*, 1998) were correct in reporting that the RGS-insensitive mutation does not modify GTP binding characteristics of the Gα subunit, the observed decrease in coupling was likely due to RGS proteins increasing the pool of $G\alpha_o$ proteins available for this activation. As in the present study, the use of a fusion protein between the GPCR and the G protein α subunit would give a 1:1 stoichiometry of the GPCR to the Gα subunit. Experiments using D_{2S} receptor fused in-frame to the N-terminal of either $G\alpha_o$ or $G\alpha_o^{G184S}$ would validate the interpretation of these results.

More recently, Shi *et al*., (Shi *et al.*, 2006) investigated the effects of over-expression of RGS-insensitive $G\alpha_q$ on $5HT_{2A}$ receptor signalling in transgenic rats. GTPγS-stimulated PLC activity was higher in rats over-expressing wild-type $G\alpha_q$ compared to $G\alpha_q^{G188S}$. Importantly, these researchers also speculated that the point mutation on $G\alpha_q$ (^{G188S}) might reduce the ability of GTPγS to bind and activate the PLC. However, it was also suggested that RGS proteins might favour G protein cycling by allowing the activation and deactivation of the G protein without receptor dissociation. Therefore, the absence of RGS binding in these transgenic rats may lead to a decrease in G protein signalling and ultimately a decrease in receptor mediated GTPγS loading (Ross and Wilkie, 2000).

Downstream signal transduction was also investigated in the present study to demonstrate the effect of the predicted RGS-insensitive $G\alpha_{11}$ subunit on agonist-mediated elevated $[Ca^{2+}]_i$. The co-expression of RGS4 with the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein in EF88 cells reduced agonist-stimulated $[Ca^{2+}]_i$. Absence of RGS activity on $G\alpha_{11}^{G188S}$ was

hypothesised to have a positive effect on Gα subunit activation and increase agonist-mediated $[Ca^{2+}]_i$. However, compared to the wild-type fusion protein, phenylephrine-stimulated $[Ca^{2+}]_i$ was diminished in EF88 cells expressing the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein. A similar result was observed in EF88 cells expressing the TRHR-1R-$G\alpha_{11}^{G188S}$ fusion protein, indicating a genuine effect of $G\alpha_{11}^{G188S}$ to abolish agonist-mediated $[Ca^{2+}]_i$ signalling.

In EF88 cells the agonist-mediated elevation of $[Ca^{2+}]_i$ is a measure of Gβγ release and function (Stevens *et al.*, 2001). Co-expression of α-transducin, which is an effective Gβγ sequestering agent, resulted in blocking of the agonist activated elevation of $[Ca^{2+}]_i$ (Liu *et al.*, 2002). It could therefore be speculated that $G\alpha_{11}^{G188S}$ has a reduced ability to release Gβγ. Expression of a Gα mutant with a deficiency in Gβγ release would result in a construct unable to elevation $[Ca^{2+}]_i$ in EF88 cells. Indeed, the reduced binding of $[^{35}S]$GTPγS to $G\alpha_{11}^{G188S}$ in the present study could also be due to the reduced ability of the mutated Gα subunit to release Gβγ. If $G\alpha_{11}^{G188S}$ was less able to release the Gβγ subunit, then binding of GTPγS would also subsequently be reduced.

Mutant forms of $G\alpha_{11}$ have previously been demonstrated to poorly elevate $[Ca^{2+}]_i$ in EF88 cells (Liu *et al.*, 2002). Mutations of the corresponding residues in $G\alpha_q$ had already been inferred to lack the capacity to bind Gβγ effectively (Evanko *et al.*, 2000). Lui *et al.*, therefore used co-immunoprecipitation studies, and clearly showed that the α_{1b}-adrenoceptor fused to the wild-type $G\alpha_{11}$ subunit associated with co-expressed β_1, but only small amounts of β_1 were present along with the mutant $G\alpha_{11}$ containing-fusion proteins. It was reasoned that this alteration was a reflection of the reduced effectiveness of Gβγ binding to the mutant $G\alpha_{11}$ (Liu *et al.*, 2002). Fusion proteins containing a mutation in the α_{1b}-adrenoceptor, which generated constitutive activity of the receptor, with reduced ability to bind $[^{35}S]$GTPγS have also been described (Carrillo *et al.*, 2002). This mutant was reasoned to be unable to adopt the conformational change required to dissociate the G protein subunits. It now therefore seems essential to conduct co-immunoprecipitation studies using the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ to quantify the amount of co-expressed β_1 associated with the mutant Gα subunit. The results of this study would elucidate if indeed $G\alpha_{11}^{G188S}$ had a reduced ability to bind Gβγ or more likely, infer an inability of the mutant to dissociate Gβγ.

Alternatively, the observed reduction of agonist-stimulated $[Ca^{2+}]_i$ following expression of the adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein or the TRHR-1R-$G\alpha_{11}^{G188S}$ fusion protein is

perhaps due to an decreased availability of free Gβγ. If RGS proteins and Gβγ cannot bind to the Gα subunit at the same time, then the RGS-insensitive mutation would presumably lead to less free Gβγ to elevate $[Ca^{2+}]_i$. Co-expression of the Gβγ complex with the mutant $G\alpha_{11}$ containing-fusion protein would perhaps give sufficient Gβγ to allow $[Ca^{2+}]_i$ mobilisation in EF88 cells. No increase in agonist-stimulated $[Ca^{2+}]_i$ would suggest that $G\alpha_{11}^{G188S}$ has good interactions with endogenous Gβγ and that the mutant fusion protein has lost the ability to dissociate the Gβγ complex. However, a resultant increase in agonist-stimulated $[Ca^{2+}]_i$ would suggest that a reduction in available Gβγ is responsible for the observed reduction in agonist-stimulated $[Ca^{2+}]_i$.

PLCβ not only mediates agonist-stimulated $[Ca^{2+}]_i$ but is also a GAP for the $G\alpha_q$ subfamily of Gα subunits. RGS4 can act as a receptor shield for $G\alpha_q$ (Hepler *et al.*, 1997) and binding of RGS4 to $G\alpha_{11}$ could interfere with the GAP activities of PLCβ, and have a positive effect on Gα protein activation. If indeed RGS4 is endogenously expressed in EF88 cells, the transient expression of the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein might have no interaction with RGS4 but allow PLCβ to interact with the fusion protein without restraint. The Gα subunit would be subjected to maximal GAP activity from PLCβ and abolish second messenger signalling. Indeed, biochemical characterisation demonstrated that $G\alpha q^{G188S}$ responds to the GAP activity of PLCβ, but does not respond to the GAP activity of RGS4 (Clark and Lambert, 2006).

Multifunctional protein complexes comprising of receptor, G protein and RGS protein have also been described (Ross and Wilkie, 2000). Direct and specific binding of RGS proteins to Gα subunits and other components of the cellular signalling complex can contribute to signal transduction. Preliminary research indicates that RGS proteins can act as scaffolds to assemble signalling complexes. Perhaps the absence of RGS binding to the RGS-insensitive Gα subunits could abolish the scaffolding properties of RGS proteins and reduce signalling. A specific interaction between RGS2 and the α_{1b}-adrenoceptor has been shown to include the scaffold, spinophillin (Wang *et al*., 2005). It is perhaps such an interaction which is prevented in RGS-insensitive Gα mutants and abolishes the rise in $[Ca^{2+}]_i$.

The PDZ domain of RGS12 has been found to selectively bind the chemokine receptor CXCR2 (Snow *et al.*, 1998b). A number of other RGS proteins contain this PDZ domain and are likely to show binding selectivity to other relevant receptors and it could be presumed that RGS proteins with multiple domains would provide the best scaffolding

properties. It has also been suggested that RGS proteins form stable complexes with inactive G proteins to form quaternary complex (Benians *et al*., 2005). Although many RGS proteins show affinity for $G\alpha_{11}$, the precise RGS proteins in EF88 cells remains unknown. Total RNA prepared from cells was subject to reverse transcription-PCR using RGS specific primers that were tested by amplifying RGS cDNA plasmids as a positive control (data not shown). Despite the presence of mRNA for the positive control (glyceraldehyde-3-phosphate dehydrogenase), available primers specific for RGS3, RGS4 and RGS7 did not identify endogenous expression of any of these RGS proteins in EF88 cells. Despite these results, it is possible to assume that these RGS proteins, and others, are expressed in EF88 cells but at undetectable levels.

The localisation of RGS4-eYFP in cells expressing the RGS-insensitive fusion proteins suggest that the mutation in $G\alpha_{11}$, as previously predicted prevents RGS4 from binding to the Gα subunit and subsequently prevents RGS4 from translocating from the cytoplasm to the plasma membrane. In this study, HEK293T cells are used because EF88 cells are difficult to transfect and yield minimal protein for determining the percentage fluorescence of RGS4-eYFP in the plasma membrane. As mentioned previously, the RGS-insensitive Gα subunit mutations are under the control of receptors to enhance the overall efficacy of the G protein cycle. Agonist-stimulation, therefore, promoted RGS4 to bind to the active conformation of the G protein at the plasma membrane. Presence of the RGS-insensitive Gα subunit perhaps did not allow RGS4 to bind, reducing the presence of RGS4 at the plasma membrane. Biochemical studies have shown that a $G\alpha_{i1}$-RGS-insensitive mutant does not bind to RGS4 (Lan *et al.*, 1998). The RGS-insensitive mutation is predicted to be in the switch region I of $G\alpha_{i1}$ and provide a substantial contribution to the buried surface area between the Gα subuit and RGS4 (DiBello *et al.*, 1998). Introduction of the hydromethyl side chain of serine would sterically hinder the formation of a tight complex between $G\alpha_{i1}$ and RGS4 (Lan *et al.*, 1998) suggesting that RGS4 would not be translocated to the plasma membrane in the presence of agonist-activated RGS-insensitive $G\alpha_{i1}$.

The use of RGS-insensitive Gα subunits provide novel insights into subtype-selective signalling by Gα subunits. Other approaches, such as expression of constitutively active Gα subunits, will not reveal key functions mediated by Gβγ release. In addition, any non-GAP effects must consequently involve the ^{G188S} mutation site. The results from this study suggest that introduction of serine at position 188 in $G\alpha_{11}$ may well disrupt G protein activation, Gβγ binding or release, effector antagonism and/or RGS scaffolding properties. The inability to identify which RGS proteins are responsible for these differences is a

limitation in this present study but RGS-insensitive mutants will prove useful tools in future investigations into the role of endogenous RGS proteins.

7 Final Discussion

RGS proteins modulate G protein mediated signalling pathways by acting as GAPs for $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$ heterotrimeric G proteins. Often comprising of a diverse combination of signalling domains, RGS proteins are regulated by a variety of assorted mechanisms. Discovering how specific RGS proteins modulate signal transduction remains vitally important, as pathological conditions have been linked to abnormal RGS expression. Pharmaceutical intervention of RGS activity may therefore impact the treatment of these conditions (Mittmann *et al.*, 2002). The precise role of RGS4, one of the most extensively studied RGS proteins, is still uncertain in the face of complex biochemical activity and overlapping patterns of expression.

In this study, the first objective was to investigate the subcellular localisation of RGS4. This was done using several techniques including microscopy, immunoblotting, and fluorescence analysis. Each technique demonstrated that transiently expressed RGS4 is located within the cytosol of HEK293T cells. Co-expression of $G\alpha_{i1}$, or the α_{2A}-adrenoceptor, or agonist-activated α_{2A}-adrenoceptor, clearly translocated transiently expressed RGS4 from the cytosol to the plasma membrane. Previous studies investigating the cellular localisation of RGS4 proteins also demonstrated that RGS4 could be recruited to the plasma membrane by the incorporation of a specific $G\alpha_i$- or $G\alpha_q$-associated signalling partner (Druey *et al.*, 1998;Roy *et al.*, 2003). Unlike other RGS proteins such as RGS9-2, which has been reported to be largely membrane associated (Song *et al.*, 2006) and RGS2 which has been reported to localise in the nucleus (Heximer *et al.*, 2001), the majority of transfected RGS4 was found in the cytoplasm of HEK293T cells.

The transfection of RGS proteins is typically conducted in order to overcome the low natural abundance of endogenous RGS proteins. However, substantial differences between endogenous RGS4 and heterologously over-expressed RGS4 have been suggested (Krumins *et al.*, 2004). An alternative start site for synthesis of RGS4 from methionine 19 has previously been predicted (Davydov and Varshavsky, 2000). RGS4 lacking the first 18 amino acids is typically produced by *in vitro* translation and in contrast, the longer full length form of RGS4 is expressed endogenously in tissue or cultured cells. As previously mentioned, the N-terminal of RGS4 is particularly important to the subcellular localisation of RGS4. Therefore, it is perhaps not surprising that Krumins *et al.*, found endogenous full length RGS4 predominantly in the membrane fraction of cells. Endogenous RGS4 at the plasma membrane would seem reasonable to preclude the necessity for translocation in

physiological systems. The physiological relevance of the differences between the transcription and localisation of endogenous and transfected RGS proteins therefore needs to be carefully considered.

In this study, it was noticed that a relatively small portion of RGS4-eYFP was membrane associated at any given time. This most likely reflects the transient over-expression of RGS4. Over-expression of RGS4 could flood the cell's cytoplasm and subsequently transfected RGS4 is pushed to the plasma membrane. Alternatively, the RGS4 could perhaps be recruited to the plasma membrane by the endogenous pool of Gα subunits in HEK293T cells. It was also noticed that on co-expression of $G\alpha_i$, or indeed any of the other signalling proteins that were co-expressed, a proportion of RGS4 is always left residing in the cytosol. Again, this is most likely due to the transient over-expression of RGS4 and possibly the saturation of RGS4 binding sites at the plasma membrane. The amount of RGS4 cDNA transfected was initially optimised but perhaps re-visiting this area and modulating the level of expression of RGS4 and the relative co-expression levels of Gα subunits and GPCRs would now allow this concept to be further explored. When Roy *et al*., (2003) decreased the amount of receptor cDNA co-transfected with RGS-GFP to a level at which RGS-GFP did not significantly localise to the plasma membrane, addition of agonist also failed to recruit RGS4-GFP to the plasma membrane (Roy *et al.*, 2003). This reinforces that a careful approach is needed to work out the precise transfection ratio of RGS and signalling proteins.

RGS proteins have been reported to physically interact with the transition state conformation of Gα subunits (Tesmer *et al*., 1997). Localisation of RGS4 by a protein partner has therefore been most clearly demonstrated by studies transiently expressing a constitutively active GTPase-deficient Gα subunit. Constitutively active Gα subunits are locked in an active state and are transition state models of Gα subunits. RGS proteins are therefore predicted to bind to these proteins but are unable to modulate intrinsic GTPase activity. Over-expression of the constitutively active GTPase-deficient $G\alpha_{i2}$ ($G\alpha_{i2}^{Q205L}$) showed the recruitment of the majority of the cytosolic RGS4 to the membrane surface (Druey *et al.*, 1998). Data from the current study, using the constitutively active $G\alpha_{i1}$ ($G\alpha_{i1}^{Q204L}$), was consistent with this hypothesis and indicated that RGS4 can be cytosolic and then be recruited to the plasma membrane in the presence of a constitutively active $G\alpha_{i1}$ subunit. RGS proteins must still bind to the constitutively active Gα subunit, moving between different signalling complexes on the plasma membrane depending on the availability of interaction sites. RGS8 translocates to the plasma membrane from the

nucleus on co-expression of $G\alpha_o$. Co-expression with a constitutively active $G\alpha_o$ also resulted in the translocation of RGS8 protein to the plasma membrane (Masuho *et al*., 2004). Thus, the expression of a GTPase deficient Gα subunit still recruits RGS proteins and furthermore, even the over-expression of a Gα subunit in the inactive state can translocate transfected RGS4 to the plasma membrane. Gα subunits in the inactive state are perhaps momentarily activated, in constant transition between inactive and active conformation, thus in the active state able to irreversibly bind RGS4 and cause its translocation to the plasma membrane.

The confirmation of an N-terminal GOF mutant in a mammalian RGS by Hill *et al*., (personal communication) was carried out using an *in vitro* high-affinity GTPase assay. $RGS4^{S30C}$ enhanced the α_{2A}-adrenoceptor-activated increase in high-affinity GTPase activity of $G\alpha_{o1}$ despite equal expression and comparable subcellular localisation compared to wild type RGS4. $Serine^{30}$ is conserved in many other members of the B/R4 family of RGS proteins and the conversion of this residue to cysteine in RGS16 also demonstrated a significant increase in high-affinity GTPase activity of $G\alpha_{o1}$. The conserved $serine^{30}$ residue in the B/R4 subfamily of RGS proteins must be important for the GAP activity of these proteins. The N-terminus, and in particular, residue 30 may help correctly position RGS4 at the receptor where it can optimally inactivate the Gα subunit via the GAP activity of the RGS domain. Mutation of this residue may serve to change the orientation of RGS4 and subsequently the GAP activity of the RGS domain.

The observed simultaneous significant decrease in potency of adrenaline to increase α_{2A}-adrenoceptor-activated high-affinity GTPase activity of $G\alpha_{o1}$ in the presence of RGS4 or RGS16 demonstrated that these RGS proteins must interact with the α_{2A}-adrenoceptor. If the RGS proteins were to interact only with the G proteins the agonist binding site on the receptor would remain unchanged and no change would be expected in the potency of the agonist. In accordance with other published results (Ward and Milligan, 1999;Cavalli *et al.*, 2000), the potency of adrenaline to stimulate the α_{2A}-adrenoceptor high-affinity GTPase activity of $G\alpha_{o1}$ on addition of purified RGS16 was lower than on the addition of RGS4 . This perhaps provides yet further evidence for the selective interactions between RGS proteins and GPCRs. RGS4 and RGS16 must interact and alter the conformation of the α_{2A}-adrenoceptor in a different way. These closely related RGS proteins do not function equally and the specificity of the interaction is perhaps fundamental to the GAP activity of the RGS protein and the ability to control biological functions.

The GOF enhancing GAP activity of $RGS4^{S30C}$ and $RGS16^{S30C}$ are selective for $G\alpha_{o1}$ over $G\alpha_{i1}$. The addition of purified RGS4 or RGS16 did, however, decrease the potency for adrenaline to stimulate high-affinity GTPase activity of $G\alpha_{i1}$, suggesting the validity of the assay and the ability of the RGS proteins to perform. However, the potency for adrenaline to stimulate high-affinity GTPase activity of $G\alpha_{i1}$ was not further decreased by $RGS4^{S30C}$ or $RGS16^{S30C}$. It has previously been established that the B/R4 subfamily of RGS proteins show selectivity for $G\alpha_{o1}$ over $G\alpha_{i1}$ (Cavalli *et al.*, 2000;Riddle *et al.*, 2005). It is probable that both RGS4 and RGS16 interact with the α_{2A}-adrenoceptor but are not able to act as efficient GAPs for $G\alpha_{i1}$.

The subcellular localisation of $RGS4^{S30C}$-eYFP in the presence of exogenous $G\alpha_{i1}$, the α_{2A}-adrenoceptor or agonist-activated α_{2A}-adrenoceptor was also investigated. Despite similar expression levels when compared to wild type RGS4-eYFP, as monitored by immunological detection with an anti-GFP antibody, no difference in subcellular localisation was detected in this study. The knowledge of the selectivity of RGS^{S30C} to enhance the GAP activity of $G\alpha_{o1}$, indicates that an important next step would be to study the subcellular localisation of $RGS4^{S30C}$-eYFP in the presence of exogenous $G\alpha_{o1}$.

The subsequent mutation of residue 30 of RGS4 to a range of other amino acids revealed that $RGS4^{S30K}$ and $RGS4^{S30F}$ were the most active and $RGS4^{S30P}$ was the least active of the mutants that were studied. No consensus side chain was identified that conferred a specific enhancement or loss of GTPase activity. Both RGS5 and RGS18 contain a proline at residue 30. Preliminary results suggest that $RGS5^{P30S}$ is also GOF (Hill *et al.*, personal communication) and it would now be of great interest to investigate the GAP activity of $RGS18^{P30S}$. Clearly, it is also now important to mutate residue 30 of RGS4 to threonine and tyrosine to identify if the hydroxyl side chain at position 30 in RGS4 is crucial to maintain wild type GAP activity.

The N-terminal of RGS4 is particularly important for the membrane localisation of the protein. Using a peptide corresponding to the first 33 residues of RGS4 in yeast provided direct evidence for the localisation of RGS4 to a signalling complex to be determined solely by the N-terminal domain (Bernstein *et al.*, 2000). Site-directed mutagenesis of hydrophobic and basic residues within the N-terminal domain of RGS4 also revealed that the GAP activity of RGS4 is strongly correlated with the ability of the protein to bind to anionic liposomes and the tendency of an N-terminal peptide to adopt an α-helical conformation (Bernstein *et al.*, 2000). In addition, extensive mutational analysis of RGS16

has demonstrated that the N-terminal of RGS16 plays a critical role in membrane association and also may be important for the biological function of RGS16 (Chen *et al.*, 1999). Secondary structure analysis suggested that there may be two putative α-helices in the N-terminal of RGS16. The first α-helix (amino acids 1-6) was not relevant for biological activity, however, the rest of the N-terminus (amino acids 7-32), which consists of predominantly the second putative α-helix from amino acids 12-30, has been defined as the core membrane-association domain. This membrane-targeting domain for RGS16, is likely shared by RGS4 and circular dichroism spectroscopy data directly demonstrated that amino acid substitutions within residues 12-30 profoundly affect α-helical structure (Bernstein *et al.*, 2000). However, in the current study it was demonstrated that the subcellular localisation of $RGS4^{S30X}$-eYFP was not different to wild type RGS4. This suggests that residue 30 in RGS4, although important for modulating the GAP activity of the protein, is not important for targeting RGS4 to the plasma membrane. Perhaps gathering circular dichroism spectroscopy data for $RGS4^{S30X}$ would reveal changes in secondary structure for different mutants and confirm that mutation of residue 30 of RGS4 alters the helical structure of the protein serving to alter the orientation of the protein and subsequently the GAP activity of the RGS domain.

It has previously been suggested that the receptor and not the G protein dictate the function of RGS proteins. Indeed, the translocation of RGS4 and $RGS4^{S30X}$ to the plasma membrane in the presence of the α_{2A}-adrenoceptor would support this suggestion. The significant reduction in potency of adrenaline of RGS4 and RGS16 to enhance the high-affinity GTPase activity of the activated α_{2A}-adrenoceptor-$G\alpha_{o1}$ would also agree with this hypothesis. It could be envisaged in live cells, endogenous RGS proteins would not be free to find any G proteins, but instead the receptors would orientate the RGS protein at the plasma membrane and dictate specificity. However, the current findings suggest that RGS4 has selectivity for $G\alpha_{o1}$ over $G\alpha_{i1}$ on activation of the same receptor, the α_{2A}-adrenoceptor, perhaps against suggesting that the α_{2A}-adrenoceptor orientates RGS4 in a more advantageous position to interact with $G\alpha_{o1}$.

Interaction of RGS proteins with receptors may occur within lipid rafts or caveolae, specialised microdomains that may well modulate signalling events. Indeed, the pre-treatment of cells with the detergent methyl-β-cyclodextrin to deplete the membrane of cholesterol and disrupt such lipid rafts, weakens the interactions with RGS4 (Ishii *et al.*, 2005). Compartmentalisation of several signal transduction pathways and proteins occurs in lipid rafts (Shaul and Anderson, 1998;Simons and Toomre, 2000;Brown and London,

2000) and disruption of these lipid rafts can prevent signalling in many of these pathways (Miura *et al*., 2001). RGS9 in a complex with Gβ5 translocates to lipid rafts after activation of photoreceptor outer segments by illumination (Nair *et al*., 2002). However, the lipid raft localisation was not critical for the GAP activity of RGS16 but may be necessary for palmitoylation of an internal residue (Hiol *et al.*, 2003). To investigate the potential interaction of RGS4 with the α_{2A}-adrenoceptor, specific residues involved in the interaction would first have to become apparent, but would allow further investigation into the involvement of lipid rafts in the regulation of RGS proteins

From functional data (Srinivasa *et al.*, 1998) and structural data (Tesmer *et al.*, 1997) the principle function of asparagine^{128} of RGS4 is to bind and stabilise switch region I and II of Gα subunits and to contribute to the overall stability of the RGS-Gα transition state complex. Evidence showed that RGS4 stimulates GTP hydrolysis primarily, if not exclusively by binding and stabilising the transition state conformation of Gα subunits. Correspondingly, in the present study, RGS4^{N128A} had ablated GAP activity towards $G\alpha_{11}$. RGS4^{N88S} also had ablated GAP activity towards $G\alpha_{11,}$ demonstrating that no single residue seemed to exclusively catalyse GTP hydrolysis. The double mutant (RGS4^{N88S,N128A}) also had ablated GAP activity towards $G\alpha_{11}$ and any additive effect of GAP ablation was concealed due to the complete ablation of GAP activity with each single mutation. No further inhibition of $[Ca^{2+}]_i$ mobilisation with the disruption of two binding interactions along the RGS4-$G\alpha_{11}$ binding interface was possible. The complete ablation of GAP activity suggests that each single mutation was sufficient at causing a global conformation change and ablating GAP activity of the protein. The lack of modulation of $[Ca^{2+}]_i$ signalling by these GAP impaired RGS4 mutants suggests that the catalysis of GTPase activity is the dominant mechanism by which RGS4 regulates Ca^{2+} signalling.

Single cells positively transfected with RGS4 were selected for use in the $[Ca^{2+}]_i$ mobilisation assays by using RGS4-eYFP fusion proteins. Only fluorescent cells and thus those cells expressing RGS4 were selected. The brightest fluorescent cells were selected for analysis but in this way over-expression of RGS protein may have obscured small differences in RGS GAP activity amongst the RGS4 mutants. Perhaps employing a different strategy, selecting cells with the least fluorescence in a field of view and therefore a lower expression of RGS4, would reveal differences in the GAP activity of these mutants. It is also noteworthy that the GAP activity of the fluorescently-tagged RGS4 and untagged RGS4 were previously shown to be identical. This is consistent with other

studies in which RGS4 has been similarly tagged without consequence (Berman *et al.*, 1996a;Chen *et al.*, 1997)

It has previously been demonstrated that $RGS4^{N128A}$ caused severe defects in binding to $G\alpha_{i2}$ (Srinivasa *et al.*, 1998). To this end, the subcellular localisation of these GAP defected mutants would also have been worthy of investigation. Asparagine 128 is the only RGS4 residue that projects into the active site of $G\alpha_{i1}$ and together with its location at the binding interface and interaction with three $G\alpha_{i1}$ residues at the active site (glutamine^{204}, glutamate ^{207} and lysine^{180}) further underlines the importance of asparagine^{128} to the GAP activity of RGS4. Co-immunoprecipitation studies may provide further evidence that these GAP defective mutants have reduced binding to the transition state of $G\alpha_{i1}$. In addition, co-immunoprecipitation studies could elucidate the binding of these GAP defective mutants to $G\alpha_{11}$.

The third and final objective of this study was to investigate a potential RGS-insensitive $G\alpha_{11}$ mutant. A point mutation originally identified in yeast, has been shown to be RGS-insensitive in $G\alpha_q$, $G\alpha_{i1}$ and $G\alpha_o$. This mutation was introduced to $G\alpha_{11}$ ($G\alpha_{11}{}^{G188S}$) and receptor- $G\alpha_{11}{}^{G188S}$ fusion proteins failed to translocate RGS4 to the plasma membrane. Moreover, agonist-activation of the fusion proteins also failed to translocate RGS4 to the plasma membrane suggesting that indeed $G\alpha_{11}{}^{G188S}$ cannot bind and subsequently translocate RGS4 to the plasma membrane. However, fusion proteins have been reported not to be localised in lipid rafts (Hiol *et al.*, 2003). As mentioned previously, association of RGS4 in lipid rafts might be involved in the physical regulation of the protein (Ishii *et al.*, 2005). The inability of the fusion protein to associate with the lipid rafts may therefore affect the translocation of RGS4. Future translocation studies using $G\alpha_{11}{}^{G188S}$ should perhaps consider the use of separate receptors and Gα subunits.

Despite this shortcoming, the present results are consistent with the finding that, RGS-insensitive Gα subunits do not promote RGS association with the plasma membrane. RGS7 belongs to a subfamily of RGS proteins that exist as dimers with the Gβ5 subunit. When expressed in HEK293 cells, Gβ5-RGS7 was found to be cytoplasmic and soluble. Expression of $G\alpha_o$ promoted a strong redistribution of Gβ5-RGS7 to the plasma membrane. The constitutively active mutant $G\alpha_o{}^{R179C}$, like wild type $G\alpha_o$, strongly recruited Gβ5-RGS7 to plasma membrane, however, RGS-insensitive $G\alpha_o{}^{G184S}$ was defective in the ability to promote plasma membrane localization of Gβ5-RGS7 (Takida *et al.*, 2005). It has also been demonstrated that RGS-insensitive Gα subunits containing an

additional point mutation that conferred constitutive activity to the G proteins did not promote RGS4-GFP association with the plasma membrane ($G\alpha_{i2}^{Q205L/G184S}$, $G\alpha_q^{Q209L/G188S}$) (Roy *et al.*, 2003). It has been further implied that RGS4-GFP binds directly to G proteins in the plasma membrane and that the recruitment of RGS proteins to the plasma membrane is not caused by events that occur after G protein activation. Agents that promote events downstream of G protein activation did not alter RGS localization (Roy *et al.*, 2003). The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (which mimic events down-stream of $G\alpha_q$), forskolin (which bypasses $G\alpha_s$ to directly activate AC), and the tyrosine phosphatase inhibitor vanadate failed to promote RGS4 translocation to the plasma membrane in HEK293T cells. Further use of these agents may be informative about the signal transduction pathway following $G\alpha_{11}$ activation and RGS4 translocation.

RGS-insensitive mutations have been reported to only have an effect on RGS binding (Lan *et al.*, 1998). In the present study, however, $G\alpha_{11}^{G188S}$ had significantly reduced agonist-stimulated [^{35}S]GTPγS binding compared to wild type $G\alpha_{11}$. A subsequent review of the literature uncovered two additional independent reports that the RGS-insensitive mutation attenuated [^{35}S]GTPγS binding (Boutet-Robinet *et al.*, 2003;Shi *et al.*, 2006). Boutenet-Robinet *et al.*, reasoned that the reduction in [^{35}S]GTPγS binding by the RGS-insensitive Gα subunit was caused by RGS proteins increasing the pool of endogenous $G\alpha_o$ proteins available for interaction with the activated receptor. An RGS-insensitive Gα subunit would not be able to bind RGS protein and subsequently not be able to increase the pool of endogenous Gα subunits and increase G protein signalling. However, this reasoning has been contradicted by the present study. Using an equivalent number of the α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein and the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein, as established by [^{3}H]prasozin binding, made it clear that the endogenous pool of Gα subunits in HEK293T cells would be having a the same effect with both the RGS-sensitive and -insensitive $G\alpha_{11}$. The reduction in [^{35}S]GTPγS binding to the RGS-insensitive Gα subunit was therefore not caused by availability of action of endogenous G proteins.

Shi and co-workers suggested that GTPγS-stimulated PLC activity was lower in rats over-expressing the $G\alpha_q^{G188S}$ compared to its wild type counterpart because RGS proteins favour G protein cycling (Shi *et al.*, 2006). The absence of RGS proteins may lead to a decrease in G protein signalling and a decrease in receptor mediated GTPγS loading (Ross and Wilkie, 2000). This hypothesis may also be true for the present study but is difficult to prove and investigating other possibilities about the properties of $G\alpha_{11}^{G188S}$ would be less problematic.

It has been well established that co-expression of RGS4 reduces agonist-stimulated mobilisation of $[Ca^{2+}]_i$ (Tovey and Willars, 2004). In the present study, single-cell imaging techniques demonstrated that RGS4 inhibited both the magnitude and rate of the immediate, agonist-induced generation of $[Ca^{2+}]_i$. In EF88 cells expressing the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein or the TRHR-1-$G\alpha_{11}^{G188S}$ fusion protein, agonist-induced generation of $[Ca^{2+}]_i$ was ablated. In EF88 cells the agonist-mediated elevation of $[Ca^{2+}]_i$ is a measure of Gβγ release and function (Stevens *et al.*, 2001). The predicted RGS-insensitive mutation in $G\alpha_{11}$ may therefore also have a deficiency in Gβγ release. It could also be reasoned that the reduced binding of $[^{35}S]GTP\gamma S$ was also due to a reduced ability of the mutant Gα subunit to release Gβγ and subsequently bind $[^{35}S]GTP\gamma S$. The expression of α-transducin to sequester free Gβγ would investigate this new hypothesis.

The ability of RGS4 to bind to $G\alpha_{11}^{G188S}$ is clearly an important future experiment. Co-immunoprecipitation studies using the predicted RGS-insensitive and –sensitive $G\alpha_{11}$ subunits would determine if RGS4 interacted with this mutant Gα subunit. Future studies could also transfer the predicted RGS-insensitive mutation into $G\alpha_q$. $G\alpha_q$ and $G\alpha_{11}$ share 98% homology but RGS5 and RGS16 can discriminate between the two highly related $G\alpha_q$ family subunits (Ladds *et al.*, 2007).

When the N-terminal of RGS4 targets the protein to particular GPCRs, RGS4 can occupy a position that can prevent coupling between $G\alpha_{q/11}$ and PLCβ (Zeng *et al.*, 1998). RGS proteins could therefore act as negative regulators of $G\alpha_{q/11}$ signalling by serving as effector antagonists, competing with PLCβ for binding to active G proteins (Hepler *et al.*, 1997). Thus, it is not clear to what extent RGS proteins are essential for terminating transient $G\alpha_{q/11}$-mediated signals and it is important to consider the possibility that the RGS-insensitive mutation introduced into $G\alpha_{q/11}$ may have caused an altered kinetic response of PLCβ. A recent study used over-expression of constitutively active $G\alpha_q$ protein to investigate the functional importance of GTPase activation to mediate the inhibitory effect of RGS proteins. Cells were transiently transfected with either the M3 muscarinic receptor or $G\alpha_q^{Q209L}$. Comparing the inhibitory effect of RGS proteins demonstrated that RGS5 and RGS16 did not exert any inhibitory effect when they were unable to act as a GAP, whereas RGS2 and RGS3 markedly blunted $G\alpha_q$-mediated signalling even in the absence of GAP, suggesting that other mechanisms, such as effector antagonism, are sufficient to mediate their inhibitory effect (Anger *et al.*, 2004). Future Ca^{2+} signalling experiments in EF88 could also use the over-expression of constitutively

active $G\alpha_{q/11}$. Any observed differences in kinetics or amplitude of $[Ca^{2+}]_i$ could be attributed to the effector antagonism properties of endogenous RGS4.

Alternatively, the observed reduction of agonist-stimulated $[Ca^{2+}]_i$ release following expression of the α_{1b}-adrenoceptor-$G\alpha_{11}^{G188S}$ fusion protein or the TRHR-1-$G\alpha_{11}^{G188S}$ fusion protein is perhaps due to an decreased availability of free $G\beta\gamma$. If RGS proteins and $G\beta\gamma$ cannot bind to the $G\alpha$ subunit at the same time, then the RGS-insensitive mutation would presumably lead to less free $G\beta\gamma$ to elevate $[Ca^{2+}]_i$. Co-expression of the $G\beta\gamma$ complex with the mutant $G\alpha_{11}$ containing-fusion protein would perhaps give sufficient $G\beta\gamma$ to allow $[Ca^{2+}]_i$ mobilisation in EF88 cells.

The predicted RGS-insensitive mutant has great potential to more fully understand the specific functions of endogenous RGS proteins. However, the specific RGS proteins endogenously expressed in HEK293T cells and EF88 cells remain unclear, most likely due to very low expression. Others too have also experienced difficulty in detecting endogenous RGS proteins. Immunoblotting of COS, murine neuro-2A neuroblastoma, and NG108 neuroblastoma/glioma cells, with a specific anti-RGS4 antiserum failed to identify endogenous RGS4. A PCR-based screen was performed to "semi quantitatively" examine the level of RGS4 mRNA in various cell types. Strong signals were obtained for rat PC12M and human AtT-20 cells but little or no signal was produced from murine neuro-2A neuroblastoma, rat pituitary GH3, rat RBL-2H3, rat C6 glioma, CHO, or NG108 neuroblastoma/glioma cells (Krumins *et al.*, 2004). Perhaps future studies carrying out RGS RNA interference, the specific knock-down of mRNA, with rat PC12M or human AtT-20 cells would allow the role of endogenous RGS proteins to be determined in these cells.

The current study has attempted to further elucidate the function of RGS4 in signal transduction. Future studies will undoubtedly uncover as yet unidentified mechanisms of regulation and functions for this complex protein. For example, the recent discovery of an Arabidopsis protein (AtRGS1) containing both a GPCR and a RGS domain within the same protein is an exciting breakthrough (McCudden *et al.*, 2005). Perhaps by having conjoined guanine nucleotide exchange factors and GAP capabilities, the AtRGS1 forms a precisely controlled signalling complex. Alternatively, an agonist (or an inverse agonist) could regulate the activity of the RGS domain or the membrane spanning N-terminal GPCR domain may simply anchor the protein to the membrane.

The translocation of RGS4 from the cytoplasm to the plasma membrane will always be of particular interest. Electron microscopy has previously been used to study RGS localisation (Druey *et al.*, 1998;Fischer *et al.*, 1999;Hiol *et al.*, 2003) but there have been no reports recording the real-time live images of translocating RGS4. Efforts to assess this are beyond the scope of the current study but new advances in high-resolution electron microscopy to visualise the macromolecular arrangements in cells and improved methods for rapid freezing to capture transient processes may improve the understanding of RGS4 translocation. The ability to dynamically define the 3-D locations of signalling components will be central to unravelling the function and regulation of RGS4.

At the next level of investigation, a capillary electrophoresis assay could be employed to further compare the functional activity of RGS mutants. Capillary electrophoresis using a fluorescent, hydrolysable GTP analogue to detect GPCR-stimulated G protein GTPase activity in cell membranes expressing the α_{2A} adrenoreceptor-$G\alpha_{o1}$ fusion protein has recently been described (Jameson *et al*., 2007). Separation of fluorescent GTP from fluorescent GDP by capillary electrophoresis can show the accumulation of product or substrate and hence, the relative GTPase activity of the G protein. Addition of RGS mutants would allow the comparison of agonist-stimulated substrate levels and the development of a high throughput method of investigating the GAP activity of RGS mutants.

Polymorphisms in RGS4 loci have been linked to schizophrenia in humans (Chowdari *et al.*, 2002). Remarkably, RGS4 expression levels are also modulated by cocaine and morphine in brain regions known to be involved in drug behaviour (Bishop *et al.*, 2002). It will be extremely interesting to determine whether small molecules designed to inhibit RGS4 from interacting with the Gα subunit (Jin *et al.*, 2004) will be directly involved in regulating drug sensitivity and physiological processes in mammalian tissues. If RGS proteins were unrestrictedly active, they would completely suppress G protein mediated cell signalling. Therefore, it is important to understand how the activity of RGS proteins are regulated. RGS4 is considered the prototypical member of the B/R4 subfamily and therefore discoveries about candidate regions that could serve as targets for drugs to alter RGS function may be transferred to a number of related RGS proteins. The GOF mutants in RGS4 could be used directly to decrease signalling and current small molecule inhibitors (Ingi *et al.*, 1998) could be tested to examine their ability to inhibit the enhanced RGS activity of these mutants.

It will be important in future investigations to determine whether conservation of distinct regulatory and functional properties exists in various RGS proteins. The expression of RGS proteins is, in many cases, considerably more restricted than that of most G proteins or GPCRs. RGS proteins therefore represent novel and potentially exciting targets for the development of new pharmaceuticals. RGS proteins and the regulation of signal transduction will certainly remain an intensely studied area of investigation.

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9 Additional Material

The following paper was published as a result of the studies carried out for this thesis.