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MODULATION OF REGULATOR OF G PROTEIN SIGNALLING (RGS) FUNCTION BY 14-3-3 PROTEINS

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MODULATION OF REGULATOR OF G PROTEIN SIGNALLING (RGS) FUNCTION BY 14-3-3 PROTEINS

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ABSTRACT

Regulator of G protein signalling (RGS) proteins are primarily known to attenuate G protein function within G protein-coupled receptor (GPCR) signalling pathways, however they can interact with additional proteins. We identified an interaction between RGS proteins (RGS4, RGS5, RGS16) and the multifunctional protein 14-3-3. Two isoforms, 14-3-3β and 14-3-3ε, interact with all three purified RGS proteins. Data from *in vitro* steady state GTP hydrolysis assays show that 14-3-3 inhibits the GTPase activity of RGS4 and RGS16, but has no appreciable effect on RGS5. Furthermore in a competitive pull-down experiment, 14-3-3ε competes with Gαo for RGS4 but not for RGS5. Thus 14-3-3 proteins may prevent RGS from interacting with Gα and ultimately prolong signalling. Tyrosine 167 within the conserved 14-3-3 binding motif does not play a significant role in RGS inhibition by 14-3-3. In conclusion, 14-3-3 proteins appear to indirectly promote GPCR signalling via their inhibitory effects on RGS function.

KEYWORDS: G protein-coupled receptor (GPCR) signalling pathway, Regulator of G protein signalling protein (RGS protein), GTPase activating protein (GAP), 14-3-3 proteins, scaffolding protein, signalling complex.

CO-AUTHORSHIP

Parts of Chapter 1 (sections 1.3., 1.4., and 1.5.) were adapted from the following review: Abramow-Newerly, M, Roy, AA, Nunn, C, and Chidiac, P (2005). RGS proteins have a signalling complex: Interactions between RGS proteins and GPCRs, effectors and auxiliary proteins. *Cell. Signal.* DOI:10.1016/j.cellsig.2005.08.010. In Press.

All experiments described in Chapter 2 were carried out in the laboratory of Dr. Peter Chidiac in the Department of Physiology and Pharmacology at the University of Western Ontario. I performed all the experiments described, except for the yeast 2-hybrid screen that was carried out by a former member of the laboratory, Hong Ming.

DEDICATION

THIS THESIS IS DEDICATED TO MEMBERS OF MY FAMILY,
WANDA, JAROSLAW, HELENA AND FRANKY,
FOR THEIR UNCONDITIONAL LOVE AND CONSTANT SUPPORT

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Living in London has truly been an enjoyable experience and for that, I am obliged to my close friends for all their thoughtfulness and awesome memories. I am truly indebted to my family who has always been extremely supportive and understanding. Thank you for teaching me how to live life with a smile. Finally, I am grateful for having such a wonderful best friend and life companion, Chad who always has the ability to make me laugh.

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LIST OF ABBREVIATIONS

AMF	$\underline{Al}Cl_3$, $\underline{Mg}Cl_2$, $\underline{N}aF$
14-3-3ε	14-3-3 epsilon
14-3-3β	14-3-3 beta
DEP	Disheveled/EGL-10/Plextrin homology domain
DMAP1	DNA methyltransferase-associated protein 1
G proteins	Heterotrimeric guanine-nucleotide binding proteins
Gβ5	Subtype 5 of the heterotrimeric G protein beta subunit
GAIP	G alpha interacting protein
GAP	GTPase activating protein
GDP	Guanosine-5'-diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GGL	G gamma-like domain
GIPC	RGS-GAIP interacting protein C terminus
GoLoco	G-protein regulatory (GPR) motif that acts as a guanine dissociation inhibitor (GDI) on G alpha
GPCR	G protein-coupled receptor
GST	Glutathione-S-transferase
GTP	Guanosine-5'-triphosphate
HA	Hemagglutinin epitope of the human influenza virus
HEK293	Human embryonic kidney cell line
HIS	Hexa-histidine

IB	Immunoblot
NES	Nuclear export sequence
PDZ	PSD95/DLG/ZO-1 domain
PTB	Phosphotyrosine-binding domain
RID	Rap interaction domain
RGS	Regulators of G protein signalling
SCG10	A neuronal growth-associated protein
SNARE	Soluble *N*-ethyl maleimide-sensitive factor attachment protein
WT	Wild-type

CHAPTER 1

LITERATURE REVIEW

1.1. **GPCR Signal Transduction Pathway**

The superfamily of G-protein coupled receptors (GPCRs) represents the largest class and the most diverse type of cell surface receptors existing in mammalian genomes (Schioth and Fredriksson, 2005). These receptors are also known as 7 transmembrane-spanning proteins based on their common membrane topology that consists of an amino-terminal extracellular domain, a seven hydrophobic transmembrane domain, and a carboxyl-terminal intracellular domain (Kristiansen, 2004; Schioth and Fredriksson, 2005). GPCRs participate in a diversity of important physiological functions and use distinct domains to bind to a wide range of ligands (eg. ions, organic odorants, amines, peptides, proteins, lipids, nucleotides, and photons) (Bockaert *et al.*, 2003). This type of receptor has been found to be an important drug target for the treatment of a number of diseases including cardiovascular, neurological, digestive, skeletal and respiratory disorders. In fact, GPCRs are targets for approximately 60% of currently available therapeutics (Leurs *et al.*, 1998).

Based on physiological and structural features, GPCRs can be classified into at least six families (A–F groups) that show little inter-family sequence similarity (Kolakowski, Jr., 1994). The primary role of GPCRs is to bind to specific ligands found outside the cell and to convert an extracellular signal into an intracellular response via heterotrimeric guanine-nucleotide binding proteins (G proteins), consisting of Gα, Gβ, and Gγ subunits (Neer, 1995). G proteins are segregated into four families based on sequence homology of the Gα subunit and on their effector interaction: (1) Gs (stimulates adenylyl cyclase); (2) Gi (inhibits adenylyl cyclase); (3) Gq (stimulates phospholipase Cβ); (4) G12 (activates Rho) (Downes and Gautam, 1999). The signal transduction pathway is initiated by the

binding of a ligand to the receptor, followed by a conformational change of the receptor that enables it to act as a guanine nucleotide exchange factor (GEF), thereby promoting the exchange of GDP for GTP on the Gα subunit of the heterotrimeric G protein. This leads to the activation of the Gα subunit, and a conformational change within (and possible dissociation of) the heterotrimeric G protein (Kjeldgaard *et al.*, 1996; Chidiac, 1998). Both GTP-bound Gα and the Gβγ subunits promote downstream signalling by activating a number of effectors and second messengers. The diversity within the heterotrimeric G protein subunits and the subtle variations in expression between tissues may be important in partly determining GPCR signal specificity (Downes and Gautam, 1999). The amplitude and duration of the receptor-mediated signal is highly dependent on the length of time Gα remains in the active GTP-bound state. The rate of its self-inactivation *in vitro* is often slow compared to the fast physiological responses seen *in vivo.* This apparent discrepancy in many cases can be accounted for by the intracellular regulators of G protein signalling (RGS) proteins (Chasse and Dohlman, 2003) (Figure 1.1).

1.2. **RGS Proteins**

RGS proteins play a key role in the desensitization of GPCR-mediated signals and are primarily known as negative regulators of G protein-mediated signalling pathways (Wilkie and Ross, 2000) (Figure 1.2). In mammals, twenty distinct genes for RGS proteins have been identified (numbered RGS1-21, omitting 15), some of which have splice variants, as well as there are a similar number of structurally divergent "RGS-like"

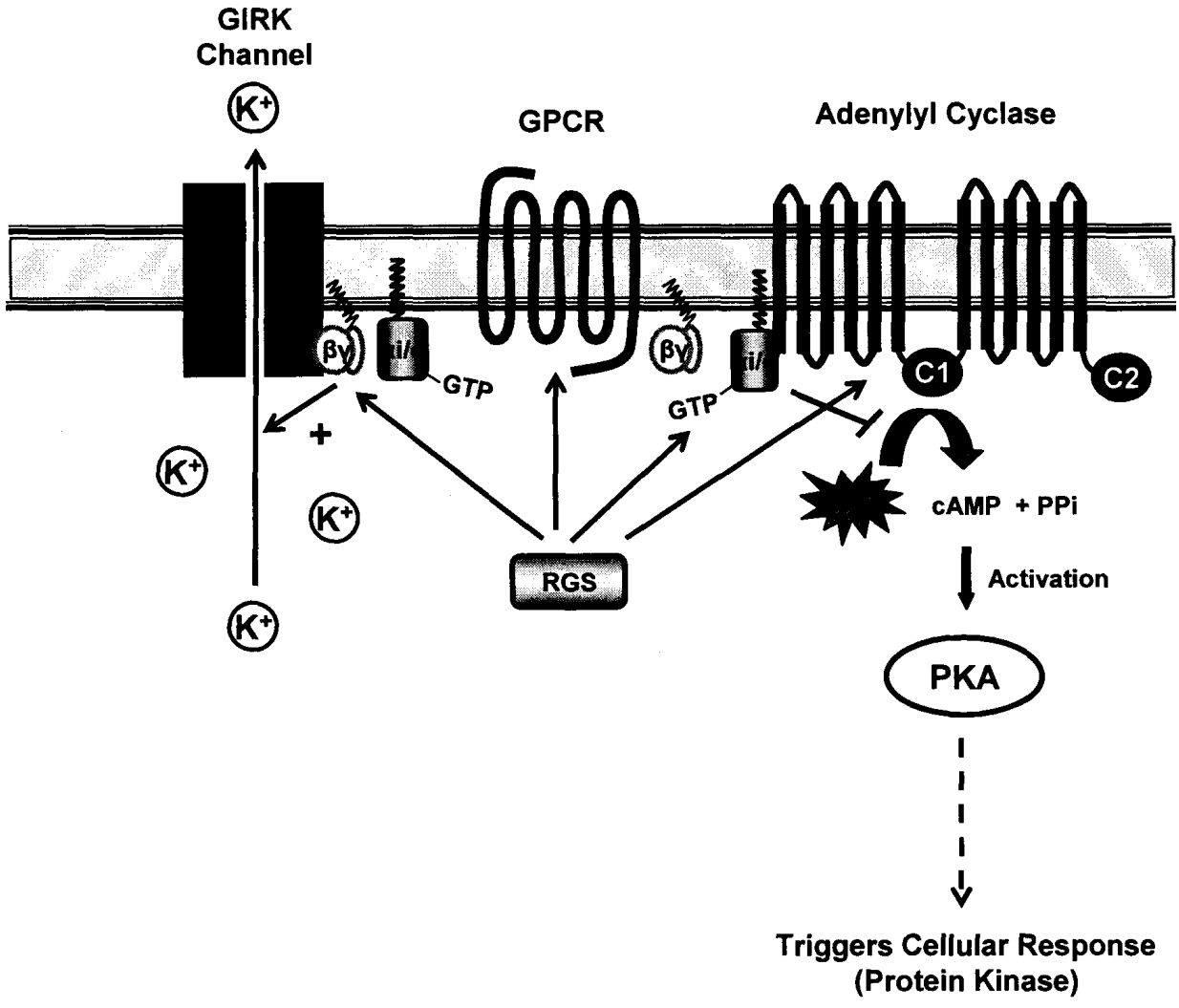

Figure 1.1. *RGS proteins and an example of Gi/o cellular targets.* Once the GPCR has been activated, the GTP-Gαi/o subunit can function as an inhibitor of the effector adenylyl cyclase, while the Gβγ subunit can elicit the protein-gated inwardly rectifying potassium channels (GIRK), both significantly redirecting cellular responses. RGS proteins are known to interact with both subunits of the heterotrimeric G protein, as well as GPCRs and effectors to negatively modulate their activities (Sadja *et al.*, 2003; Abramow-Newerly *et al.*, 2005). C1 and C2 = cytosolic domains of adenylyl cyclase; cAMP = cyclic AMP; PKA = Protein kinase A.

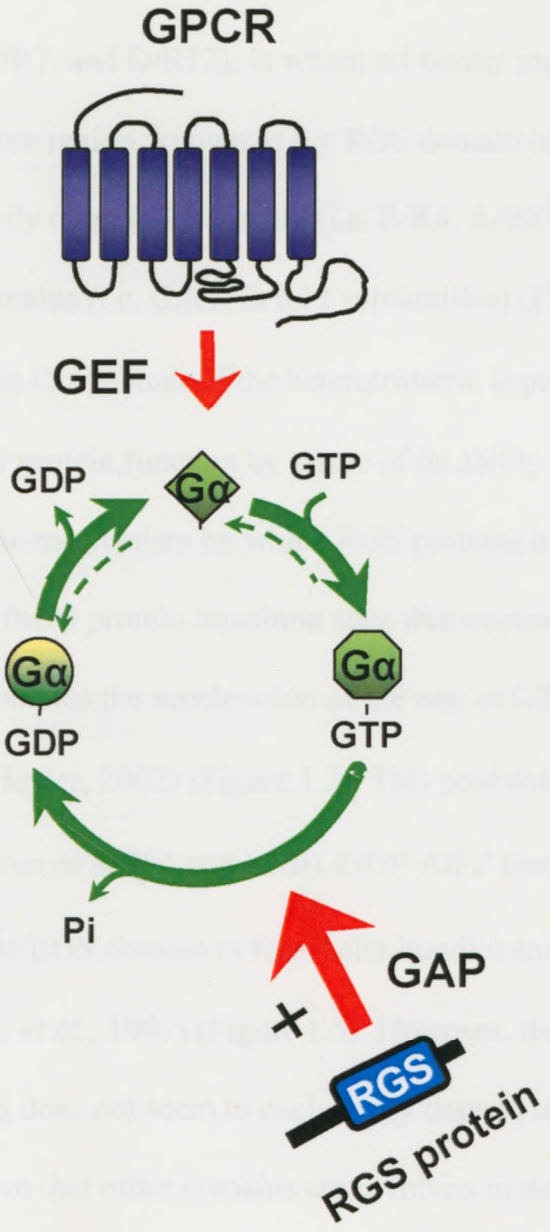

Figure 1.2. *RGS proteins, negative regulators of GPCR-mediated signal transduction pathways.* The diagram illustrates the cycle of activation and inactivation of the Gα subunit of the heterotrimeric G protein. The binding of agonist activates the GPCR which acts as a GEF, catalyzing the exchange of GDP for GTP. RGS proteins function as GAPs that increase the rate of hydrolysis of GTP to GDP, resulting in the inactivation of Gα and the reformation of the inactive heterotrimeric G protein.

proteins, a few of which have GAP activity (Hollinger and Hepler, 2002; Riddle *et al.*, 2005). Based on sequence similarities, RGS proteins can be classified into four subfamilies (B/R4, A/RZ, C/R7, and D/R12), in which all family members possess a ~120-amino acid (15kDa) core region, known as the RGS domain (or RGS box). Some RGS proteins consist primarily of an RGS domain (i.e. B/R4, A/RZ subfamilies), while others contain additional domains (i.e. C/R7, D/R12 subfamilies) (Figures 1.3 and 1.4). The RGS domain binds to the Gα subunit of the heterotrimeric G protein and is necessary and sufficient to terminate G protein function by virtue of its ability to act as a GTPase activating protein (GAP). The mechanism by which RGS proteins inactivate G proteins involves the stabilization of the G protein transition state that occurs immediately prior to GTP hydrolysis, and this promotes the acceleration of the rate of GTP hydrolysis on the Gα subunit (Hollinger and Hepler, 2002) (Figure 1.2). This postulated mechanism is supported by the crystallization of RGS4 with Gαi1-GDP-AlF_4^- that clearly illustrates a direct interaction between the RGS domain (a four-helix bundle) and the Gα subunit at three switch regions (Tesmer *et al.*, 1997) (Figure 1.5). However, the ability of RGS proteins to impede signalling does not seem to exclusively depend on the RGS domain, in fact recent studies have shown that other domains are involved in determining receptor specificity, intracellular localization, and also provide additional functions (Siderovski and Willard, 2005).

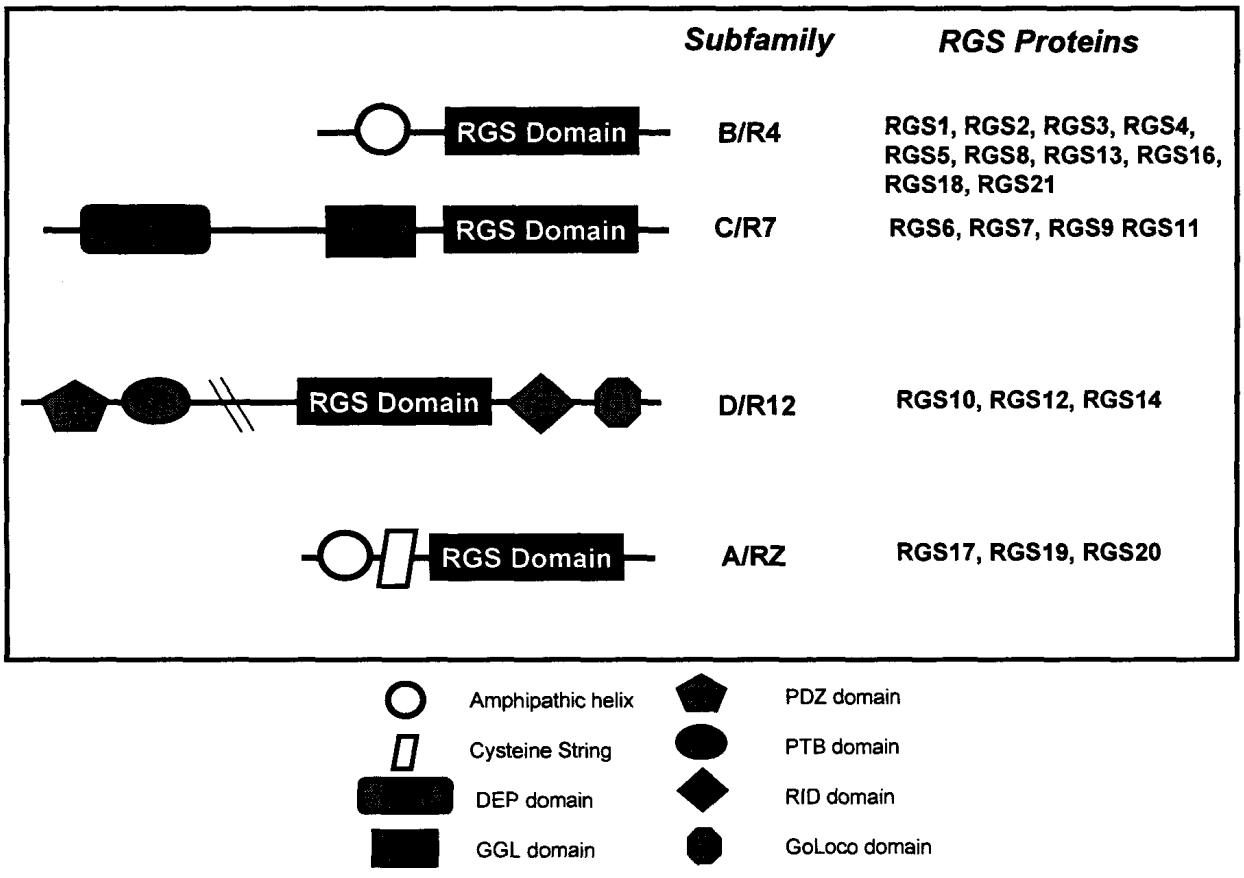

Figure 1.3. *Summary of RGS structure.* The schematic illustrates common RGS protein domains classified according to subfamilies (B/R4, C/R7, D/R12, A/RZ). Variance may exist between isoforms or splice variants. Note that not all domains indicated are found in all members within a given subfamily. Adapted from (Riddle *et al.*, 2005).

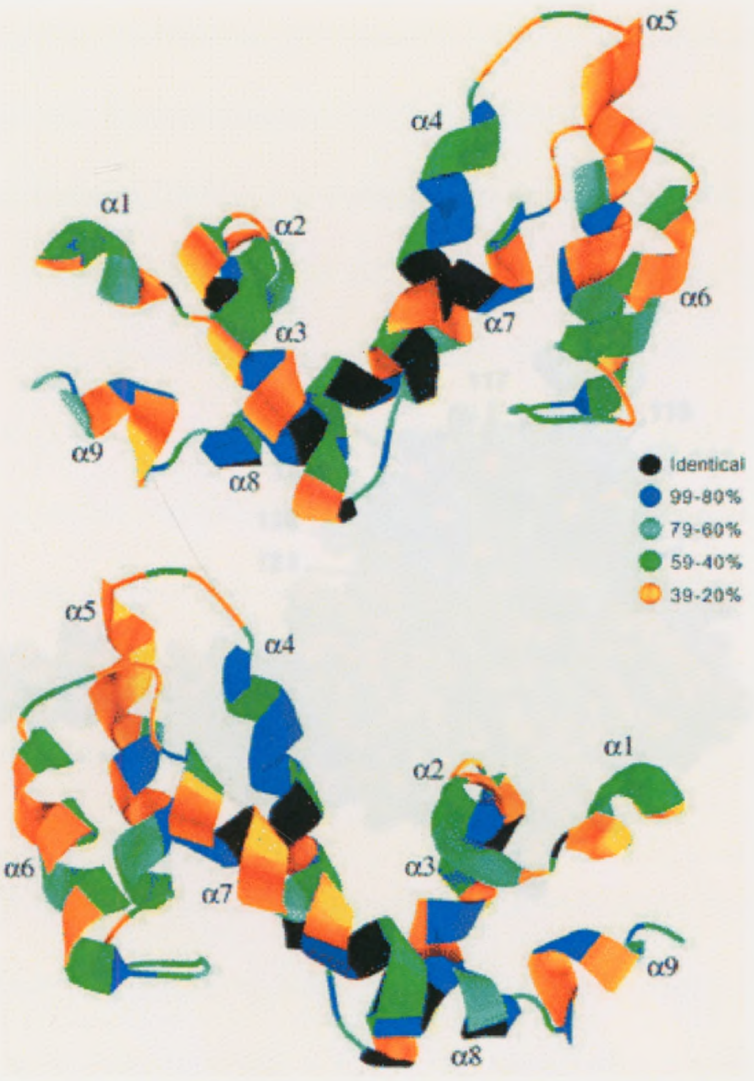

Figure 1.4. *Conserved regions in the RGS family mapped onto the structure of the RGS domain of RGS4.* The amino acid sequence of all members of the A/RZ, B/R4, C/R7, and D/R12 subfamilies were aligned and the relative conservation of each residue was mapped onto the structure of RGS4. *Top panel*: the RGS domain with the Gα contact surface below. *Bottom panel*: 180° rotation of the top image about the vertical axis. The α-helices are numbered according to (Tesmer *et al.*, 1997). Reprinted, with permission, from the Annual Review of Biochemistry, Volume 69 © 2000 by Annual Reviews, www.annualreviews.org.

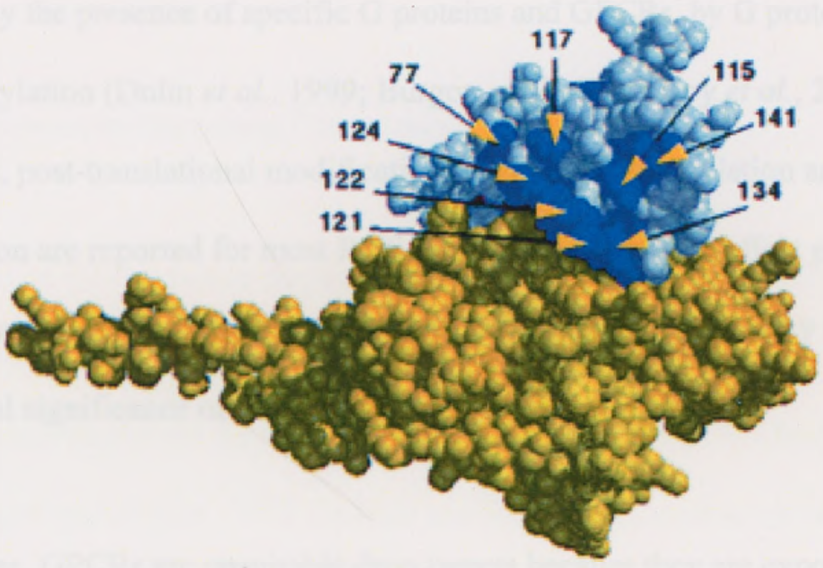

Figure 1.5. *Crystal structure of RGS4 in complex with Gαi1 protein.* The Gα protein is shown in yellow and the RGS protein in light blue. The evolutionary trace analysis identified a cluster of residues (blue; numbered) that includes the RGS-Gα binding interface. The following work is published in: Sowa ME, He W, Wensel TG, Lichtarge O. A regulator of G protein signaling interaction surface linked to effector specificity. *Proc Natl Acad Sci U.S.A.* 2000 Feb 15; 97(4):1483-8. Copyright (2000) National Academy of Sciences, U.S.A.

As they are potent regulators of GPCR-mediated signalling pathways, RGS protein levels and activities are highly controlled within the cell via various mechanisms including regulation of subcellular localization, post-translational modifications and their association with protein binding partners (Song *et al.*, 1999; Kim *et al.*, 1999; Schiff *et al.*, 2000; Ishii and Kurachi, 2004). For example, the subcellular localization of several RGS proteins including RGS2, RGS3, RGS4 and RGS10 has been shown to be modulated by the presence of specific G proteins and GPCRs, by G protein activation and by phosphorylation (Dulin *et al.*, 1999; Burgon *et al.*, 2001; Roy *et al.*, 2003). Furthermore, post-translational modifications such as phosphorylation and/or palmitoylation are reported for most RGS proteins and appear to affect protein stability, localization and GAP activity; however additional studies are necessary to determine the physiological significance of these alterations (Riddle *et al.*, 2005).

In many cases, GPCRs are unsuitable drug targets because they are expressed in many tissues, and treatment of one disease can lead to unwanted side effects in other organs. Thus, RGS proteins have the potential of becoming distinct drug targets partly due to the fact that some of them have unique tissue and organ distribution (i.e. RGS9), and transcriptional modulation profiles (De Vries and Gist-Farquhar M., 1999; Larminie *et al.*, 2004). RGS protein functions may differ according to tissue type, signalling mechanism, and (patho)-physiological state (Neubig and Siderovski, 2002; Chasse and Dohlman, 2003; Ghavami *et al.*, 2004). The role of several RGS proteins *in vivo* has been studied using RGS-deficient mouse models. For instance, RGS2 knock-out mice possess an increased blood pressure (Gross *et al.*, 2005), RGS9-2 knock-out mice demonstrate abnormal involuntary movements that resemble drug-induced dyskinesia (Kovoor *et al.*,

2005), and the loss of RGS14 expression in the mouse embryonic genome leads to developmental arrest (Martin-McCaffrey *et al.*, 2004). On the other hand, RGS4 null mice appear to be viable, fertile and fail to display any significant developmental and behavioral anomalies (Grillet *et al.*, 2005). Thus, understanding the mechanism(s) by which RGS proteins alter signalling and/or are themselves modulated is challenging, albeit necessary in predicting potential RGS-drug interactions.

1.3. **RGS Proteins in Signalling Complexes**

Initial studies on RGS function postulated that these proteins catalytically turned off activated, free Gα subunits *in vivo*, under the assumption that Gα upon GTP binding would dissociate from both the Gβγ dimer and the activated GPCR (Wilkie and Ross, 2000; Hollinger and Hepler, 2002; Chidiac and Roy, 2003). In this context, RGS proteins would "intercept" Gα-GTP and shuttle between GPCRs and effector proteins (Chidiac, 1998). However recently, this shuttling dogma has been questioned (Chidiac, 1998; Levitzki and Klein, 2002), and an alternative interpretation is that signals are transduced via protein complexes that consist of GPCRs, G proteins, effectors, and possibly other proteins (Rodbell, 1980; Chidiac, 1998; Levitzki and Klein, 2002; Rebois and Hebert, 2003). In addition to the latter proteins, signalling complexes can contain a variety of other structural and modulatory protein components and although a lot still remains to be elucidated, there is mounting evidence that RGS proteins may be at least transiently involved in these signalling complexes (Kreienkamp, 2002; Benians *et al.*, 2005).

It is now becoming evident that RGS proteins are targeted to more than just G proteins. Data suggest that members of all four RGS protein subfamilies are able to interact with GPCRs. Several mammalian GPCRs have been identified as real or putative RGS protein targets and these include the M_1 (Bernstein *et al.*, 2004) and M_2 muscarinic (Ingi *et al.*, 1998; Roy *et al.*, 2003), D_2 (Cabrera-Vera *et al.*, 2004; Jeanneteau *et al.*, 2004b; Kovoor *et al.*, 2005) and D_3 dopaminergic (Jeanneteau *et al.*, 2004a), α_{1A}- (Hague *et al.*, 2005), β_1- (Hu *et al.*, 2003) and β_2-adrenergic (Roy *et al.*, 2003), angiotensin AT_1 (Roy *et al.*, 2003) interleukin-8 B (CXCR2) (Snow *et al.*, 1998) and μ opioid receptors (Garzon *et al.*, 2004). Often, the GPCR-RGS protein interaction involves regions outside the conserved RGS domain and in some cases requires auxiliary proteins. Overall these data suggest that RGS protein targeting to G proteins can be regulated by GPCRs.

RGS proteins have also been observed to bind to a variety of effector proteins including adenylyl cyclase, GIRK channels, cGMP phospho-diesterase (PDEγ), phospholipase C-β (PLC-β) and Ca^{2+} channels (Wilkie and Ross, 2000; Chidiac and Roy, 2003). In some cases, the RGS-effector protein interaction can have a negative effect whereby RGS proteins act as effector antagonists and bind to either the effector protein or the Gα subunit to prevent the physical interaction between the two. In other cases, it can have a positive effect on GPCR signalling, whereby it creates a complex between the effector and the activated G protein resulting in the fast transduction of the activated Gα signal (Wilkie and Ross, 2000).

1.3.1. *RGS interactions with auxiliary proteins*

In addition to the primary participants in G protein-mediated signal transduction (i.e., receptors, G proteins and effectors), RGS proteins have been found to interact with a wide variety of other proteins (De Vries *et al.*, 2000) and these associations can influence their subcellular localization, function and stability. Novel binding interactions via both non-RGS and RGS domains have introduced more complexity to our understanding of the potential role of RGS proteins *in vivo*. For example, it has been observed that the RGS domain, primarily known for its binding to Gα proteins, also interacts with small GTPases, protein kinase A, and components of the Wnt signalling pathway (De Vries *et al.*, 2000). In fact, the GAP effects of RGS proteins are sensitive to a wide variety of factors, including cations [i.e. sodium, potassium and magnesium (Wang *et al.*, 1997; Cladman and Chidiac, 2002)], and phospholipids [i.e. phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Ishii *et al.*, 2002) and phosphatidylserine (Tu *et al.*, 2001)]. These interactions have functional implications, for instance: the RGS-box GAP activity has been shown to be modulated by other molecules which can act either as potentiators of RGS GAP activity (eg. calmodulin) or allosteric inhibitors which reduce RGS GAP activity (eg. phosphatidylinositol-3,4,5-trisphosphate) (Neubig and Siderovski, 2002).

1.3.2. *GGL and DEP domains*

A number of RGS proteins have been found to interact with intracellular proteins to create scaffolding complexes. Notably, the C/R7 RGS family (RGS6, RGS7, RGS9 and RGS11) contains two additional functional regions, the Gγ-like (GGL) and the Disheveled/EGL-10/Plextrin homology (DEP) domains, and these serve as binding

interfaces for other signalling proteins (Druey, 2001; Chatterjee *et al.*, 2003; Civera *et al.*, 2005). The function of C/R7 RGS proteins may be dependent upon the SNARE-like proteins R9AP and R7BP, which bind to the DEP domain. R9AP serves a scaffolding function and is required for the stability, localization and function of RGS9-1 in the retina (Martemyanov *et al.*, 2003). Likewise, R7BP may play a similar role in the nervous system by forming part of a regulatory complex with C/R7 RGS proteins themselves (Keresztes *et al.*, 2004; Martemyanov *et al.*, 2005).

The GGL domains of C/R7 RGS proteins may bind to GPCRs, and are known to interact with other proteins to produce effects distinct from those that they have on G protein-mediated signalling. For instance, a Gβ5-independent function of the GGL domain in RGS localization has been described recently, wherein both the long N-terminus and the GGL domain sequence prevented nuclear/nucleolar accumulation of several distinct transcripts of human RGS6 (Chatterjee *et al.*, 2003). Moreover, RGS6, through a motif in the GGL domain that is distinct from the binding site of Gβ5, is able to bind to the transcriptional repressor protein DNA methyltransferase-associated protein 1 (DMAP1) and thus become part of a complex with DNA methyltransferase 1 (Liu and Fisher, 2004). In this context, RGS6 seems to decrease DNA methylation through the inhibition of the transcriptional repressor activity of DMAP1. These data suggest that RGS proteins can be involved in transcriptional regulation and have a G protein-independent function in the nucleus.

Finally, it was observed that the GGL domain of RGS6 can also interact with SCG10, a neuronal growth-associated protein. Co-expression of these proteins results in their co-

localization and a synergistic enhancement of PC12 cell differentiation induced by NGF (Liu *et al.*, 2002). Thus, this example emphasizes a role for RGS6 in neuronal differentiation via a G protein-independent mechanism. To complicate matters further, SCG10 also appears to bind to RGS20 (RGSZ1) which has no GGL domain, and this interaction has the opposite effect, resulting in the blockage of microtubule disassembly (Nixon *et al.*, 2002).

1.3.3. *GIPC*

GIPC belongs to a central PDZ (PSD95/DLG/ZO-1) domain-containing group of proteins (Katoh, 2002; Bockaert *et al.*, 2004). It was first identified by its ability to bind to the C terminus of RGS19/GAIP (hence its name RGS-GAIP interacting protein C terminus) through a PDZ-binding motif (De Vries *et al.*, 1998b; De Vries and Gist-Farquhar M., 1999; Lou *et al.*, 2002). However, since then it has been shown that GIPC-binding partners are not defined by a common, prototypical PDZ-binding motif, but rather GIPC interactions are flexible and for this reason, it is able to associate with a variety of proteins with divergent PDZ-binding motifs (Cai and Reed, 1999; Lou *et al.*, 2001; Hirakawa *et al.*, 2003). Many GIPC binding partners have been identified and these include GPCRs [(D_2 and D_3 dopaminergic and β_1 adrenergic, (Hu *et al.*, 2003; Jeanneteau *et al.*, 2004a; Jeanneteau *et al.*, 2004b)] and tyrosine kinase receptors [(IGF-1 and TGF beta type III, (Blobe *et al.*, 2001; Booth *et al.*, 2002)].

PDZ-containing proteins are known to (1) stabilize large functional complexes; (2) spatially cluster and anchor transmembrane proteins to specific subcellular domains; (3)

act as adaptors/scaffolds; (4) regulate trafficking of cytosolic proteins to and from the plasma membrane and (5) interact with the cytoskeleton (Fanning and Anderson, 1999). Therefore the GPCR-GIPC complex can be viewed as part of a dynamic protein network, necessary for fine-tuning downstream signalling and introducing functional diversity within different cell types, by acting as a bridge between GPCRs and other types of signalling molecules (Bockaert *et al.*, 2004). Evidence for these diverse interactions has been demonstrated by immunoprecipitation, pull-down assays and yeast-two hybrid screens in which GIPC has been found to bind to the cytoplasmic domain of a number of membrane proteins, such as the glucose transporter Glut-1 (Bunn *et al.*, 1999), semaphorin-F and its receptor neuropilin-1 (Cai and Reed, 1999; Wang *et al.*, 1999), syndecan-4 (Gao *et al.*, 2000), and the nerve growth factor receptor TrkA (Lou *et al.*, 2001). These findings imply that GIPC has an important function as a scaffolding protein, capable of spatially clustering and assembling receptors and signalling molecules in a particular cellular domain.

1.3.4. *GIPC and RGS proteins*

It has been well established that GIPC forms a complex with RGS19 (A/RZ family) that is membrane-anchored by its N-terminus and is mostly localized in clathrin-coated vesicles (De Vries *et al.*, 1998a). This localization supports a role for the RGS19-GIPC complex in the regulation of vesicular trafficking and endocytosis (De Vries *et al.*, 1998b; Wylie *et al.*, 1999; De Vries and Gist-Farquhar M., 1999; Fischer *et al.*, 2000; Fischer *et al.*, 2003). It is possible that the function of GIPC in this complex is simply to promote RGS19-mediated Gαi-GTP hydrolysis, ultimately terminating G protein signalling.

However, this seems unlikely since overexpression of both RGS19 and Gαi3 (the preferred substrate of RGS19) produces inhibition of vesicular trafficking (Hollinger and Hepler, 2002), suggesting that the mechanism is primarily independent of RGS19's GAP activity. Thus, the real significance of the RGS19-GIPC interaction is probably to create a complex which can further associate with other proteins (Druey, 2001; Lou *et al.*, 2001).

The RGS19-GIPC complex may be important in clustering transmembrane receptors with signalling molecules. As mentioned previously, it has been shown for the dopamine D_2 receptor that, once stimulated by dopamine agonists, the signal is fine-tuned by the GIPC-dependent protein complex, consisting of D_2 receptor and RGS19, in which the GIPC-receptor interaction actively recruits and clusters RGS19 to the plasma membrane (Jeanneteau *et al.*, 2004b). As yet, it is unclear whether this complex involves GIPC dimerization and/or other accessory proteins (i.e. G proteins) and/or post-translational modifications (Garzon *et al.*, 2004; Jeanneteau *et al.*, 2004a). However, it has been suggested that GIPC may accomplish this function by forming homo-oligomers which contain multiple PDZ-binding sites through interactions at its N terminus (Gao *et al.*, 2000; Hirakawa *et al.*, 2003; Jeanneteau *et al.*, 2004a).

The interaction between GIPC and RGS19 is also able to promote cross-talk between non-G protein and G protein signalling networks (Cai and Reed, 1999; Wang *et al.*, 1999; Lou *et al.*, 2001; Lou *et al.*, 2002). It has been observed that the RGS19-GIPC complex is likely to associate with the phosphorylated NGF receptor TrkA, where GIPC co-localizes with the receptor in retrograde transport vesicles and inhibits MAP kinase activation by NGF. In this example, GIPC facilitates a link between NGF tyrosine kinase pathways and

G proteins (Lou *et al.*, 2001). Similarly, Lou *et al.* (2002) presented evidence that megalin, belonging to the LDL (low-density lipoprotein) receptor family, binds to GIPC in clathrin-coated pits in the renal proximal tubule epithelium. Megalin was shown to be concentrated in endocytic compartments of the proximal tubule along with Gαi3, RGS19 and GIPC. Hence, it may be regulated partially by Gαi3, RGS19 and GIPC, suggesting a model in which G protein-mediated signalling modulates megalin's endocytic function and/or trafficking.

1.3.5. *Spinophilin*

Interactions between RGS and scaffolding proteins are not limited to the larger, more complex RGS proteins. A recent report demonstrated that spinophilin, a multi-domain protein is able to interact with RGS1, RGS2, RGS4, RGS16 and RGS19 in pull-down assays (Wang *et al.*, 2005). Spinophilin also binds to the 3rd intracellular loop of a number of GPCRs including α_{1B}-, α_{2A}-, α_{2B}-, and α_{2C}-adrenergic and D_2 dopaminergic receptors (Wang *et al.*, 2005). This suggests a possible scaffolding role, as was demonstrated using Xenopus oocytes, where spinophilin was found to enhance the ability of RGS2 to inhibit Ca^{2+} signalling via the α_{1B} adrenoceptor (Wang *et al.*, 2005). Spinophilin also contains a PDZ domain and interacts with protein phosphatase-1 and the cytoskeletal proteins F-actin (Smith *et al.*, 1999) and doublecortin (Tsukada *et al.*, 2003). Moreover spinophilin binds to the nucleotide exchange factor Tiam-1, where it promotes the activation of p70 S6 kinase by the small GTPase Rac (Buchsbaum *et al.*, 2003), further implying a role for spinophilin as an organizer of signalling complexes.

1.3.6. *Ca^{2+}/calmodulin*

The calcium sensor calmodulin undergoes a pronounced conformational change in response to the binding of calcium and regulates multiple signalling proteins (Berridge *et al.*, 2000). Ca^{2+}/calmodulin directly binds to RGS1, RGS2, RGS4, RGS10, RGS16, and RGS19, in a Ca^{2+}-dependent manner (Popov *et al.*, 2000). This binding does not seem to affect the GAP activity of the RGS proteins (Popov *et al.*, 2000), despite the finding that intracellular RGS activity can be increased by binding to Ca^{2+}/calmodulin. For instance in cardiomyocytes, RGS action on GIRK channels was facilitated via an increase in intracellular Ca^{2+} in a Ca^{2+}/calmodulin dependent manner (Ishii *et al.*, 2001). Ca^{2+}/calmodulin competes with PIP_3 for binding to RGS4 and this is significant since PIP_3 binding has been shown to inhibit GAP activity in a concentration-dependent manner (Popov *et al.*, 2000). Therefore it seems likely that calmodulin positively regulates RGS4 activity in cells not by increasing GAP activity per se, but by preventing the inhibition of GAP activity by PIP_3.

Calmodulin and PIP_3 both bind to the C-terminal portion of helix 4 of the RGS domain of RGS4 (Ishii *et al.*, 2005). This binding site is well conserved in different RGS proteins, suggesting that reciprocal regulation by PIP_3 and Ca^{2+}/calmodulin may be important for the physiological control of multiple RGS subtypes. The mutually exclusive binding of Ca^{2+}/calmodulin and PIP_3 to RGS proteins implies an elegant mechanism for RGS protein-mediated modification of intracellular Ca^{2+} oscillations *in vivo* (Luo *et al.*, 2001; Hollinger and Hepler, 2002). PIP_3 may initially prevent the RGS protein from inhibiting PLCβ activity, allowing an increase in intracellular Ca^{2+} and activation of Ca^{2+}/

calmodulin. Ca^{2+}/calmodulin will then compete for the PIP_3 binding site on the RGS protein, thereby promoting the RGS inhibitory effect on PLCβ activation. This in turn will decrease intracellular Ca^{2+}/calmodulin activation, thereby allowing PIP_3 to rebind to the RGS protein. In this way, the dual regulation of RGS activity can cause Ca^{2+} oscillations (Sierra *et al.*, 2000; Popov *et al.*, 2000).

1.4. **14-3-3 Proteins**

The name "14-3-3" has no functional significance and simply represents the elution fraction following DEAE-cellulose chromatography and its observed migration position on starch gel electrophoresis (Yaffe, 2002). 14-3-3 proteins are small (27-32 kDa) proteins which, despite having no detectable catalytic or functional domains (Tzivion *et al.*, 2001), appear to be involved in diverse cellular processes including signal transduction pathways, adhesion, cellular proliferation, survival and apoptosis (van Hemert *et al.*, 2004). 14-3-3 proteins function primarily as chaperones, adaptors and scaffolds (Jones *et al.*, 1995; Ferl *et al.*, 2002; Dougherty and Morrison, 2004; Wilker and Yaffe, 2004). They were initially thought to bind to either of two specific phosphorylated motifs (RSXpSXP and RXY/FXpSSXP) (Muslin *et al.*, 1996; Wilker and Yaffe, 2004), however it is now recognized that 14-3-3 binding sites vary widely among the many (~200) binding partners that have been identified, with some interactions occurring in a phosphorylation-independent manner (Aitken *et al.*, 2002; Pozuelo *et al.*, 2004). These binding partners include a number of regulatory proteins and integral components of signal transduction, such as GPCRs ($GABA_B$, (Couve *et al.*, 2001), α_2-adrenergic (Prezeau *et al.*, 1999) and parathyroid hormone receptors (Tazawa *et al.*, 2003)) (Figure

1.6), as well as tyrosine kinase receptors (Craparo *et al.*, 1997; Furlanetto *et al.*, 1997; Spence *et al.*, 2003), kinases (Irie *et al.*, 1994; Reuther *et al.*, 1994; Bonnefoy-Berard *et al.*, 1995; Camoni *et al.*, 1998; Light *et al.*, 2002), phosphatases (Conklin *et al.*, 1995), apoptosis-related proteins (Datta *et al.*, 2000; Bae *et al.*, 2003) and protooncogene products (Liu *et al.*, 1996).

The biological importance of 14-3-3 proteins would appear to be reflected in their high degree of sequence conservation, in their ubiquitous expression among eukaryotes and in their remarkable ligand binding flexibility (Aitken *et al.*, 2002). The manner in which 14-3-3 proteins regulate such a large number of substrates is still under investigation, however it has been shown that they are able to assemble oligomeric complexes (Tzivion *et al.*, 2001), act as phosphoprotein adaptors (Yaffe and Cantley, 1999; McGonigle *et al.*, 2001; McGonigle *et al.*, 2002; Ferl *et al.*, 2002), affect intracellular localization (Lopez-Girona *et al.*, 1999; Zhang *et al.*, 1999), and regulate apoptosis (Clark *et al.*, 1998; Datta *et al.*, 2000; Xing *et al.*, 2000; Bialkowska *et al.*, 2003).

The molecular consequences of 14-3-3 binding are diverse, and in most cases, are poorly understood. However, a few functions have been described including inhibition or promotion of protein interactions, alteration of enzymatic activity, sequestration of proteins in the cytoplasm and enhanced post-translational modifications (i.e. phosphorylation). Thus, it has been postulated that disturbances in 14-3-3 binding may be a factor in the occurrence of diseases such as cancer, Miller-Dieker syndrome, Alzheimer's and Parkinson's disease (Dougherty and Morrison, 2004; Wilker and Yaffe, 2004; Mhawech, 2005).

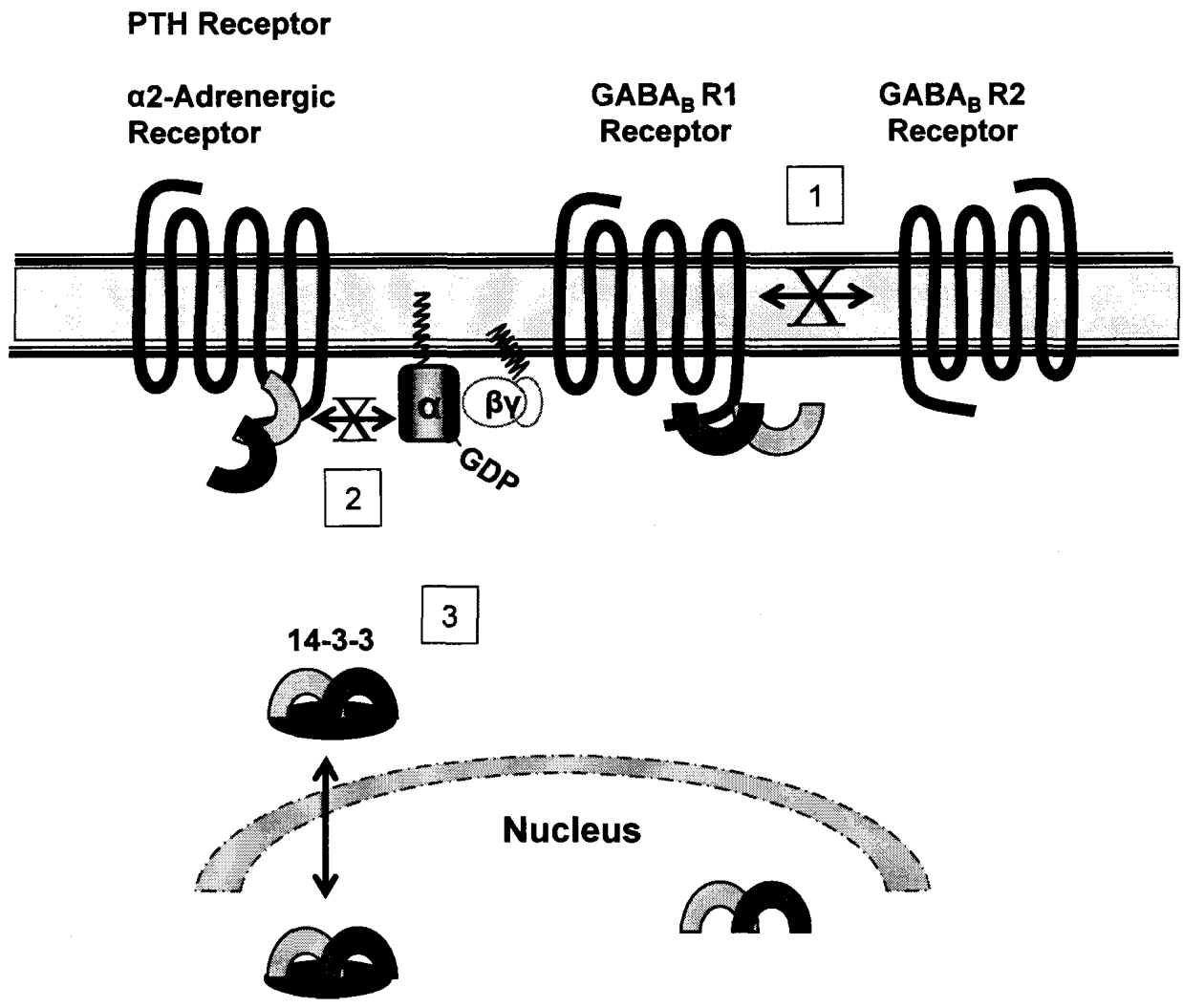

Figure 1.6. *Functions of 14-3-3 proteins.* 14-3-3 proteins (1) prevent heterodimerization of receptors and (2) can directly bind to several GPCRs to potentially inhibit signalling by impeding the GPCR – G protein interaction. Moreover, 14-3-3 proteins (3) may shuttle in and out of the nucleus due to their NES signal, and may act as chaperones by affecting the localization and function of some binding target proteins. PTH = Parathyroid hormone.

1.4.1. *14-3-3 structure*

In mammals, there are seven 14-3-3 isoforms (β, γ, ζ, σ, ε, η and τ,), in each of which there is 96-100% identity in the same isoform across different mammalian species (Ferl *et al.*, 2002; Dougherty and Morrison, 2004). 14-3-3 proteins are 'cup-shaped' dimers, in which monomers each containing 9 antiparallel α-helices are able to bind to a single phosphorylated target protein (Rosenquist *et al.*, 2000; Yaffe, 2002). Based on the crystal structure of 14-3-3ζ, four helices (3,5,7,9) form a negatively charged amphipathic groove and three conserved basic residues (Lys-49,Arg-56, Arg-127) constitute a pocket that may explain the high affinity of 14-3-3 for phosphorylated proteins (Yaffe, 2002) (Figure 1.7). This core region is highly conserved among 14-3-3 subtypes and species, and represents the main functional domain of the protein. Most importantly, it includes the 14-3-3 recognition sequences that bind to phosphoserine/threonine motifs on target proteins (Ferl *et al.*, 2002). Since the majority of the conserved residues are located inside the alpha-helices facing the interior of the protein, it is believed that one function of these residues is to stabilize the monomer and prevent degradation (Rosenquist *et al.*, 2000).

Under physiological conditions, 14-3-3 proteins primarily exist as homo- and hetero-dimers that are very stable and do not exchange monomers readily (Chaudhri *et al.*, 2003). Its dimerization is the result of an interaction between the N-terminus of two monomers, forming a conserved dimer interface. In most cases, 14-3-3 dimerization is required for its function (Tzivion *et al.*, 2001) and despite the fact that the mechanism underlying the modulation of 14-3-3 dimerization is unclear, it appears to be an important phenomenon (Aitken, 2002; Powell *et al.*, 2002; Shen *et al.*, 2003; Woodcock *et al.*, 2003). Dimerization of 14-3-3 not only stabilizes its structure, but it allows the protein to

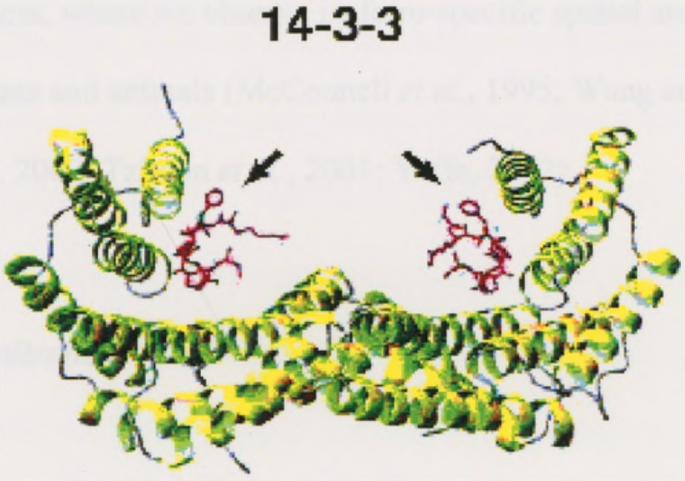

Figure 1.7. *14-3-3 protein structure*. This model is based on the crystal structure of 14-3-3ζ dimer. The arrows indicate the location where phosphoserine peptides bind within the ligand-binding groove (Dougherty and Morrison, 2004). Reprinted with permission from: J Cell Sci 2004 117: 1875-1884 and Company of Biologists Ltd.

function as an adaptor wherein each monomer can interact with a different protein and promote a novel protein complex (Jones *et al.*, 1995; Aitken *et al.*, 2002; Ferl *et al.*, 2002). Moreover, 14-3-3 heterodimerization may partially account for its specificity towards some target binding proteins. This is supported by the observation that heterodimerization of 14-3-3 protein appears to be tightly regulated *in vivo*, especially during development, where we observe isoform-specific spatial and temporal gene expression in plants and animals (McConnell *et al.*, 1995; Wang and Shakes, 1996; Rosenquist *et al.*, 2000; Tzivion *et al.*, 2001; Yaffe, 2002).

1.4.2. *14-3-3 localization*

14-3-3 proteins were first identified in brain tissue where they demonstrate the highest abundance, but in fact they seem to be present in all tissues (Ferl *et al.*, 2002; Dougherty and Morrison, 2004). 14-3-3 proteins have been observed in both the cytoplasm and in the nucleus, and have been found to be associated with the cytoskeleton, centrosomes and membranes. As of yet, there is no definite explanation of the requirement for the seven highly conserved 14-3-3 isoforms in mammals (Wang and Shakes, 1996; Fu *et al.*, 2000; Tzivion *et al.*, 2001). In fact, it still remains unclear whether individual 14-3-3 isoforms have specialized functions or whether their actions depend entirely on tissue-specific regulation and/or temporal and developmental control (Rosenquist *et al.*, 2000; Aitken, 2002; Sehnke *et al.*, 2002; Yaffe, 2002). Despite the significant structural similarity between 14-3-3 isoforms, there is evidence to support the notion that 14-3-3 proteins might slightly differ from one another based on their

differential subcellular localization and expression, and their specificity towards different protein binding partners (Tzivion and Avruch, 2002).

1.4.3. *Phosphorylation*

Signal transduction events often involve phosphorylation-dependent transition states of a protein and it is becoming apparent that such changes are not always spontaneous but in many cases, are caused by the phosphorylation-induced association with accessory proteins (McGonigle *et al.*, 2001; Foschi *et al.*, 2001; McGonigle *et al.*, 2002). Phosphorylation of the 14-3-3 target protein generally seems to increase its interaction with 14-3-3 (Zenke *et al.*, 2004) and this phosphorylation-dependent protein interaction may differentially affect the function of the target protein (Muslin *et al.*, 1996; Yaffe, 2002; Dumaz and Marais, 2003; Tazawa *et al.*, 2003).

Several 14-3-3 isoforms have been shown to be phosphorylated by kinases including PKC (Van Der Hoeven *et al.*, 2000), casein kinase I (Dubois *et al.*, 1997) and a serine kinase sphingosine-dependent protein kinase (SDK1) (Megidish *et al.*, 1998; Woodcock *et al.*, 2003), and it has been hypothesized that phosphorylation is a mechanism by which 14-3-3 function can be regulated. Thus, it is thought that the phosphorylation of 14-3-3 may alter its ability to dimerize with itself and/or to bind to its target proteins. However the physiological significance of 14-3-3 phosphorylation and how 14-3-3 proteins are regulated *in vivo* still remains to be elucidated (Tzivion *et al.*, 2001).

1.4.4. *14-3-3 mechanisms of action*

14-3-3 proteins demonstrate a range of functions that are highly dependent on the target protein itself (Mackintosh, 2004). For example 14-3-3 proteins can bind to a single protein and affect its conformation and/or enzymatic activity, as well as reveal or mask functional motifs that can ultimately alter a protein's localization, stability, activity and/or phosphorylation state (Yaffe, 2002). In other cases, 14-3-3 proteins may bind to two proteins simultaneously and promote cross-talk between different networks and/or recruit effector enzymes for downstream signalling (El Far and Betz, 2002; Olayioye *et al.*, 2003), as is observed with the α_2-adrenergic receptor (Prezeau *et al.*, 1999).

All 14-3-3 proteins contain a nuclear export signal (NES) that behaves as a regulator of subcellular localization, whereby 14-3-3 alters the cytoplasmic/nuclear localization of its target proteins and via spatial control modulates their function in signalling pathways (Gorlich and Mattaj, 1996; Lopez-Girona *et al.*, 1999; Rittinger *et al.*, 1999; Zhang *et al.*, 1999; Sehnke *et al.*, 2002). This is clearly seen in the glucocorticoid signalling pathway, where 14-3-3σ acts as a negative regulator of the glucocorticoid receptor by interacting with and promoting cytoplasmic subcellular localization of the ligand-free receptor (Kino *et al.*, 2003). Finally, 14-3-3 proteins are able to bind to and sequester proteins in subcellular compartments, preventing further interactions with their targets. This occurs in the phosphorylation-dependent binding of 14-3-3 to BAD (a pro-apoptotic member of the Bcl-2 family), in which 14-3-3 blocks apoptosis by relocating BAD from the mitochondria (Datta *et al.*, 2000). In fact, it has been hypothesized that a similar mechanism may be responsible for the inhibitory action of 14-3-3 on RGS proteins.

1.5. **14-3-3 and RGS Proteins**

Recently, RGS1, RGS3, RGS7, RGS8, RGS9 and RGS16 have all been identified as 14-3-3 binding partners, and thus far it appears that 14-3-3 proteins impede RGS function by such binding (Benzing *et al.*, 2000; Benzing *et al.*, 2002; Niu *et al.*, 2002; Garzon *et al.*, 2005; Ward and Milligan, 2005). A study carried out by Benzing *et al.* (2000) demonstrated that 14-3-3τ was able to abolish the inhibitory effect of RGS3 on the carbachol-mediated MAP kinase activation in HEK293 cells. Subsequently, Schreiber *et al.* (2001) observed that 14-3-3τ suppressed the cystic fibrosis transmembrane conductance regulator (CFTR) by antagonizing the inhibitory effects of RGS3 on Gαi2 protein. Finally, the fast RGS7-mediated deactivation kinetics of G protein-coupled inwardly rectifying K^+ channels (GIRKs) were slowed by the 14-3-3τ isoform (Benzing *et al.*, 2002).

The foregoing observations suggest that 14-3-3 may impede RGS protein GAP activity, and indeed this has been demonstrated with 14-3-3-dependent decreases in the inhibitory effects of both RGS3 and RGS7 on G protein-mediated signals, and a reduced GAP effect of RGS7 on free Gαi1 (Benzing *et al.*, 2000) (Figure 1.8). However, a recent study failed to show inhibition of RGS7 and RGS16 GAP activity by 14-3-3 proteins in a GPCR-dependent, steady state GTP hydrolysis assay using membranes from HEK293 cells. The reason for this discrepancy is unclear and the effects of 14-3-3 proteins on RGS GAP activity still remain to be established in a receptor-activated G protein model system.

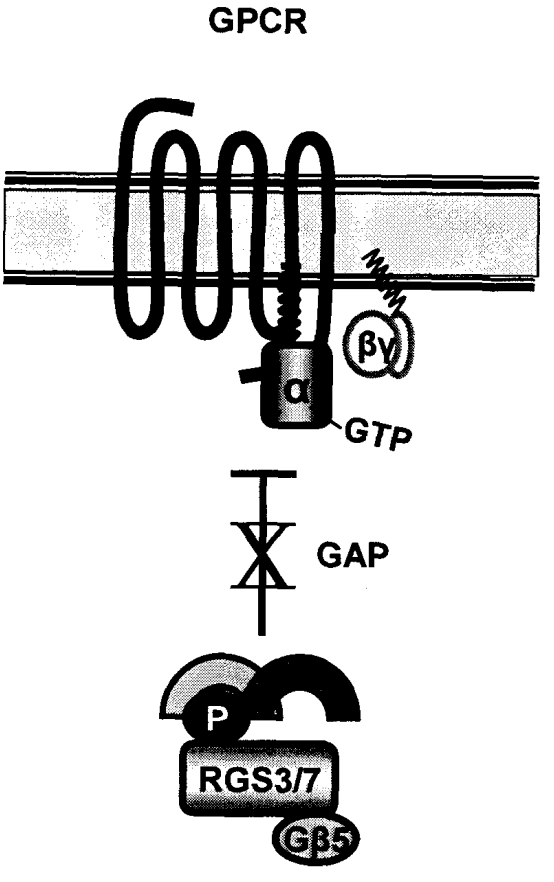

Figure 1.8. *14-3-3 and RGS proteins.* 14-3-3 proteins have been observed to bind to phosphorylated RGS3 and RGS7 and inhibit their GAP activity. , 14-3-3 protein; P, phosphorylation; Gβ5, Gβ5 subunit.

The primary 14-3-3-binding site on RGS7 and RGS3 appears to be located within the Gα-binding RGS domain at a conserved ^{434}SYP motif (Benzing *et al.*, 2000; Benzing *et al.*, 2002). Interestingly, the SYP motif is present in about half of all RGS proteins, suggesting that other RGS isoforms may also be subject to14-3-3 regulation (Figure 1.9). According to Benzing *et al.* (2002), divergent sequences are thought to preclude 14-3-3 – RGS interactions. For example, the SYR-containing RGS4 was found by this group to lack apparent sensitivity to 14-3-3, but it did appear to interact when a proline residue was substituted for the divergent arginine. In contrast to these findings, recent data suggest that 14-3-3 – RGS protein binding interactions may be less limited than originally perceived. Another study found that RGS9-2, which has an alanine substituted for the conserved proline, can bind to 14-3-3 (Garzon *et al.*, 2005) (Figure 1.9). Similarily, RGS3 appears to have a second 14-3-3-binding site that is outside the RGS domain, located near the N-terminus (Niu *et al.*, 2002; Ward and Milligan, 2005). Despite the differences in the putative 14-3-3-binding site within RGS proteins, it is clear that 14-3-3 proteins seem to modulate RGS function by interfering with its binding to Gα proteins.

14-3-3 proteins are primarily known to bind to phosphorylated target proteins and previous reports have claimed that phosphorylation of the RGS protein is required for 14-3-3 binding. Indeed, phosphorylation of RGS3 and RGS7 increases their apparent affinities for 14-3-3, and the protein interaction appears to be dependent upon the phosphorylation of serine 434 (SYP motif) within RGS7 and analogously of serine 264 (N-terminus) within RGS3 (Benzing *et al.*, 2000; Niu *et al.*, 2002; Ward and Milligan, 2005). In support of this concept, cellular phosphatases appear to increase the pool of

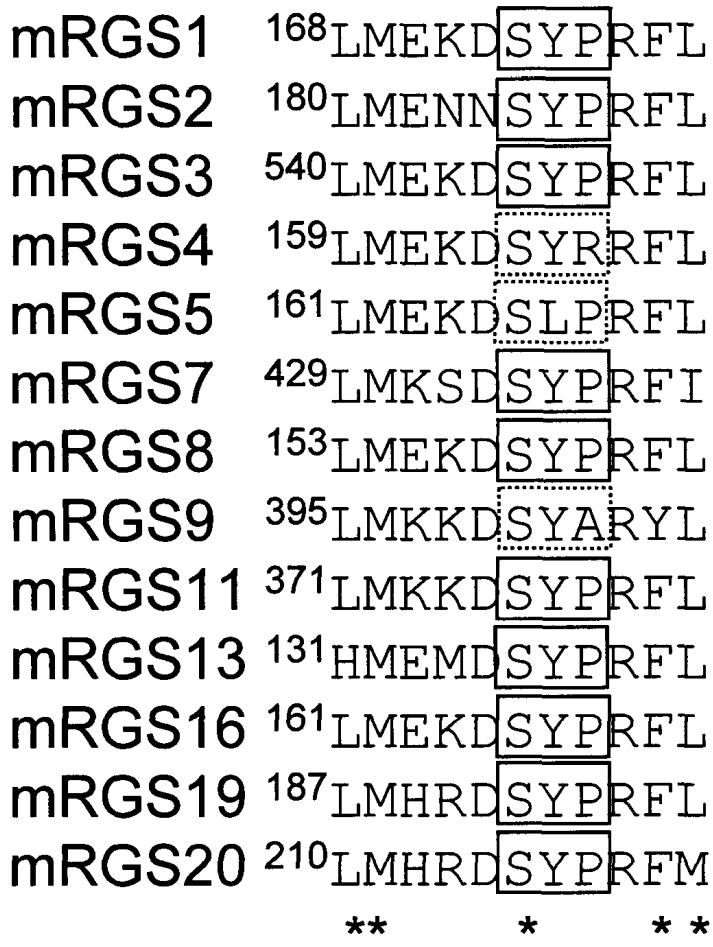

Figure 1.9. *The SYP motif in RGS proteins.* The sequences represent the alignment of the end portion of the RGS domain and illustrate all mouse RGS proteins that possess the conserved putative 14-3-3 binding motif [SYP], while the [.....] emphasizes divergent motifs in RGS proteins that are relevant to the discussion. * indicates the most conserved residues among mammalian RGS proteins and invertebrate homologs.

active RGS7 by impeding 14-3-3 binding to RGS proteins (Benzing *et al.*, 2000; Benzing *et al.*, 2002; Niu *et al.*, 2002). Hence, the phosphorylation-dependent interaction of RGS proteins with 14-3-3 may serve as a mechanism to rapidly modulate RGS protein GAP activity without altering their expression (Figure 1.8).

However, it is becoming apparent that phosphorylation of serine residues within the RGS binding sites may not be required for all RGS-14-3-3 interactions. Recently, it has been demonstrated that once the serine residue within the SYP motif is mutated into an aspartate, which is thought to act as a phosphoserine mimic, 14-3-3 binding to both RGS3 and RGS16 remains unchanged compared to wild-type. However, this substitution seems to be detrimental to RGS GAP activity (Ward and Milligan, 2005). These data suggest that phosphorylation of this conserved serine does not necessarily increase the binding affinity of 14-3-3 to an RGS protein, but rather might be a potential modulator of RGS function itself. In fact, phosphorylation of some RGS proteins has the ability to either decrease or increase GAP activity (i.e. RGS9) (Riddle *et al.*, 2005), as well as to affect their intracellular localization (Chidiac and Roy, 2003). Thus, it may be that phosphorylation of serine 434 is unique to RGS7, where it appears to promote 14-3-3 binding and this might not occur in all RGS proteins. In the case where phosphorylation of RGS proteins does increase 14-3-3 binding (i.e. RGS3, RGS7), additional phosphorylation sites may be involved that could contribute to the inhibition of GAP activity seen *in vitro.* Despite the controversy regarding the requirement of RGS phosphorylation for 14-3-3 association, it is becoming evident that 14-3-3 proteins are likely associated with, and important regulators of RGS function *in vivo* and *in vitro.*

1.6. **Rationale for the Present Studies**

The activity of RGS proteins can be regulated in a number of different ways and one possible mechanism is through their interaction with other proteins. Auxiliary proteins such as GIPC seem to be tightly intertwined in signal transduction pathways, and play essential roles in organizing and cross-linking various signalling components. For this reason, our interest lies in determining novel RGS binding partners that may modulate their function and ultimately affect receptor signalling.

The primary 14-3-3 binding site(s) within RGS proteins is still under investigation. Based on peptide sequence analysis, RGS3, RGS9, RGS12, RGS14 and RGS19 all possess the putative 14-3-3-binding domain in the N-terminus, while the SYP motif is present in RGS1, RGS2, RGS3, RGS7, RGS8, RGS11, RGS13, RGS16, RGS19, RGS20 (Figure 1.9). Indeed, it is possible that RGS proteins possess distinct or even multiple 14-3-3-binding sites (Yaffe *et al.*, 1997; Tzivion *et al.*, 2001), in which the additional sites may increase the affinity and stability of this protein-protein interaction. This is seen in the association of 14-3-3 with several other target proteins, such as c-Raf-1 which contains at least two 14-3-3 binding sequences (Tzivion *et al.*, 1998; Sehnke *et al.*, 2002).

A yeast 2-hybrid screen in our laboratory revealed that 14-3-3ε was indeed a putative binding partner for RGS4, when screened against a mouse brain cDNA library, in contrast to the findings of Benzing *et al.* (2000). This observation served as the impetus for the work described in this thesis, and led to the detailed investigation of the interaction between 14-3-3 and other RGS proteins belonging to the B/R4 subfamily. RGS4, RGS5

and RGS16 are highly homologous and little is known about their binding to 14-3-3 proteins. To compare 14-3-3ε to an isoform known to form homodimers, and additionally to allow comparison with previous studies on 14-3-3 and RGS proteins (Benzing *et al.*, 2000; Benzing *et al.*, 2002), we studied the effects of 14-3-3β concurrently. Based on the available literature, one may question the importance of the SYP motif within the RGS domain and its phosphorylation in terms of the binding of RGS to 14-3-3 proteins.

The main objectives of my thesis are:

(1) To characterize the direct interaction of RGS proteins (specifically RGS4, RGS5 and RGS16) with two isoforms of 14-3-3 [14-3-3 beta (β) and 14-3-3 epsilon (ε)] in different experimental systems

(2) To establish the functional significance of the protein interactions observed using *in vitro* steady state GTP hydrolysis assays and competitive pull-down experiments

(3) To investigate the role of the tyrosine residue in the SYP putative 14-3-3-binding motif within the RGS domain

We hypothesize that 14-3-3 proteins directly bind to RGS proteins and act as negative modulators of RGS GAP activity by sequestering them away from their G protein targets (Figure 1.10).

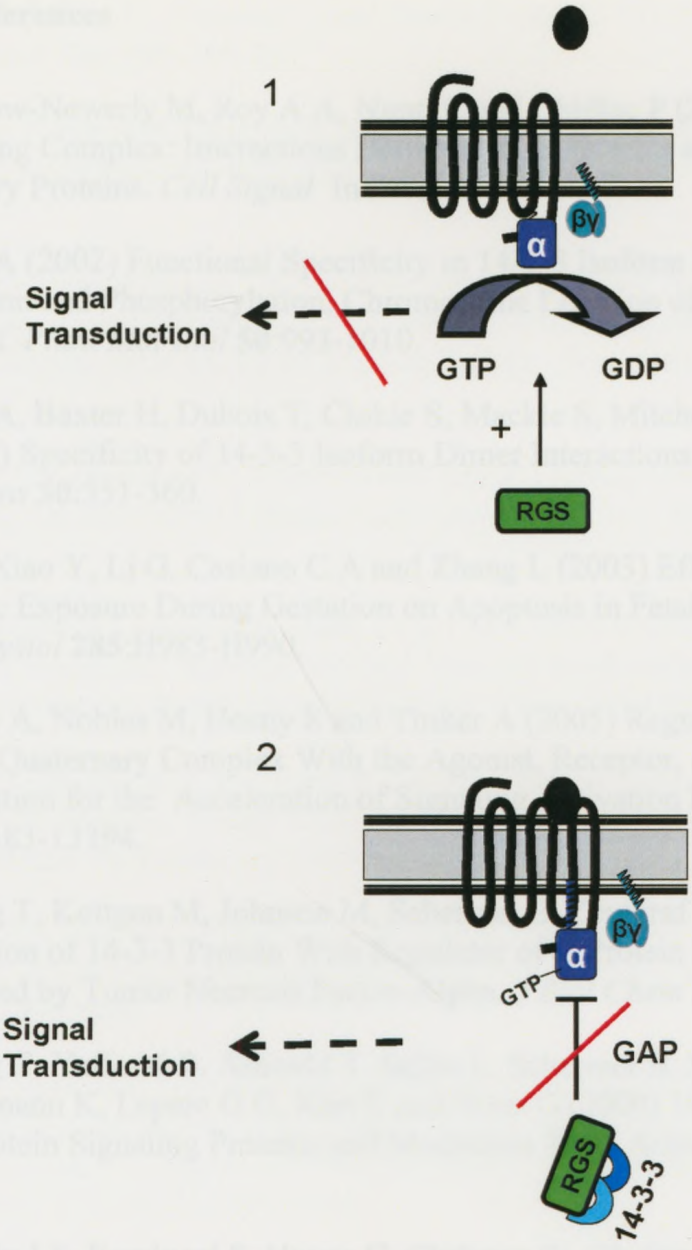

Figure 1.10. *Model mechanism for the role of 14-3-3 on RGS function.* The GPCR signalling pathway is activated by the binding of an agonist that leads to the exchange of GDP for GTP on the Gα subunit, a conformational change of the heterotrimeric G protein and the binding of Gα to the receptor. (1) In the presence of RGS alone, the signal transduction pathway is inhibited by RGS GAP activity but (2) in the presence of 14-3-3 proteins, signalling is prolonged due to the 14-3-3 - RGS complex that sequesters the RGS protein away from the G protein.

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CHAPTER 2

MODULATION OF RGS FUNCTION BY 14-3-3 PROTEINS

2.1. **Introduction**

G protein-coupled receptors (GPCRs) are the most diverse type of cell surface protein and are involved in a wide variety of important physiological functions (Bockaert *et al.*, 2003; Kristiansen, 2004). In response to specific agonist signals, GPCRs act as guanine nucleotide exchange factors (GEFs) and accelerate the exchange of GDP for GTP on the Gα subunit of heterotrimeric G proteins. This is followed by a conformational change within the G protein and activation of the Gα subunit, whereby both the GTP-bound Gα and the Gβγ subunits propagate downstream signalling via effectors and second messengers.

Regulator of G protein signalling (RGS) proteins are primarily known as negative regulators of G protein-mediated signalling pathways and function as GTPase activating proteins (GAPs) for the α subunit of hetrotrimeric G proteins (Watson *et al.*, 1996; Berman and Gilman, 1998; Wilkie and Ross, 2000; De Vries *et al.*, 2000). However, the functions of RGS proteins appear to be more complex *in vivo* than previously believed, and they should be seen as multifunctional signalling regulators due to their interaction with proteins other than G proteins through regions distinct from the RGS domain (De Vries and Gist-Farquhar M., 1999; Druey, 2001; Abramow-Newerly *et al.*, 2005). This is further supported by the observation that several RGS proteins are located in sites other than the plasma membrane, including in the nucleus (Burchett, 2003). The activity and expression of RGS proteins are highly regulated within the cell, as might be expected based on their profound effects on GPCR-mediated signalling. They appear to be modulated through various mechanisms including the regulation of their subcellular

localization, post-translational modifications and interactions with protein binding partners (Song *et al.*, 1999; Kim *et al.*, 1999; Wilkie and Ross, 2000; Schiff *et al.*, 2000; Roy *et al.*, 2003; Chatterjee *et al.*, 2003). For instance, phosphorylation of RGS proteins affects their intracellular localization, as is observed with RGS4 (Pedram *et al.*, 2000), RGS19 (Fischer *et al.*, 2000) and RGS10 (Burgon *et al.*, 2001).

14-3-3 proteins are small dimeric proteins (27-32 kDa), with seven highly conserved isoforms (β, γ, ζ, σ, ε, η and τ,) in mammals, whose functions appear to be largely similar (Fu *et al.*, 2000; Rosenquist *et al.*, 2000; Tzivion *et al.*, 2001; Aitken, 2002). 14-3-3 proteins were initially thought to bind to either of two specific phosphorylated motifs (RSXpSXP and RXY/FXpSSXP) (Muslin *et al.*, 1996), however many binding partners have been identified that lack these motifs and it is now recognized that there are more than 200 binding partners, with some interactions occurring in a phosphorylation-independent manner (Dougherty and Morrison, 2004; Pozuelo *et al.*, 2004). 14-3-3 proteins bind to a number of regulatory proteins and integral components of signal transduction, including several GPCRs ($GABA_B$, (Couve *et al.*, 2001), α_2-adrenergic (Prezeau *et al.*, 1999) and parathyroid hormone receptors (Tazawa *et al.*, 2003)), as well as tyrosine kinase receptors (Furlanetto *et al.*, 1997; Craparo *et al.*, 1997; Spence *et al.*, 2003), kinases (Reuther *et al.*, 1994; Irie *et al.*, 1994; Bonnefoy-Berard *et al.*, 1995; Camoni *et al.*, 1998; Light *et al.*, 2002), phosphatases (Conklin *et al.*, 1995), apoptosis-related proteins (Datta *et al.*, 2000; Bae *et al.*, 2003) and protooncogene products (Liu *et al.*, 1996). Despite having no detectable catalytic or functional domains (Tzivion *et al.*, 2001), 14-3-3 proteins appear to be regulators of key signalling components and function

primarily as chaperones, adaptors and scaffolds (Jones *et al.*, 1995; Ferl *et al.*, 2002; Wilker and Yaffe, 2004; Dougherty and Morrison, 2004).

RGS proteins can bind to, and be negatively modulated by 14-3-3 proteins (Schreiber *et al.*, 2001) and through a yeast 2-hybrid screen, we have identified 14-3-3ε as a putative binding partner for RGS4. Previous studies by Benzing *et al.* (2000 and 2002) identified a primary 14-3-3-binding site on RGS3 and RGS7 that is located within the Gα-binding RGS domain, at a conserved SYP motif. Moreover, another group demonstrated that RGS3 has a second 14-3-3-binding site that is outside the RGS domain, located near the N-terminus and which is dependent on the phosphorylation of serine 264 (Niu *et al.*, 2002; Ward and Milligan, 2005). Despite the differences in the 14-3-3-binding sites on RGS3 and RGS7, similar conclusions have been drawn that describe the ability of 14-3-3 proteins to interfere with the RGS-Gα protein interaction. Hence, the phosphorylation-dependent interaction of RGS with 14-3-3 proteins may serve as a mechanism to rapidly modulate intracellular GAP activity without altering RGS protein expression (Benzing *et al.*, 2000; Niu *et al.*, 2002; Ward and Milligan, 2005).

Recent reports (Garzon *et al.*, 2005; Ward and Milligan, 2005) suggest that 14-3-3 – RGS protein binding interactions may be less limited than originally perceived. Here, we identify novel interactions between RGS4, RGS5 and RGS16 of the B/R4 subfamily and two 14-3-3 isoforms [14-3-3 beta (β) and 14-3-3 epsilon (ε)]. The main objectives of this study were (1) to characterize the direct interaction of RGS proteins with two 14-3-3 isoforms in different experimental systems, (2) to establish the functional significance of

the protein interactions observed using *in vitro* steady state GTP hydrolysis assays and competitive pull-down experiments, and finally (3) to investigate the role of the tyrosine residue in the SYP putative 14-3-3-binding motif within the RGS domain. Based on our data, we suggest a mechanism wherein 14-3-3 proteins negatively modulate RGS function and act as molecular chelators that sequester RGS proteins away from both the G protein and the plasma membrane. Thus, we conclude that 14-3-3 proteins indirectly promote GPCR signalling via their inhibitory effects on RGS proteins.

2.2. **Materials and Methods**

2.2.1. *Constructs*

Human 14-3-3β and 14-3-3ε, cloned into the bacterial/mammalian expression vector pTriEX4 (HIS-tagged), were gifts from Dr. M Kahn (Department of Pathobiology, University of Washington, USA) and were subcloned in-frame into the bacterial pGEX-4T1 expression vector (GST-tagged). Briefly, 14-3-3 fragments were cut with SmaI and NotI from pTriEx4 vector and inserted into pGEX-4T1 vector at EcoRI and NotI sites. Human pGEX-5X-3-RGS16 and human pGEX-5X-3-RGS5 were generously donated by Dr. MT Greenwood (Department of Medicine, McGill University, Canada). Rat pGEX-4T-RGS4 was a gift from Dr. RR Neubig (Departments of Pharmacology and Internal Medicine/Hypertension, University of Michigan, USA). Bacterial expression vectors

encoding N-terminally hexa-histidine-tagged rat RGS4 (QE-60-RGS4) and mouse RGS16 (pET20b-RGS16) were generously provided by Dr. JR Hepler (Department of Pharmacology, Emory University School of Medicine, USA). The open-reading frame of mouse RGS5 was cloned into pCR2.1-TOPO (Invitrogen, San Diego, CA) using previously described methods (Snow *et al.*, 2002) and was provided by Dr. DP Siderovski (Department of Pharmacology, The University of North Carolina, USA). Mouse RGS5 was cut with BamHI and XbaI restriction enzymes and inserted in-frame into pET19b expression vector. 3xHA-tagged human RGS4, RGS5 and RGS16 proteins (subcloned into the mammalian pDNA3.1(+) expression vector) were obtained from the UMR cDNA Resource Center (University of Missouri-Rolla). In all cases, HIS and GST tags within the RGS and 14-3-3 fusion proteins are located on the N-terminus. All constructs were sequenced and verified (DNA Sequencing Facility, Robarts Research Institute, University of Western Ontario, Canada).

Leucine to tyrosine (CTG to TAT), and tyrosine to leucine (TAT to TTG) RGS5 and RGS16 mutants respectively, were constructed using the Stratagene QuikChange site-directed mutagenesis protocol. pET19b-RGS5 and pET20b-RGS16 were used as DNA templates and were amplified in a PCR reaction (16 cycles of amplification) using Platinum P*fx* Taq Polymerase (Invitrogen). The following oligonucleotides, and their reverse complements were used as primers: pET19b-RGS5 (sense-CCTGATGGAGA AGGATTCTTATCCCCGCTTTGTGCGCTCTG) and pET20b-RGS16 (sense-GATGG AGAAGGACTCCTTGCCGCGCTTCCTCAAGTC). The presence of the appropriate

mutations was confirmed by sequencing (DNA Sequencing Facility, Robarts Research Institute, University of Western Ontario, Canada).

2.2.2. *Protein purification*

N-terminally hexa-histidine (6xHis)-tagged RGS and 14-3-3 proteins were purified from *Escherichia coli* (*E. coli*) strain BL21/DE3 essentially as described in Cladman and Chidiac (2002). Five hundred ml of LB media containing ampicillin (final concentration, 100 μg/ml) were inoculated with 20 ml of transformed cells that had been incubated overnight at 37°C, and were grown to an $OD_{600} \geq 0.5$. Expression of the 6xHIS-tagged proteins was induced by the addition of 150 μM isopropyl-β, D-thiogalacto pyranoside (IPTG) for 3 h before harvesting the bacteria by centrifugation at 6 370 *x g* for 10 min at 4°C. Bacteria were resuspended in buffer A (final concentrations, 20 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 10 μg/ml aprotinin) (30 ml/L culture) and incubated on ice with 0.2 mg/ml lysozyme for 1 h. Twenty five μg/ml DNase and 0.5 mM $MgCl_2$ were added on ice for 30 min. After centrifugation (13 000 *x g*, 35 min, 4°C), the volume of supernatant was increased to 25 ml/L culture with buffer B (final concentrations, 50 mM Hepes, pH8.0, 150 mM NaCl, 20 mM 2-mercaptoethanol, 1% Triton X-100, 0.1 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 50% glycerol). Twenty mM imidazole and 50% Ni-NTA affinity resin (Qiagen) equilibrated in buffer B, were incubated with the supernatant (1.5 h, 4°C on a rocker). Later, the resin was loaded onto a 30 ml column and washed with 20 ml buffer C (final concentrations, 50 mM Hepes, pH8.0, 500 mM NaCl, 20 mM

2-mercaptoethanol, 1% Triton X-100, 0.1 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 20 mM imidazole) and 15 ml buffer D (final concentrations, 100 mM Hepes, pH8.0, 300 mM NaCl, 40 mM 2-mercaptoethanol, 0.2 mM PMSF, 2 μg/ml leupeptin, 20 μg/ml aprotinin, 40 mM imidazole). The proteins were eluted with 650 μl buffer E (final concentrations, 100 mM Hepes, pH8.0, 300 mM NaCl, 40 mM 2-mercaptoethanol, 0.2 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 400 mM imidazole) after a 20 min incubation (protein purified to >95%). A maximum of three protein samples from the Ni-NTA column were loaded on and eluted from a Superdex 75 HR20/30 column (Pharmacia). Peak fractions were pooled, placed in aliquots and stored at –80°C.

For the purified Glutathione-S-Transferase (GST) and GST-fusion proteins, *E. coli* strain BL21/DE3 was transformed with pGEX-RGS or 14-3-3 constructs, induced with 200 μM IPTG (4 h, 37°C). Cell were pelleted and resuspended in PBS (final concentrations, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.2 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, pH adjusted to 7.4). The pellets were then frozen at –80°C overnight. Samples were thawed and sonicated on ice (10 x 15 s bursts, allowing 5 s for cooling between bursts). Triton X-100 (final, 1%) was added (30 min on ice) and the insoluble material was removed by centrifugation at 8 500 *x g*, 10 min, 4°C. The supernatant was collected and incubated with a 50% slurry of glutathione-Sepharose 4B beads (equilibrated in PBS) for 30 min at room temperature, rotating end-over-end (Amersham Pharmacia Biotech). After centrifugation (500 *x g*, 5 min, 4°C), the glutathione-Sepharose 4B beads were washed three times with PBS and proteins were

eluted with glutathione elution buffer (0.0154 g of reduced glutathione dissolved in 5 ml of 50 mM Tris-HCl, pH8.0).

All samples were visualized by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with 0.1% Coomassie Blue. Protein concentrations were determined by Bradford assay (Bio-Rad Protein Assay) according to the manufacture's instructions.

2.2.3. *Mammalian cell transfection*

Human embryonic kidney (HEK)-293 cells were seeded onto 10 cm dishes ($7x10^5$ cells/plate) the day before transfection and at 50-70% confluency, were transiently transfected with 10 μg pcDNA HIS-tagged 14-3-3 (β and ε isoforms) or HA-tagged RGS constructs (RGS4, RGS5, RGS16), using the calcium phosphate precipitation. Briefly, 9 ml of complete Minimum Essential Medium (MEM) was added 2 h prior to precipitation. For each dish, 10 μg pcDNA was diluted in 500 μl H_2O containing $CaCl_2$ (final concentration, 0.25 M). A precipitate containing calcium phosphate and DNA was formed by slowly bubbling 500 μl 2x HEPES-buffered saline solution (HeBS) (final concentrations, 0.28 M NaCl, 0.05 M HEPES, 1.5 mM Na_2HPO_4, H_2O) and adding the DNA/$CaCl_2$ solution dropwise. The precipitate was incubated for 20 min at room temperature before being distributed evenly over the 10 cm dish. The cells were incubated with the precipitate for 5 h under standard growth conditions (37°C, 5% CO_2) and later, were washed twice with 5 ml PBS and stored in 10 ml of complete MEM.

2.2.4. *Cell lysate pull-down experiments*

Twenty to forty eight hours after transfection, the attached cells were rinsed twice with PBS, trypsinized with Trypsin-EDTA (Gibco), collected by centrifugation (514 *x g*, 5 min, 4°C), resuspended in buffer F (final concentrations, 50 mM Tris-HCl, pH7.6, 1 mM EDTA, pH8.0, 0.4 M NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM NaF, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin). The samples were sonicated (3 X 5 s), subjected to centrifugation (20 800 *x g*, 5 min, 4°C) and the supernatants were transferred to new microfuge tubes. Five hundred μl of supernatant was incubated with 50 μl of a 50% slurry of glutathione-Sepharose 4B beads (equilibrated in buffer F) for 1 h (pre-clearing step) and later subjected to centrifugation (500 *x g* for 5 min at 4°C) and transferred into fresh tubes. Cell lysates were incubated with 10 μg of GST or GST-fusion proteins of RGS4, RGS5, RGS16, 14-3-3β or 14-3-3ε for 4 h (incubations for shorter time periods were found to yield inconsistent results), followed by an overnight incubation with 30 μl of equilibrated 50% slurry of glutathione-Sepharose 4B beads at 4°C, with gentle rotation. Cell lysates were then subjected to centrifugation (500 *x g* for 5 min at 4°C) and the glutathione-Sepharose 4B beads were washed by resuspension and centrifugation three times in 1 ml buffer G (final concentrations, 50 mM Tris-HCl, pH7.6, 1 mM EDTA, 0.4 M NaCl, 0.1% Triton X-100, 0.5 mM NaF, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin). The proteins were released from the glutathione-Sepharose 4B beads by heating at 99°C for 5 min, and 25 μl of loading buffer was added (60 mM Tris-HCl, pH6.8, 24% glycerol, 2% SDS, 20 mg bromophenol blue, 2-mercaptoethanol) for immunoblot analysis. For

negative controls, each lysate was incubated with purified GST protein and glutathione-Sepharose 4B beads as appropriate to determine non-specific binding. To verify protein expression, 4% of cell lysate taken prior to the pull-down experiment was assessed by Western blot analysis.

2.2.5. *Pull-down experiments with purified proteins*

For the purified protein pull-down experiments, HIS-14-3-3ε or HIS-14-3-3β proteins [0.1 μM or 0.5 μM final concentrations] were diluted in 500 μl buffer G to which 5 μg of GST [0.4 μM] or GST-RGS4, GST-RGS5 or GST-RGS16 [0.2 μM] had been added. To activate Gα proteins, the latter were pre-incubated for 1 h in the presence of AMF (final concentrations, 10 mM NaF, 10 mM $MgCl_2$ and 20 μM $AlCl_3$) in buffer F and later, GST-tagged proteins were added as described above. For G protein pull-down experiments (Figure 2.10), HIS-Gαo or HIS-Gαi1 proteins [0.2 μM] in the presence or absence of AMF, were combined with purified GST [0.4 μM] or GST-RGS5, GST-14-3-3β or GST-14-3-3ε [0.2 μM] in 500 μl buffer F. For protein loading controls, 1 μg of Gαi1 and 0.1 μg of Gαo were diluted in H_2O and loading buffer. For competitive pull-down experiments, GST-RGS4 and GST-RGS5 [0.2 μM] were incubated with AMF-activated HIS-Gαo proteins [0.01 μM] or HIS-14-3-3ε [0.5 μM] or both in 500 μl buffer F. For protein loading controls, 0.1 μg of Gαo, 1 μg of RGS, and 0.01 μg of 14-3-3 proteins were all assessed by Western blot analysis. For negative controls, samples were incubated with purified GST protein and glutathione-Sepharose 4B beads as appropriate to determine non-specific binding.

After proteins were combined, the solutions were incubated for 4 h and subsequently, 30 µl of a 50% slurry of glutathione-Sepharose 4B beads (equilibrated in either buffer F or G) was added into each tube overnight (4°C), with gentle rotation. The glutathione-Sepharose 4B beads were pelleted by centrifugation (500 *x g*, 5 min, 4°C) and washed by resuspension and centrifugation three times with 1 ml buffer G. The proteins were released from the glutathione-Sepharose 4B beads by heating (99°C for 5 min) and 25 µl of loading buffer was added for Western blot analysis.

2.2.6. *Immunoblot Analysis*

Samples were resolved on a 12% SDS-PAGE gel and transferred onto a Polyvinylidene Fluoride Transfer (PVDF) membrane (Pall Corporation), followed by an incubation in blocking buffer for 1 h (Tris buffered-saline (TBST) with 5% nonfat milk, 0.1% Tween-20 final concentrations). To visualize protein-protein interactions, membranes were probed with rabbit anti-HIS (diluted 1:1000) (Santa Cruz Biotechnology) or mouse anti-HA (diluted 1:2000) (12CA5, Roche) primary antibodies overnight at 4°C in blocking buffer. Membranes were washed three times with TBST and probed with horseradish peroxidase (HRP)-conjugated IgG anti-rabbit or anti-mouse secondary antibody (diluted 1:2000) (Promega). The immunoblots were visualized by chemiluminescence using a digital camera (FluorChem 8000 Advanced Chemiluminescence and Visible Light Imaging, AlphaEaseFC software, Alpha Innotech Corporation). Subsequently, to visualize the GST-fusion proteins eluted from the glutathione-Sepharose 4B beads, membranes were stripped at 53°C for 30 min (final concentrations, 62.5 mM Tris-HCl, pH6.8, 2% SDS, 100 mM 2-mercaptoethanol), and reprobed with rabbit anti-GST (diluted

1:2000) (Santa Cruz Biotechnology) primary antibody. Following this, membranes were washed three times with TBST and probed with HRP-conjugated IgG anti-rabbit secondary antibody (diluted 1:2000). The immunoblots were visualized as described above. In some experiments, a band corresponding to free GST was observed with purified GST-fusion proteins; this suggests that a fraction of these proteins was cleaved and implies that in some cases the concentrations of GST-fusion proteins may have been lower than the calculated values based on mass.

2.2.7. *Densitometry*

Densitometry of unsaturated immunoblot images was carried out using the AlphaEaseFC software (FluorChem 8000 Advanced Chemiluminescence and Visible Light Imaging, Alpha Innotech Corporation). Statistical differences in protein binding were determined by a one-tailed unpaired Student's *t* test for all cell lysate and purified protein pull-down experiments, with exception of the competitive pull-down experiment, where a two-tailed unpaired Student's *t* test was used. Values of $p < 0.05$ were considered significant.

2.2.8. *Receptor and G protein expression in Sf9 cells and membrane preparation*

Sf9 insect cells were multiply infected for 48 h with baculoviruses encoding N-terminal c-myc-tagged M_2 muscarinic receptor, Gαo, Gβ1and Gγ2, and membranes from these cells were prepared as described in Cladman and Chidiac (2002).

2.2.9. *Steady state GTP hydrolysis assay*

The *in vitro* steady-state hydrolysis of [γ^{32}P]GTP by agonist stimulated G proteins in Sf9 membranes was measured in the presence or absence of RGS and/or 14-3-3 proteins. Each reaction tube consisted of a 50 µl mixture, containing 40 mM HEPES (pH 7.5), 2 mM EDTA, 4 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 1 µg/ml leupeptin, 10 µg/ml aprotinin and was incubated at 30°C for 5 min with 1 µM GTP, 500 µM ATP, [γ^{32}P]GTP ($1x10^6$ cpm/assay), either 100 µM carbachol (agonist) or 10 µM tropicamide (inverse-agonist), and membranes (5 µg/assay) (Cladman and Chidiac, 2002). The assay was stopped by adding 950 µl of ice-cold 5% (w/v) Norit in 0.05 M NaH_2PO_4. The mixture was subjected to centrifugation and the amount of $^{32}P_i$ in the supernatant was determined by liquid-scintillation counting. The nonspecific membrane GTPase signal was estimated by adding 1 mM unlabeled GTP to one set of reaction tubes and this value was subtracted from the total counts per minute (CPM). In each experiment, separate controls were added to identify the GTPase activity attributed to trace contaminants in the protein preparations; these included samples lacking membranes. Agonist-dependent GTPase activity was determined by subtracting the signal observed in the presence of tropicamide from that observed with carbachol.

All data are expressed as means ± S.E.M. and statistical significance was determined with one-way ANOVA, followed by a Dunnett's or a Tukey's Multiple Comparison Test. Values of $p < 0.05$ were considered significant.

2.3. Results

2.3.1. *Purified RGS proteins bind to 14-3-3 proteins expressed in HEK293 cells*

In this study, we examined the protein-protein interactions between 14-3-3 proteins and RGS proteins belonging to the B/R4 subfamily. To that end, we transiently transfected HEK293 cells with HIS-14-3-3ε or HIS-14-3-3β and examined their binding to purified GST-RGS4, GST-RGS5 or GST-RGS16 in pull-down experiments using cell lysates. As demonstrated in Figures 2.1 and 2.2, we found that both cytosolic 14-3-3β and 14-3-3ε readily interacted with all three purified RGS proteins (data summarized in Table 2.1).

2.3.2. *Purified 14-3-3 proteins bind to RGS proteins expressed in HEK293 cells*

To further establish the binding of 14-3-3 and RGS proteins observed in Figures 2.1 and 2.2, we carried out a similar cell lysate pull-down experiment to that described above, whereby we investigated the interaction between transiently transfected HA-RGS proteins and purified GST-fusion proteins of both 14-3-3 isoforms. As expected, 14-3-3β and 14-3-3ε proteins consistently bound to cytosolic RGS5 (Figure 2.3). However, the interaction between 14-3-3 proteins and cytosolic RGS4 and RGS16 could not be verified using this approach because both of these RGS proteins were prone to binding non-specifically to GST protein and/or glutathione-Sepharose 4B beads. Taken together, the results in Figures 2.1-2.3 indicate that members of the B/R4 subfamily of RGS proteins are able to

interact with 14-3-3 when either is expressed inside the cell, implying that RGS4, RGS5 and RGS16 might interact with 14-3-3 *in vivo*.

2.3.3. *Purified RGS proteins bind to purified 14-3-3 proteins*

We next examined whether the interaction observed between RGS and 14-3-3 proteins in HEK293 cell lysate pull-down experiments was direct, and not dependent on additional proteins or other unknown intracellular factors. To address this question, a pull-down experiment using glutathione-Sepharose 4B beads was carried out with the addition of bacterially expressed, purified GST-RGS and HIS-14-3-3 proteins. Under these conditions, any observed protein-protein interactions presumably would be independent of post-translational modifications that may occur within mammalian cells. An example of these experiments is shown in Figures 2.4 and 2.5 (data summarized in Table 2.2), where RGS4, RGS5 and RGS16 were found to directly bind to both 14-3-3ε (2.4.A) and 14-3-3β (2.5.A, 2.5.B) in the absence of any other proteins or factors. Moreover, these RGS proteins interacted with 14-3-3 in a concentration-dependent manner, as demonstrated in Figures 2.6 and 2.7. The two 14-3-3 isoforms bound similarly to all three RGS proteins tested (Table 2.2), however these RGS proteins appeared to have greater affinity for 14-3-3ε than for 14-3-3β. This was consistent between 2 separate batches of purified 14-3-3β and 3 separate batches of purified 14-3-3ε proteins and thus probably does not reflect differences in the quality or activity of the purified proteins. Overall, the binding data clearly indicate that indeed 14-3-3 proteins bind directly to RGS4, RGS5 and RGS16 in the absence of any other proteins or factors.

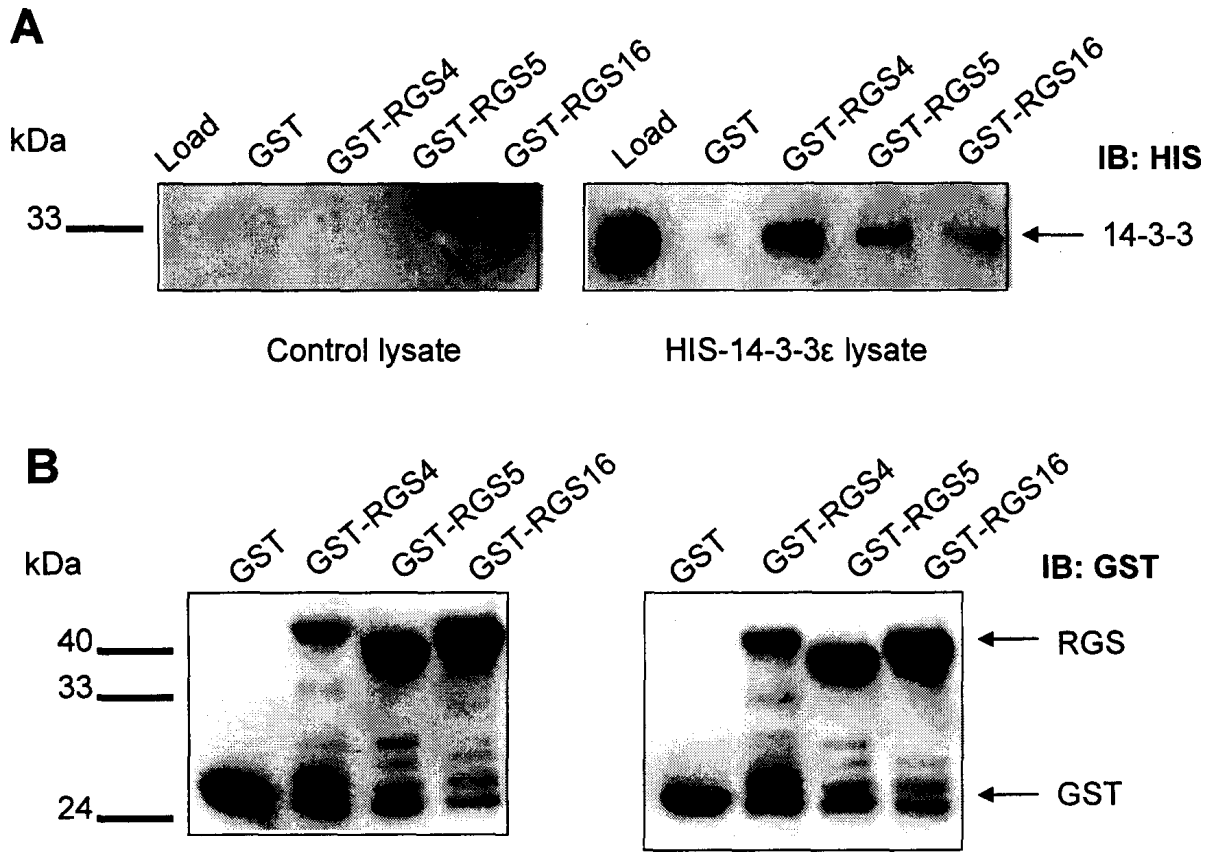


Figure 2.1. *14-3-3ε in cytosolic extracts interacts with purified RGS4, RGS5 and RGS16.* Western blot analysis of a pull-down experiment using lysates of HEK293 cells expressing His-14-3-3ε or mock-transfected control. Cell lysates were incubated with GST (N=5) [0.8 μM] or GST-RGS4 (N=3), GST-RGS5 (N=4) or GST-RGS16 (N=5) [0.4 μM] for 4 h, followed by an overnight incubation with a slurry of glutathione-Sepharose 4B beads at 4°C. The eluted samples were resolved on a 12% SDS-PAGE gel. The blot was probed with anti-HIS antibody (A), after which the membrane was stripped and reprobed with anti-GST antibody (B). The immunoblots were visualized by chemiluminescence (data summarized in Table 2.1).

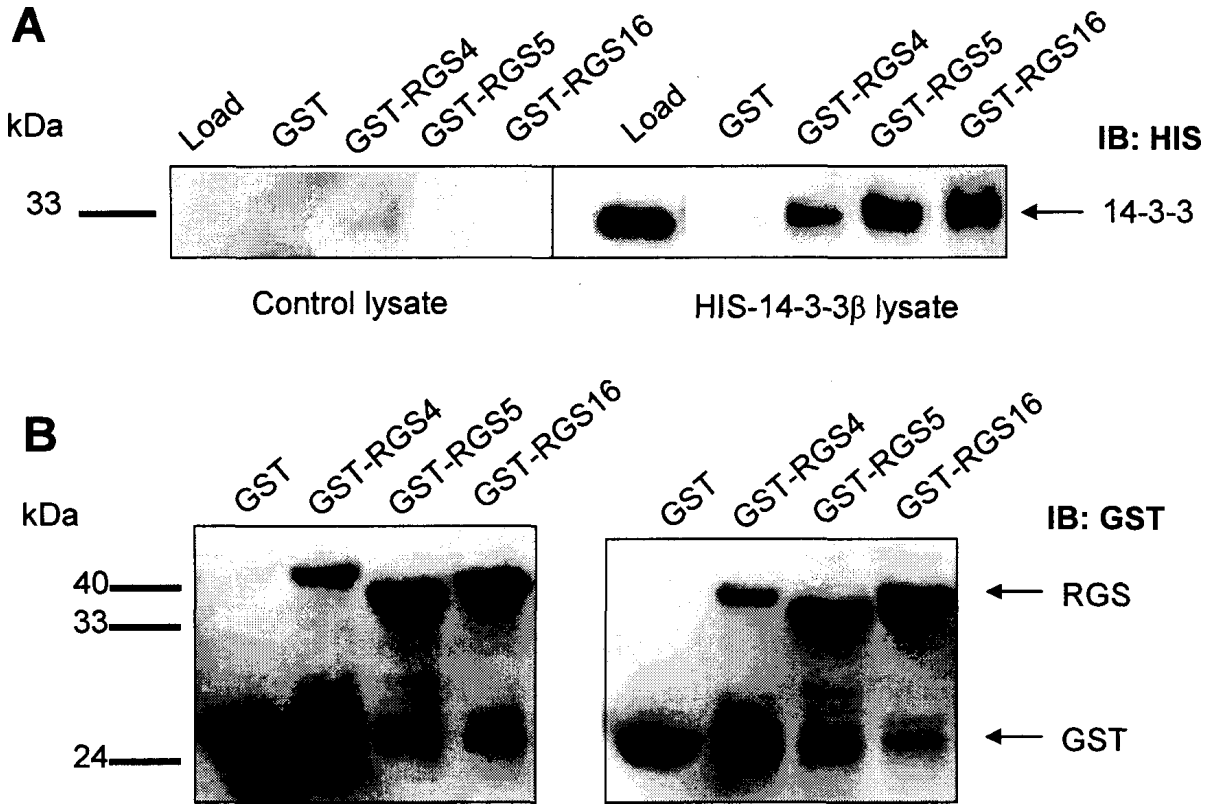
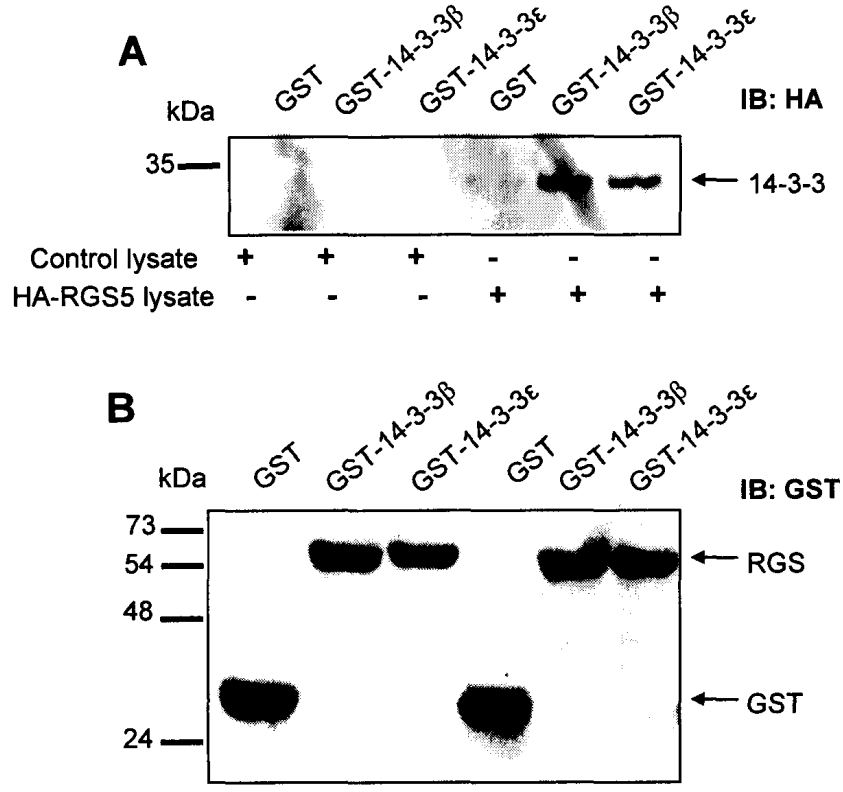


Figure 2.2. *14-3-3β in cytosolic extracts interacts with purified RGS4, RGS5 and RGS16.* Western blot analysis of a pull-down experiment using lysates of HEK293 cells expressing His-tagged 14-3-3β or mock-transfected control. Cell lysates were incubated with GST (N=4) [0.8 μM] or GST-RGS4 (N=3), GST-RGS5 (N=4) or GST-RGS16 (N=4) [0.4 μM] for 4 h, followed by an overnight incubation with a slurry of glutathione-Sepharose 4B beads at 4°C. The eluted samples were resolved on a 12% SDS-PAGE gel. The blot was probed with anti-HIS antibody (A), after which the membrane was stripped and reprobed with anti-GST antibody (B). The immunoblots were visualized by chemiluminescence (data summarized in Table 2.1).

Table 2.1. *14-3-3 in cytosolic extracts interacts with purified RGS4, RGS5 and RGS16.* Densitometry of immunoblots was carried out to determine binding of transiently transfected HIS-14-3-3 proteins with purified GST-RGS4, GST-RGS5 and GST-RGS16. Binding data correspond to Figures 2.1 and 2.2 plus replicate experiments, and are expressed as signal relative to GST control for each cell lysate (means ± S.E.M.). Statistical significance was assessed by the use of an unpaired one-tailed Student's *t* test (* indicate statistically significant binding of 14-3-3 to GST-RGS, compared to GST control).

Binding of Intracellular 14-3-3 Proteins to Purified RGS Proteins

RGS Protein	**HIS-14-3-3ε Lysate** [GST-RGS/GST ± S.E.M. (P, N)]	**HIS-14-3-3β Lysate** [GST-RGS/GST ± S.E.M. (P, N)]
GST-RGS4	18.1 ± 3.9 (0.0005, 3)*	6.9 ± 6.0 (0.17, 3)
GST-RGS5	10.2 ± 1.7 (0.03, 4)*	3.4 ± 2.2 (0.40, 4)
GST-RGS16	4.7 ± 2.3 (0.10, 5)	3.1 ± 1.7 (0.19, 4)



C

Control Lysate [GST-14-3-3/GST ± S.E.M.]	HA-RGS5 Lysate [GST-14-3-3/GST ± S.E.M.]
0.86 ± 0.05	26.67 ± 11.79*

Figure 2.3. *RGS5 in cytosolic extracts binds to purified 14-3-3 proteins.* HEK293 cells were transfected with the cDNA for HA-RGS5 or no cDNA plasmid (control lysate). The cells were lysed and incubated with GST [0.8 μM] or GST-14-3-3β or GST-14-3-3ε [0.36 μM] (N=4). Cell extracts were subjected to a pull-down experiment with glutathione-Sepharose 4B beads. The blot was probed with anti-HA antibody (A), after which the membrane was stripped and reprobed with anti-GST antibody (B). Table (C), densitometry of immunoblots was carried out to determine total 14-3-3 binding to RGS5 expressed in HEK293 cell lysates. The data (signal relative to GST control, mean ± S.E.M.) were obtained from 4 independent experiments. Statistical significance was assessed by the use of an unpaired one-tailed Student's *t* test. * indicates statistically significant binding of GST-14-3-3 to intracellular RGS5, compared to control lysate from mock transfected cells ($p < 0.05$).

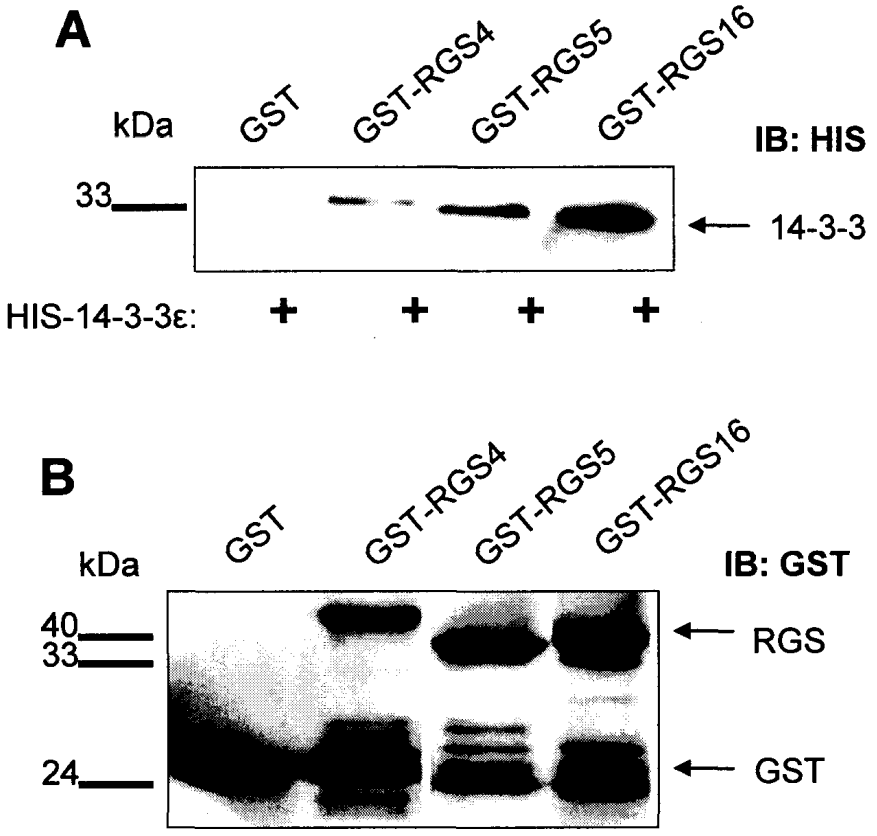


Figure 2.4. *Direct interaction between RGS proteins and 14-3-3ε.* Western blot analysis of protein binding experiment between GST (N=5) [0.4 μM] or GST-RGS4 (N=5), GST-RGS5 (N=3) or GST-RGS16 (N=3) [0.2 μM], and HIS-14-3-3ε [0.5 μM]. Proteins were incubated with glutathione-Sepharose 4B beads. The blot was probed with anti-HIS antibody to detect the 14-3-3 proteins (A), after which the membrane was stripped and reprobed with anti-GST antibody (B) (data summarized in Table 2.2). All lanes shown are taken from a single membrane (probed once with anti-HIS and once with anti-GST).

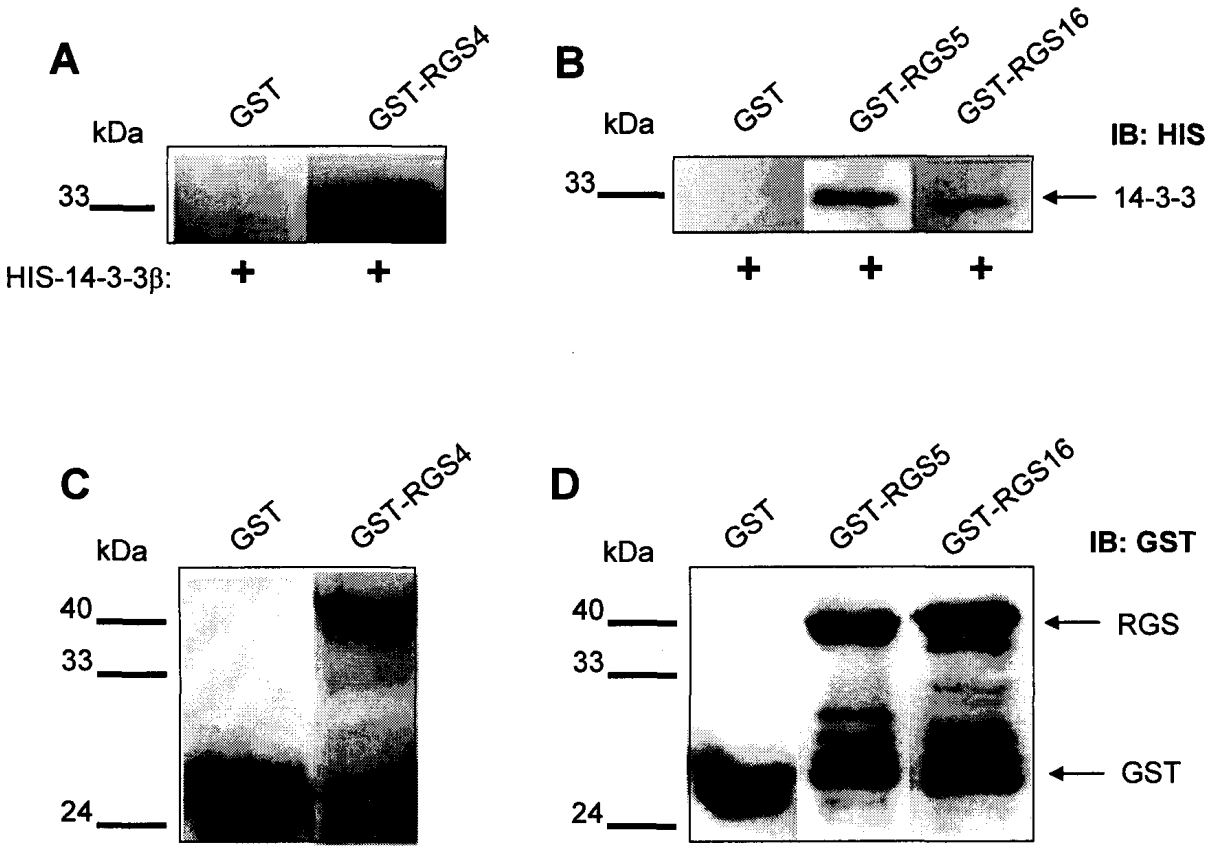


Figure 2.5. *Direct interaction between RGS proteins and 14-3-3β.* Western blot analysis of protein binding experiments between GST [0.4 μM] or GST-RGS4, GST-RGS5 or GST-RGS16 [0.2 μM], and HIS-14-3-3β [0.5 μM]. Proteins were incubated with glutathione-Sepharose 4B beads. The blots were probed with anti-HIS antibody to detect the 14-3-3 proteins (A, B), after which membranes were stripped and reprobed with anti-GST antibody (C, D). These results are representative of 3 independent experiments (data summarized in Table 2.2). All lanes shown in panel A (and C) and in panel B (and D) were taken from a single membrane, respectively (each probed once with anti-HIS and once with anti-GST).

Table 2.2. *Direct interaction between RGS proteins and 14-3-3.* Densitometry of Immunoblot analysiss was carried out to determine binding of HIS-14-3-3β and HIS-14-3-3ε with GST-RGS4, GST-RGS5 and GST-RGS16. 14-3-3 binding to GST-RGS proteins is expressed as signal relative to GST control (means ± S.E.M.). Statistical significance was assessed by the use of an unpaired one-tailed Student's *t* test. * indicates statistically significant binding of 14-3-3 to GST-RGS, compared to GST control ($p < 0.05$).

Binding of Purified 14-3-3 Proteins to Purified RGS Proteins

RGS Protein	**HIS-14-3-3ε** [GST-RGS/GST ± S.E.M (P, N)]	**HIS-14-3-3β** [GST-RGS/GST ± S.E.M (P, N)]
GST-RGS4	10.0 ± 4.4 (0.09, N=5)	2.5 ± 0.9 (0.18, N=3)
GST-RGS5	24.7 ± 18.3 (0.08, N=3)	5.3 ± 2.5 (0.03, N=3)*
GST-RGS16	30.5 ± 22.4 (0.11, N=3)	5.0 ± 1.1 (0.07, N=3)

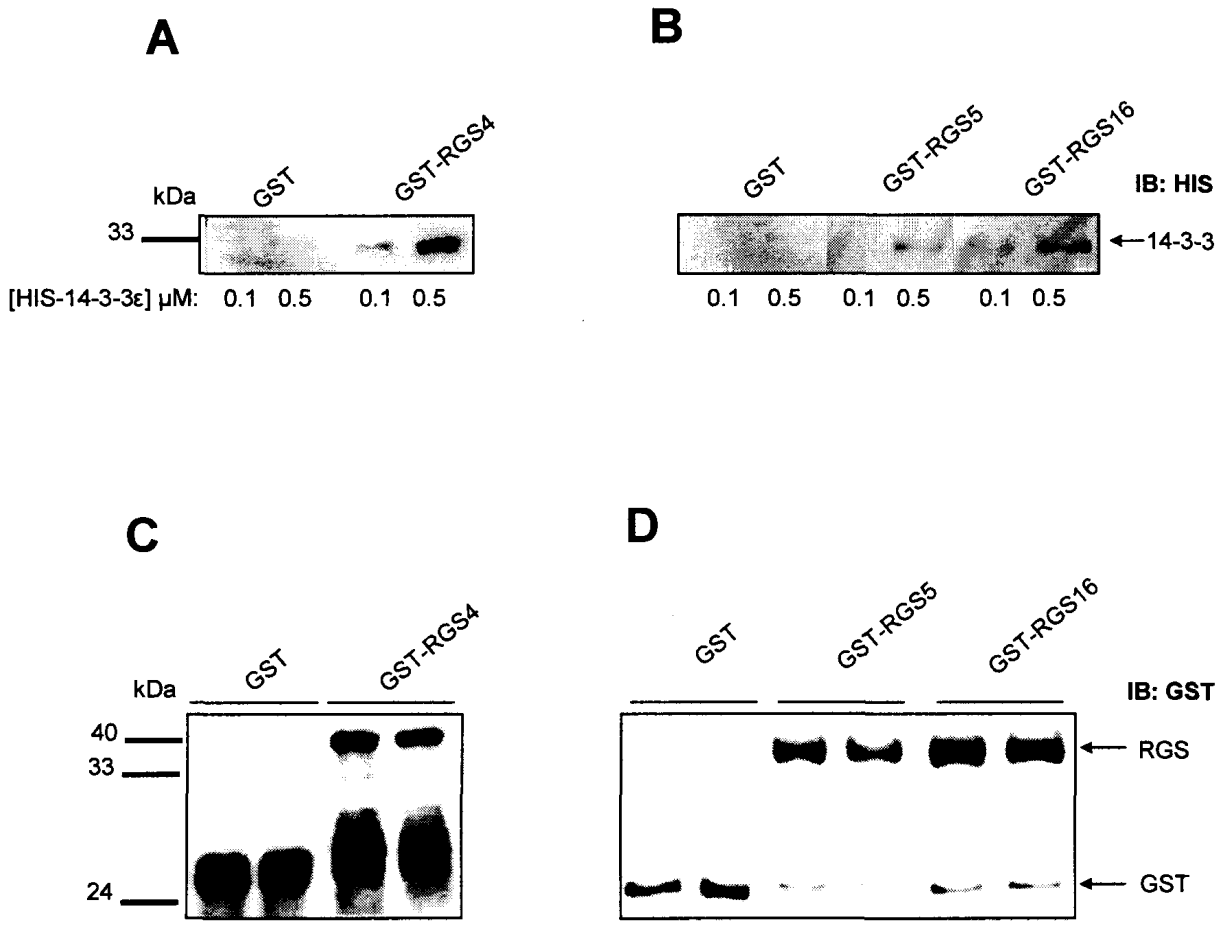


Figure 2.6. *RGS proteins bind to 14-3-3ε in a concentration-dependent manner.* Western blot analysis of protein binding experiments between GST (N=3) [0.4 μM] or GST-RGS4 (N=3), GST-RGS5 (N=2) or GST-RGS16 (N=2) [0.2 μM], and HIS-14-3-3ε at two different concentrations [0.1 μM, 0.5 μM]. The proteins were incubated with glutathione-Sepharose 4B beads. The blots were probed with anti-HIS antibody (A, B), after which membranes were stripped and reprobed with anti-GST antibody (C, D). All lanes shown in panel A (and C) and in panel B (and D) were taken from a single membrane, respectively (each probed once with anti-HIS and once with anti-GST).

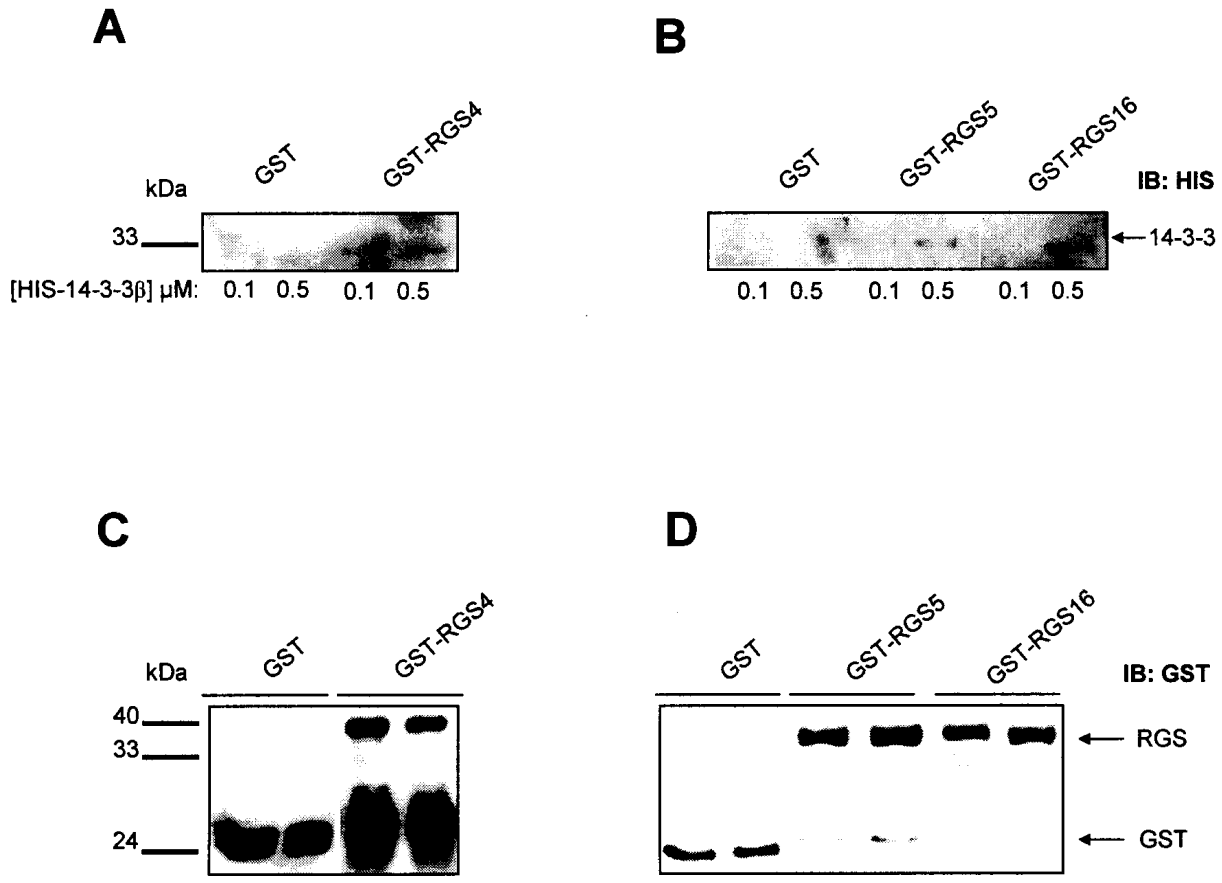


Figure 2.7. *RGS proteins bind to 14-3-3β in a concentration-dependent manner.* Immunoblot analysiss of protein binding experiments between GST [0.4 μM] or GST-RGS4, GST-RGS5 or GST-RGS16 [0.2 μM], and HIS-14-3-3β at two different concentrations [0.1 μM, 0.5 μM]. The proteins were incubated with glutathione-Sepharose 4B beads. The blots were probed with anti-HIS antibody (A, B), after which membranes were stripped and reprobed with anti-GST antibody (C, D). These results are representative of 3 independent experiments. All lanes shown in panel A (and C) and in panel B (and D) were taken from a single membrane, respectively (each probed once with anti-HIS and once with anti-GST).

2.3.4. *14-3-3 proteins inhibit the GAP activity of RGS4 and RGS16, but not RGS5*

Since 14-3-3 and RGS proteins bind to each other, it is possible that 14-3-3 might interfere with the GAP effects of the RGS proteins on their target G proteins. We therefore investigated whether such a regulatory mechanism might exist, using an *in vitro* assay in which RGS proteins promote receptor-dependent, steady state GTP hydrolysis using membranes derived from Sf9 insect cells expressing M_2 muscarinic receptor plus Gαo, Gβ1 and Gγ2 proteins. RGS GAP activities were determined from the hydrolysis of [γ-^{32}P]GTP in the presence of the muscarinic agonist carbachol. Tropicamide, an inverse-agonist, inhibits the intrinsic activity of the GPCR in the absence of agonist and was used in the assay to identify the receptor-dependent signal. 14-3-3 alone had no apparent effect on the G protein GTPase activity in the absence of RGS protein (Figure 2.8). The GAP activity of both RGS4 and RGS16 was significantly inhibited, in a concentration-dependent manner by both 14-3-3 isoforms. This inhibition was more pronounced with 14-3-3β than with 14-3-3ε. Surprisingly, 14-3-3 had little or no effect on RGS5 GAP activity (Figure 2.9) not withstanding the observed binding of RGS5 to 14-3-3. Moreover, 14-3-3 concentrations as high as 4 μM still failed to significantly inhibit the activity of RGS5 (data not shown). These results indicate that RGS proteins can be negatively regulated by 14-3-3, as seen with RGS4 and RGS16. In the case of RGS5, one possible explanation for the lack of statistically significant inhibition by 14-3-3 proteins is that the affinity of RGS5 for 14-3-3 is low relative to its affinity for G proteins.

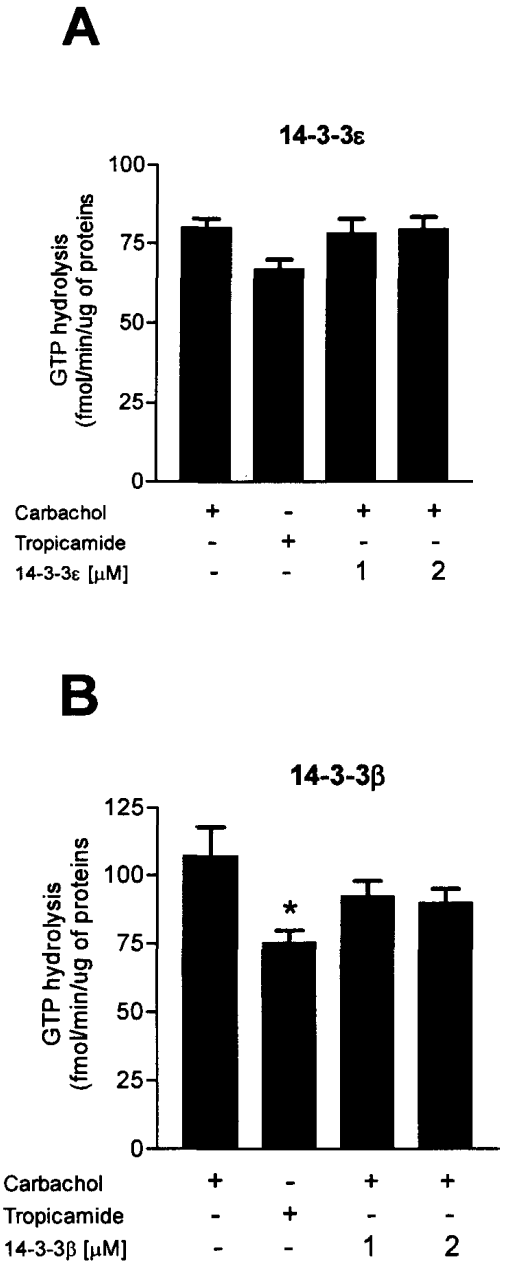
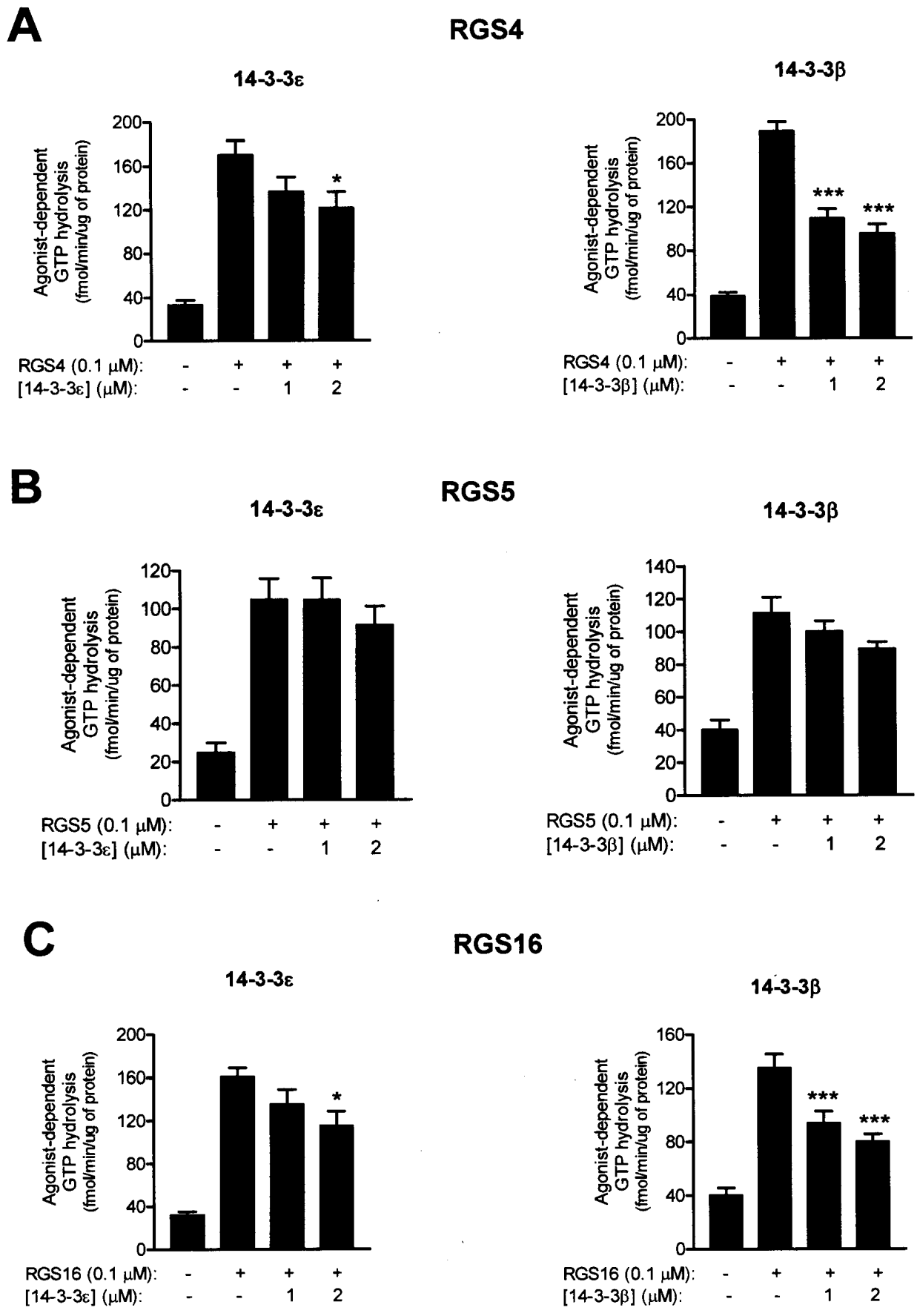


Figure 2.8. *14-3-3 has no effect on the Gαo protein-coupled M_2 cholinergic receptor signalling pathway in the absence of RGS proteins. In vitro* steady state GTP hydrolysis assay with Gαo protein-coupled M_2 cholinergic receptors, stimulated with carbachol [100 μM]. (A) 14-3-3ε (N=5) or (B) 14-3-3β (N=8) were used in the assay at two final concentrations [1 μM, 2 μM]. Each condition was performed in triplicate and data represent means ± S.E.M. Statistical significance was assessed by the use of a one-way ANOVA, followed by a Tukey's Multiple Comparison Test. *, $p < 0.05$.

Figure 2.9. *14-3-3 inhibits the GAP activity of RGS proteins. In vitro* steady state GTP hydrolysis assay with Gαo protein-coupled M_2 cholinergic receptors, stimulated with carbachol [100 μM]. The GAP activity of several RGS proteins was measured in the absence or presence of 14-3-3ε and 14-3-3β [1 μM, 2 μM]. (A) RGS4/14-3-3ε (N=6) and RGS4/14-3-3β (N=4); (B) RGS5/14-3-3ε (N=6) and RGS5/14-3-3β (N=6); (C) RGS16/14-3-3ε (N=6) and RGS16/14-3-3β (N=7). Each condition was performed in triplicate and data represent means ± S.E.M. Statistical significance was assessed by the use of a one-way ANOVA, followed by a Dunnett's Multiple Comparison Test to determine 14-3-3 inhibition on RGS GAP activity. *, p <0.05; ***, p <0.001 compared to RGS alone.


A
RGS4
14-3-3ε
14-3-3β
Agonist-dependent GTP hydrolysis (fmol/min/ug of protein)
200
160
120
80
40
0
*
***
***
RGS4 (0.1 μM): - + + +
[14-3-3ε] (μM): - - 1 2
[14-3-3β] (μM): - - 1 2
B
RGS5
14-3-3ε
14-3-3β
120
100
80
60
40
20
0
140
RGS5 (0.1 μM): - + + +
[14-3-3ε] (μM): - - 1 2
[14-3-3β] (μM): - - 1 2
C
RGS16
14-3-3ε
14-3-3β
RGS16 (0.1 μM): - + + +
[14-3-3ε] (μM): - - 1 2
[14-3-3β] (μM): - - 1 2

2.3.5. *14-3-3 and G proteins compete for RGS proteins*

The foregoing results suggest a mechanism wherein 14-3-3 sequesters RGS proteins, thereby preventing their GAP effects on G proteins. Thus, we investigated whether 14-3-3 could decrease RGS binding to G proteins. To address this question, we first assessed the abilities of RGS4 and RGS5 to bind to G proteins, and also investigated the possibility that 14-3-3 itself might associate with G proteins. Using a purified protein pull-down experiment, we looked at the binding of G proteins (Gαo and Gαi1) in the absence or presence of AMF ($AlCl_3$, $MgCl_2$ and NaF) to GST-RGS5, GST-14-3-3β or GST-14-3-3ε. AMF induces a conformation (Gα –GDP-AlF^-_4) thought to mimic the transition state of the Gα subunit bound to the gamma phosphate of GTP, i.e. activated Gα protein (Tesmer *et al.*, 1997). These effects have long been established with RGS4 (Berman *et al.*, 1996) and RGS16 (Chen and Lin, 1998).

The present data clearly show that RGS5 proteins have a much higher affinity for the GDP-AlF^-_4 activated form of Gα proteins (Figure 2.10), and complement a study carried out by Zhou *et al.* (2001), demonstrating that several endogenously expressed G proteins bind more strongly to purified RGS5 after HEK293 cell lysates have been treated with AMF. Furthermore, this is consistent with observations made with other RGS proteins (i.e. RGS4) and supports the established dogma that RGS proteins bind directly to Gα and facilitate the hydrolysis reaction by stabilizing the Gα protein transition state, and thereby enhancing the rate of inactivation (Watson *et al.*, 1996). Conversely, we were unable to detect any binding of 14-3-3 proteins to either form of Gαo and Gαi1 proteins (Figure 2.10). This observation strengthens our model that 14-3-3 acts directly on RGS

proteins and it is likely that the inhibitory effect seen in the *in vitro* steady state GTP hydrolysis assay is the result of a direct interaction between 14-3-3 and RGS proteins.

Since RGS proteins bind to both AMF-activated Gα and 14-3-3 proteins, we next studied in more detail the effect of 14-3-3 proteins on the binding of RGS to Gα. In a competitive pull-down experiment using glutathione-Sepharose 4B beads, GST-RGS4 and GST-RGS5 were combined with HIS-Gαo, HIS-14-3-3ε, or both. We used 14-3-3ε rather than 14-3-3β because in our hands the former seems to bind more reliably to all the RGS proteins (especially RGS4) under these experimental conditions (Figures 2.4-2.7). As demonstrated in Figure 2.11, 14-3-3ε appeared to compete with Gαo for the binding of RGS4, but not with the binding of RGS5 to Gαo. These data are consistent with the *in vitro* steady state GTP hydrolysis assay, where 14-3-3ε significantly inhibited the GAP activity of RGS4 and had no apparent affect on RGS5 (Figure 2.9). Thus, the present results imply that 14-3-3 might indeed be selective for certain RGS proteins. More importantly, at least in the case of RGS4, 14-3-3ε may act as a molecular chelator by impeding the interaction between RGS and Gαo within the signal transduction pathway.

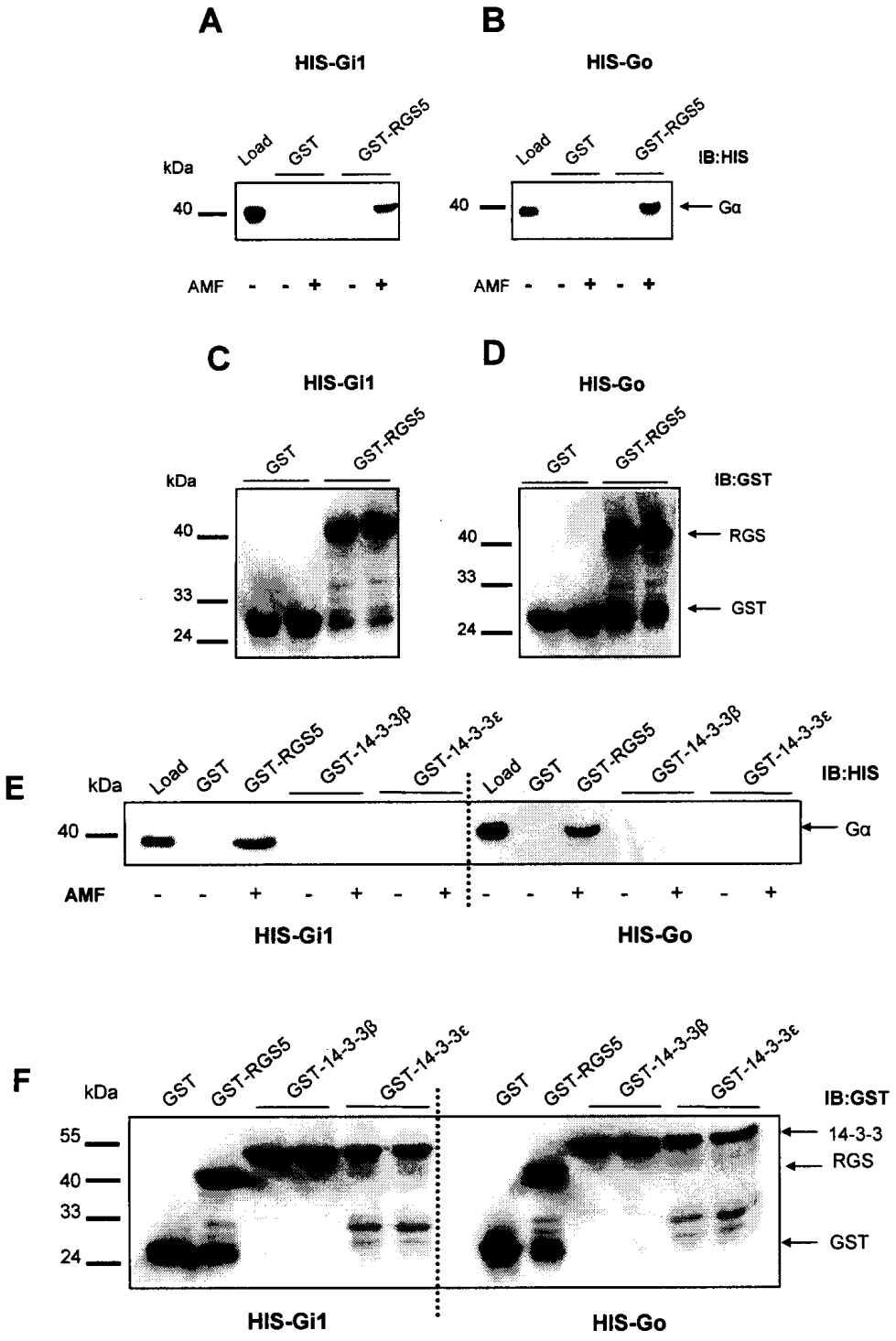
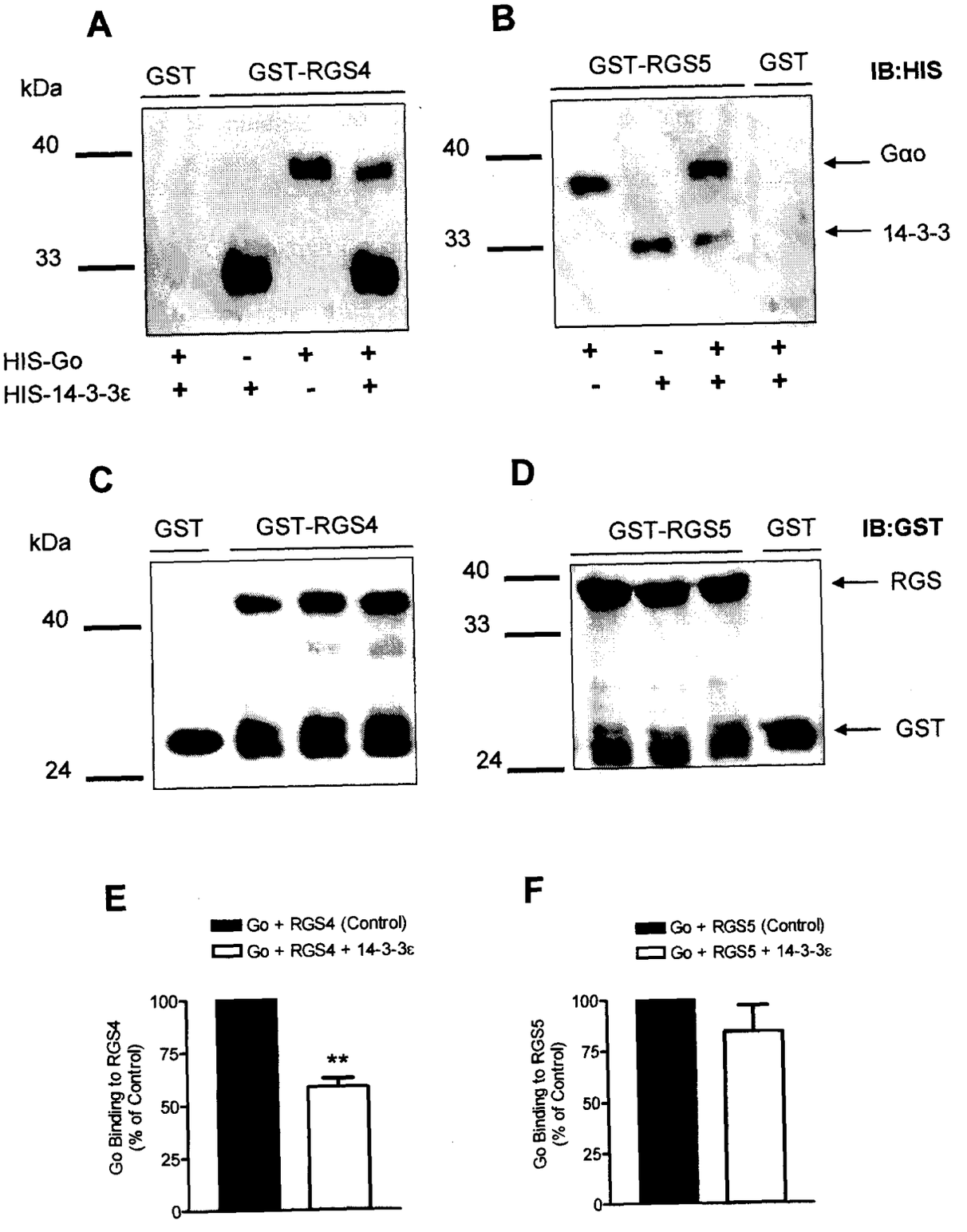


Figure 2.10. *RGS5, but not 14-3-3, binds to activated Gαo and Gαi1 proteins.* Western blot analysis of protein binding experiments between HIS-Gαi1 (A, C, E, F) or HIS-Gαo (B, D, E, F) [0.25 μM] proteins, and GST [0.4 μM] or GST-RGS5 [0.22 μM] or GST-14-3-3 [0.2 μM] in the absence and presence of AMF (MgCl, NaF, $AlCl_3$). All proteins were incubated with glutathione-Sepharose 4B beads. The blots were probed with anti-HIS antibody (A, B, E), after which membranes were stripped and reprobed with anti-GST antibody (C, D, F). Data represent one of three independent experiments.

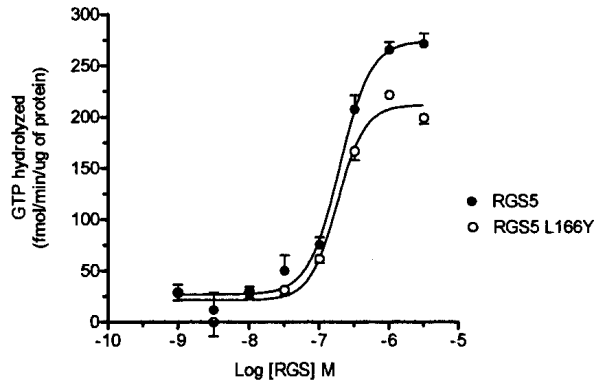
Figure 2.11. *14-3-3ε competes with activated Gαo for RGS4, but not RGS5.* Western blot analysis of binding between GST or GST-RGS4 (A, C) or GST-RGS5 (B, D) [0.2 μM] to activated HIS-Gαo [0.01 μM] (i.e. in the presence of AMF) and/or HIS-14-3-3ε [0.5 μM]. The pull-down was carried out with glutathione-Sepharose 4B beads. Purified GST with activated HIS-Gαo plus HIS-14-3-3ε was used as a negative control (Lane 1 in panel A and lane 4 in panel B). The blots were probed with anti-HIS antibody (A, B), after which membranes were stripped and reprobed with anti-GST antibody (C, D). Densitometry of Western blots was carried out to determine Gαo binding to either RGS4 (N=3) (E) or RGS5 (N=3) (F) in the absence and presence of 14-3-3ε. The data represent the average ratio between G protein pulled-down in the presence versus absence of 14-3-3 proteins. Statistical significance was assessed by the use of an unpaired two-tailed Student's *t* test. **, $p < 0.01$.


A
kDa
GST
GST-RGS4
40
33
HIS-Go
HIS-14-3-3ε
B
GST-RGS5
GST
IB:HIS
40
33
Gαo
14-3-3
C
kDa
GST
GST-RGS4
40
24
D
GST-RGS5
GST
IB:GST
40
33
24
RGS
GST
E
Go + RGS4 (Control)
Go + RGS4 + 14-3-3ε
Go Binding to RGS4 (% of Control)
100
75
50
25
0
**
F
Go + RGS5 (Control)
Go + RGS5 + 14-3-3ε
Go Binding to RGS5 (% of Control)
100
75
50
25
0

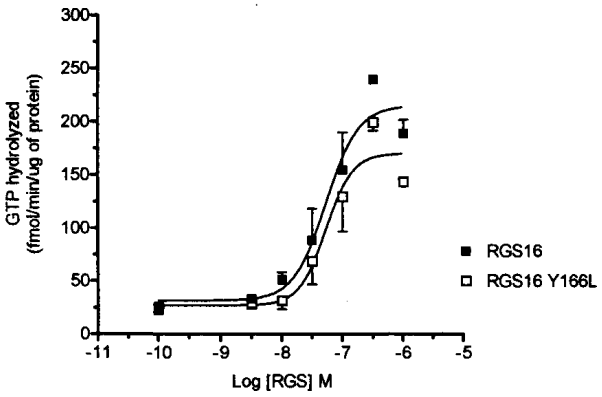
2.3.6. *14-3-3 proteins do not distinguish between leucine and tyrosine residues within the SxP motif of the RGS domain*

There exists some controversy regarding whether the SYP motif within the RGS domain is essential for 14-3-3 binding, as has been claimed in previous reports (Benzing *et al.*, 2000; Benzing *et al.*, 2002). Indeed, one potential explanation for the absence of 14-3-3 inhibition on RGS5 GAP activity, seen in the *in vitro* GTP hydrolysis assay in this study, could be that RGS16 possesses this conserved motif while RGS5 has a unique point substitution where leucine 167 takes the place of tyrosine. Using site-directed mutagenesis, we constructed mutant RGS5 L167Y and RGS16 Y167L proteins to examine whether this substitution of leucine for tyrosine within the conserved SYP motif accounts for the lack of inhibition of RGS5 GAP activity by 14-3-3. In the *in vitro* steady state GTP hydrolysis assay, RGS5 L167Y and RGS16 Y167L proteins both retained full or nearly full activities and exhibited similar potencies relative to their wild-type counterparts (comparable Km values) (Figure 2.12). The slope factor (Hill coefficient) was nearly 2 for all proteins which suggests the possibility that two or more RGS proteins interact simultaneously and in a positively cooperative manner with multiple G proteins, forming an oligomeric signalling complex (Chidiac, 1998). The tyrosine/leucine substitution within the SxP motif of RGS5 failed to render this RGS protein sensitive to the inhibitory effects of either 14-3-3β or 14-3-3ε, and correspondingly, the substitution of leucine for tyrosine did not appear to cause RGS16 to become insensitive to inhibition by 14-3-3 (Figures 2.13 and 2.14). Thus, the tyrosine/leucine substitution within the SxP motif is not accountable for the functional difference observed between RGS5 and RGS16 in the presence of 14-3-3 under these experimental conditions.

A



B



C

	pEC_{50} (M)	Slope Factor
RGS5	6.72 ± 0.05	1.95 ± 0.34
RGS5 L167Y	6.74 ± 0.08	2.21 ± 0.65
RGS16	7.27 ± 0.18	1.63 ± 0.92
RGS16 Y167L	7.27 ± 0.19	1.99 ± 1.34

Figure 2.12. *RGS5 L167Y and RGS16 Y167L mutants have similar GAP activity to wild-type RGS proteins. In vitro* steady state GTP hydrolysis assay with Gαo protein-coupled M_2 cholinergic receptors, stimulated with carbachol [100 μM]. RGS dose-response curves comparing the GAP activity of RGS5 and RGS5 L167Y (N=2) (A), and RGS16 and RGS16 Y167L (N=3) (B). Each condition was performed in triplicate and data points shown represent means ± S.E.M. (C), the averaged data were fitted by non-linear regression to a single sigmoidal function with a variable slope factor (GraphPad Prism), and the numbers indicated in the table represent the fitted parameters for each set of averaged data. The errors shown were generated during the fitting procedure and provide an estimate of the uncertainty of the fitted parameters.

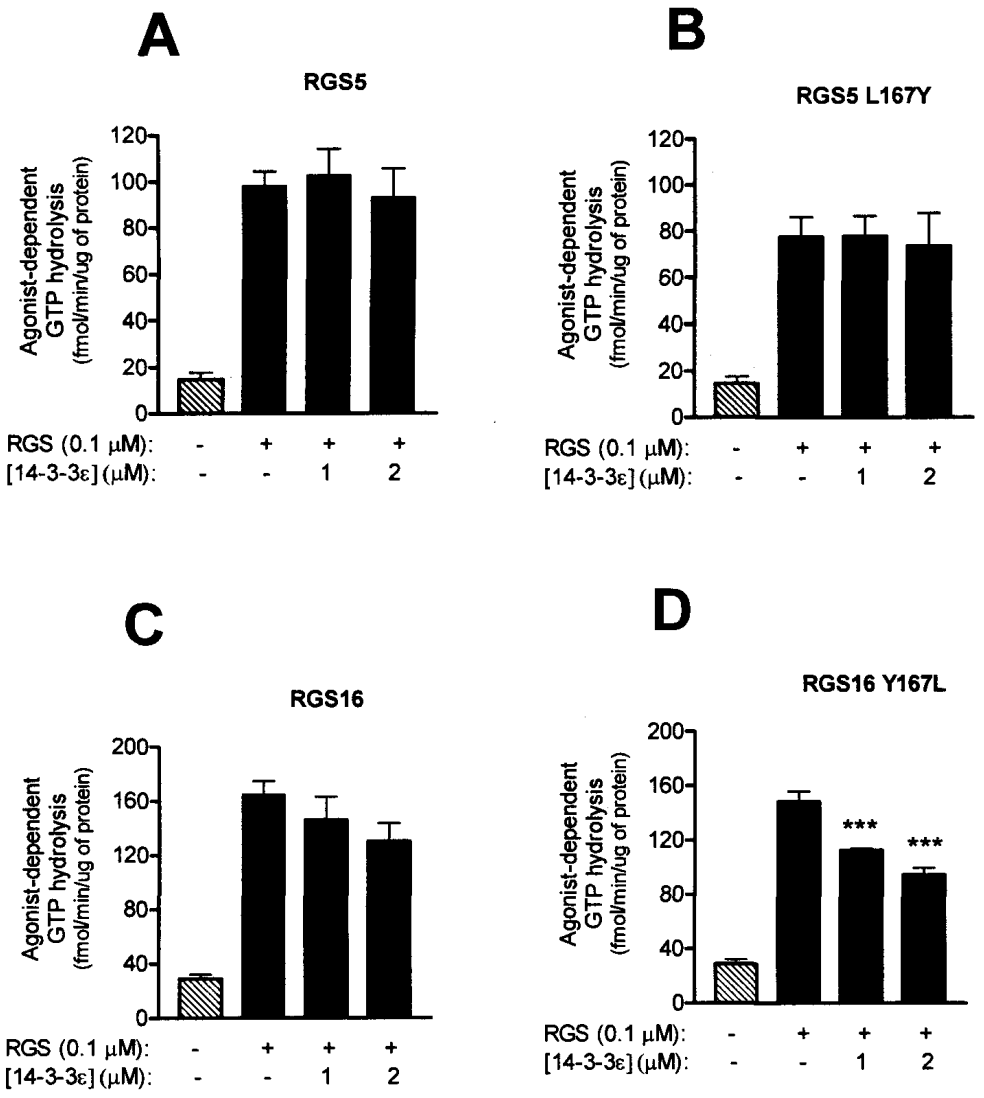


Figure 2.13. *Leucine and tyrosine residues within the SxP motif do not dictate 14-3-3ε inhibition on RGS GAP activity. In vitro* steady state GTP hydrolysis assay with Gαo protein-coupled M_2 cholinergic receptors, stimulated with carbachol [100 μM]. The GAP activities of wild-type and mutant RGS5 and RGS16 proteins were measured in the absence and presence of 14-3-3ε [1 μM, 2 μM]. (A) RGS5 (N=3); (B) RGS5 L167Y (N=3); (C) RGS16 (N=4); (D) RGS16 Y167L (N=4). Each condition was performed in triplicate and data represent means ± S.E.M. Statistical significance was assessed by the use of a one-way ANOVA, followed by a Dunnett's Multiple Comparison Test to determine 14-3-3 inhibition on RGS GAP activity. ***, p <0.001 compared to RGS alone.

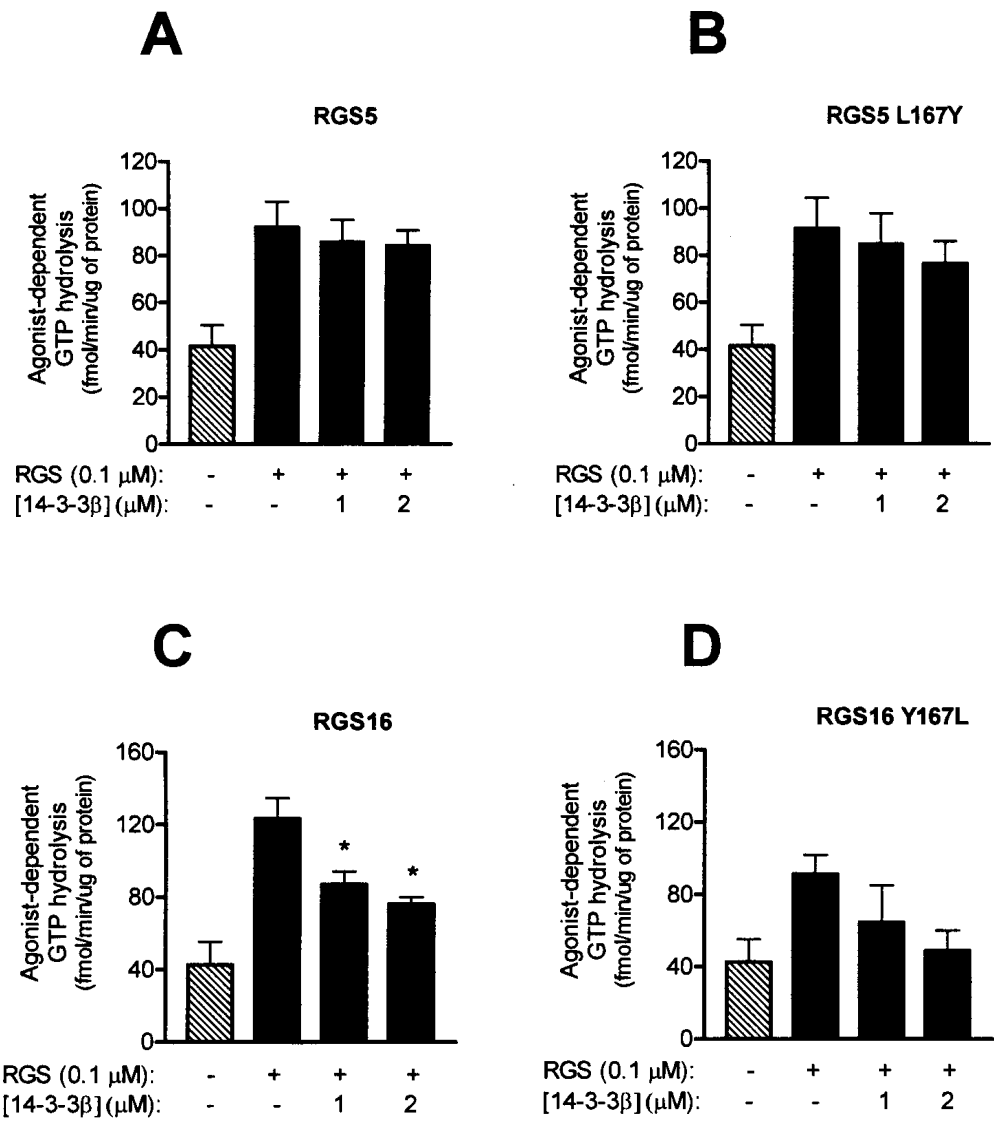


Figure 2.14. *Leucine and tyrosine residues within the SxP motif do not dictate 14-3-3β inhibition on RGS GAP activity. In vitro* steady state GTP hydrolysis assay with Gαo protein-coupled M_2 cholinergic receptors, stimulated with carbachol [100 μM]. The GAP activities of wild-type and mutant RGS5 and RGS16 proteins were measured in the absence and presence of 14-3-3β [1 μM, 2 μM]. (A) RGS5 (N=3); (B) RGS5 L167Y (N=3); (C) RGS16 (N=3); (D) RGS16 Y167L (N=3). Each condition was performed in triplicate and data represent means ± S.E.M. Statistical significance was assessed by the use of a one-way ANOVA, followed by a Dunnett's Multiple Comparison Test to determine 14-3-3 inhibition on RGS GAP activity. ***, p <0.001 compared to RGS alone.

2.4. **Discussion**

In the present study, we have identified protein-protein interactions between RGS proteins of the B/R4 subfamily (RGS4, RGS5, RGS16) and two 14-3-3 isoforms, 14-3-3β and 14-3-3ε (Figures 2.1-2.3, Table 2.1). Our results indicate that 14-3-3 directly binds to these particular RGS proteins, and other factors such as Gα proteins, are not required for the RGS-14-3-3 interaction to take place (Figures 2.4-2.7, Table 2.2). Data from the *in vitro* steady state GTP hydrolysis assay (Figure 2.8) show that 14-3-3 proteins inhibit the GTPase activity of RGS4 and RGS16, but have no apparent effect on RGS5 activity. Moreover, in a competitive pull-down experiment (Figure 2.11), 14-3-3ε proteins compete with Gαo for RGS4, but not for RGS5, implying that 14-3-3 might prevent RGS proteins from interacting with Gα and through this mechanism, act as modulators of RGS function to prolong intracellular signalling. We also examined the role of the putative SYP 14-3-3 binding motif and found that tyrosine 167 of RGS16 does not account for the observed RGS inhibition by 14-3-3. Furthermore, RGS5, in the presence of the SYP motif, still remains unaffected by 14-3-3 in the *in vitro* steady state GTP hydrolysis assay (Figures 2.13-2.14). As discussed in previous chapters, RGS proteins are potent modulators of GPCR signalling and as such, several of these proteins are known to be tightly regulated *in vivo*. For example, the availability of RGS proteins at the plasma membrane could be limited by their sequestration into other intracellular compartments (Burgon *et al.*, 2001).

Here, our combined observations suggest a mechanism wherein 14-3-3 proteins negatively modulate RGS function and act as molecular chelators that sequester RGS

proteins away from both the G protein and the plasma membrane. Thus, we conclude that 14-3-3 proteins indirectly promote GPCR signalling via their inhibitory effects on RGS proteins.

The rationale for the present study was based on an observation made in a yeast 2-hybrid screen in our laboratory of a mouse brain cDNA library, in which 14-3-3ε was identified as a putative RGS4-interacting protein. The present results confirm that this interaction exists at the protein-protein level and our study is the first to report that RGS4 interacts with both 14-3-3β and 14-3-3ε proteins. However, these findings disagree with studies carried out by Benzing *et al.* (2000 and 2002) who failed to demonstrate co-immunoprecipitation of RGS4 with 14-3-3β in HEK293 cell lysates. It is possible that their particular experimental conditions were not sufficiently sensitive to detect a protein interaction with RGS4 compared to RGS7, and/or 14-3-3 isoforms might exhibit selectivity towards different RGS proteins. Based on immunoblot densitometry of both HEK293 cell lysate (Table 2.1) and purified protein pull-down experiments (Table 2.2), it appears that RGS proteins bind more strongly to 14-3-3ε than 14-3-3β. These data support the suggestion that mammalian 14-3-3 isoforms differ slightly from one another in terms of their differential subcellular localization and expression, and their specificity towards different protein binding partners (Tzivion and Avruch, 2002; van Hemert *et al.*, 2004).

Previous reports have examined interactions between several other RGS and 14-3-3 proteins, further supporting that 14-3-3 proteins negatively modulate RGS function in

signal transduction. A study carried out by Benzing *et al.* (2000) demonstrated that 14-3-3τ was able to abolish the inhibitory effect of RGS3 on the carbachol-mediated MAP kinase activation in HEK293 cells. Subsequently, Schreiber *et al.* (2001) observed that 14-3-3τ suppressed the cystic fibrosis transmembrane conductance regulator (CFTR) by antagonizing the inhibitory effects of RGS3 on Gαi2. Finally, the fast RGS7-mediated deactivation kinetics of G protein-coupled inwardly rectifying K^+ channels (GIRKs) were slowed by the 14-3-3τ isoform (Benzing *et al.*, 2002). The foregoing effects of 14-3-3 on G protein-mediated signalling suggest a mechanism wherein 14-3-3 binds to RGS proteins and thereby limits their abilities to act as GTPase activating proteins (GAPs). Previously, inhibition of RGS GAP activity by 14-3-3 proteins has been demonstrated in a single turnover GTP hydrolysis assay, whereby *in vitro* phosphorylation of RGS7 by PKCα promoted the inhibition of GAP activity in the presence of 14-3-3 proteins (Benzing *et al.*, 2000). In contrast, Ward and Milligan (2005) were unable to detect inhibition of RGS16 GAP activity by 14-3-3 proteins in a steady state GTP hydrolysis assay using membranes from HEK293 cells expressing an α2A-adrenergic receptor-Gαo1 fusion protein, despite the fact that the authors showed an unmistakable protein interaction between RGS16 and 14-3-3τ proteins. In contrast to these findings that call into question the ability of 14-3-3 to inhibit RGS GAP effects on GPCR-activated G proteins, our data show that 14-3-3 proteins were able to inhibit the GAP activity of RGS4 and RGS16 in a concentration-dependent manner.

It is uncertain why only we were able to observe inhibition by 14-3-3 of RGS GAP effects in steady state GTP hydrolysis assays, but it seems likely that the discrepancy can

be attributed to differences in the model systems used. The study by Ward and Milligan (2005) employed GPCR-G protein fusion proteins where coupling efficiency is increased relative to that of free receptor and G protein (Bertin *et al.*, 1994; Seifert *et al.*, 1998). This stands in contrast to the model system used in our study, where the receptor and the G protein are not covalently linked to one another. Since GPCRs may promote RGS protein binding to G proteins (Chidiac and Roy 2003, Bernstein et al 2004, Hague et al 2005), it is possible that RGS protein affinity for the GPCR-G protein fusion protein may be greater than that for the free G protein, which could in turn decrease the ability of 14-3-3 to inhibit RGS activity. Furthermore, 14-3-3 has previously been shown to bind to the third intracellular loop of several α2-adrenergic receptor isoforms (Prezeau *et al.*, 1999), and consequently, the α2-adrenergic receptor may compete with the RGS protein for 14-3-3, thereby decreasing the availability of 14-3-3 to bind to the RGS protein. Thus, there are several factors that may have limited the inhibition of RGS7 and RGS16 GAP activities by 14-3-3 proteins in the study by Ward and Milligan (2005).

It has been postulated that RGS proteins can either be bound to activated G proteins or 14-3-3 proteins *in vivo*, and depending on the intracellular environment, the RGS protein can exchange binding partners. Consistent with this idea, Benzing *et al.* (2002) demonstrated that a significant fraction of endogenous RGS7 is complexed with endogenous 14-3-3 in the mouse brain. Moreover, RGS3 has been shown to bind either 14-3-3 or activated G protein in CHO cells and once RGS3 is bound to 14-3-3, it apparently loses its ability to interact with Gq (Niu *et al.*, 2002). In our study, we have shown that 14-3-3 proteins bind to several RGS proteins and this supports the idea that

14-3-3 and G proteins may compete for RGS proteins intracellularily. Indeed, we have demonstrated that 14-3-3ε inhibits the binding of Gαo to RGS4, but not to RGS5 (Figure 2.11). An analogous result was seen in the *in vitro* GTP hydrolysis assay, where 14-3-3 inhibited RGS4 and RGS16, but not RGS5 (Figure 2.9), despite readily binding to all three RGS proteins in pull-down assays. Hence, the ability of 14-3-3 to inhibit RGS GAP activity would presumably depend on the concentration of the proteins involved and on the relative affinities of 14-3-3 and of the G protein for the RGS protein. These data reveal that 14-3-3 can act as a molecular chelator, preventing RGS proteins from interacting with Gα, which may ultimately prolong the activation of the signal transduction pathway in intact cells. Furthermore, there appears to be selectivity regarding the effects of 14-3-3 on RGS proteins, where RGS-14-3-3 binding does not necessarily imply inhibition of RGS GAP activity. In the case of RGS5, one possible explanation for the absence of 14-3-3 inhibition is that the affinity of RGS5 for 14-3-3 is low relative to its affinity for Gα proteins.

Another factor that may play a role in the ability of 14-3-3 to distinguish between different RGS proteins is phosphorylation. 14-3-3 proteins are primarily known to bind to phosphorylated target proteins, however there appears to be a controversy regarding the necessity of RGS phosphorylation and its mediating role within the RGS-14-3-3 interaction. Previous studies have shown some evidence suggesting that phosphorylation of the RGS protein may be required for 14-3-3 binding. A pull-down experiment carried out in CHO cells showed an interaction between 14-3-3 and RGS3, in which the authors postulated that RGS3 was phosphorylated in the basal state and speculated that the

binding of 14-3-3 to phosphorylated RGS3 prevented its dephosphorylation (Niu *et al.*, 2002). A similar result was shown with RGS3 and RGS7, where 14-3-3 binding to RGS proteins was significantly reduced after HEK293 cells were treated with staurosporine, TNF-α or alkaline phosphatase, all agents that have the potential to reduce overall RGS phosphorylation (Benzing *et al.*, 2000; Benzing *et al.*, 2002). Previous observations, notwithstanding the present findings, imply that phosphorylation of RGS proteins is not compulsory for 14-3-3 binding, as 14-3-3 proteins are able to readily interact with non-phosphorylated RGS targets. Since the RGS proteins used in the pull-down experiments were expressed and purified from a prokaryotic system, and thus are presumably not phosphorylated (verified for RGS4 by mass spectrometry analysis, data not shown), our findings indicate that RGS4, RGS5 and RGS16 are all able to directly bind to 14-3-3 in the absence of any modifications otherwise seen in mammalian cells, such as phosphorylation. Consistent with this interpretation, phosphorylation of serine residues in RGS3 and RGS16 did not result in an increase in 14-3-3 binding (Ward and Milligan, 2005). In fact, the majority of data suggesting that RGS phosphorylation is essential for 14-3-3 binding are derived from cell-based systems; under such conditions, there is a possibility that additional proteins might be involved whose phosphorylation indirectly influences the RGS-14-3-3 interaction. Consistent with this principle, attempting to describe the precise role of TNF-α on the direct binding of 14-3-3 to RGS proteins *in vivo* becomes virtually impossible, mostly due to the pleiotropic effects of TNF-α; for example, TNF-α has been observed to inhibit RGS7 degradation in mouse brains (Benzing *et al.*, 2000), suggesting a possible mechanism for increased 14-3-3 binding that is independent of RGS phosphorylation. Notwithstanding the controversy regarding the

role of RGS phosphorylation or the lack thereof, it is abundantly clear that 14-3-3 proteins are associated with and important regulators of RGS function both *in vivo* and *in vitro*.

A putative 14-3-3 binding domain, the SYP motif, has been identified within the RGS domain of RGS3 and RGS7 (Benzing *et al.*, 2000). In fact, this motif appears to be conserved in half of all mammalian RGS protein (Figure 2.8) and based on the crystal structure of RGS4, the serine residue that is highly conserved is one of the three contact sites formed between the RGS domain and Gαi (Tesmer *et al.*, 1997). Previously, it has been shown that 14-3-3 proteins inhibited the GAP activity of phosphorylated RGS7 in a single turnover GTP hydrolysis assay, and the authors speculated that phosphorylation of serine 434 within the SYP motif provided for a critical 14-3-3 binding residue on RGS7 (Benzing *et al.*, 2000; Benzing *et al.*, 2002). In contrast to these results, another group demonstrated that once the serine residue within the SYP motif is mutated into an aspartate, thought to act as a phosphoserine mimic, 14-3-3 binding to both RGS3 and RGS16 remains unchanged compared to wild-type, but notably this substitution proves to be detrimental to RGS GAP activity (Ward and Milligan, 2005). These data suggest that phosphorylation of this conserved serine does not increase the binding affinity of 14-3-3 for RGS proteins, but rather might be a potential modulator of RGS function itself. Furthermore, preliminary work in our laboratory suggests that a serine to arginine mutation within the SYP motif of RGS4, RGS5 and RGS16, adversely affects protein stability and possibly GAP activity, reinforcing the idea that this serine residue plays an important role in the overall function of RGS proteins (data not shown). Thus, it seems

that the 14-3-3 sensitive phosphorylation of serine 434 may be unique to RGS7. It is probable that the phosphorylation of other sites on different RGS proteins might alter its association with 14-3-3 proteins, however we cannot neglect the possibility that phosphorylation of the RGS protein itself, and not 14-3-3 binding, might be sufficient to account for the inhibition of GAP activity.

It still remains unclear whether the conserved SYP motif is the primary 14-3-3 binding site on most RGS proteins. Another 14-3-3-binding site on RGS3 was identified outside the RGS domain in the N-terminal region, involving serine 264 and showed that a serine to alanine mutation at this position resulted in a loss of 14-3-3 binding and an increase in G protein binding affinity (Niu *et al.*, 2002). Similarly, Ward and Milligan (2005) observed that the predominant 14-3-3 binding site on RGS3 was serine 264 within the N-terminal domain, and not the SYP motif in the RGS domain. In our study, we showed that the GAP activity of RGS16, but not RGS5, was inhibited by 14-3-3 and we considered the possibility that this was due to the presence of the SYP 14-3-3 binding motif in RGS16 but not in RGS5, where the tyrosine residue is substituted for a leucine. Hence, we constructed reciprocal mutants RGS5 L167Y and RGS16 Y167L, and investigated whether the exchange of leucine and tyrosine residues at position 167 might account for the inhibition of RGS16 GAP activity by 14-3-3 in the *in vitro* steady state GTP hydrolysis assay. Notably, these two residues appeared to be irrelevant to GAP activity and did not account for the functional difference between RGS5 and RGS16 with respect to 14-3-3. Thus, the SYP motif may not be the primary 14-3-3 binding motif and in this case, it is possible that not all RGS proteins share a common 14-3-3 binding domain. It follows that there may be additional low affinity 14-3-3 binding sites on RGS proteins

that contribute to the binding of RGS to 14-3-3 and that influence RGS function (Tzivion *et al.*, 2001; Sehnke *et al.*, 2002). Consistent with this principle, 14-3-3 proteins have been observed to bind to several sites on the same target protein, as is the case with c-Raf-1 (Yaffe *et al.*, 1997).

In conclusion, RGS proteins of the B/R4 subfamily are capable of binding to both purified and intracellular 14-3-3, in which the protein interaction does not appear to be dependent upon any post-translational modifications. The 14-3-3 – RGS complex is relevant in the context of signal transduction, based on the observation that 14-3-3 inhibits the GAP activity of both RGS4 and RGS16, and competes with Gαo for RGS4. Taken together, we speculate that 14-3-3 proteins negatively modulate RGS function by acting as molecular chelators that sequester RGS proteins away from both the G protein and the plasma membrane.

2.5. **References**

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CHAPTER 3

3.1. **Final Conclusions and Future Perspectives**

GPCRs are historically known to be key targets for drug discovery and development, and it is now becoming evident that these receptors are closely associated with accessory proteins that significantly contribute to their function *in vivo* (Milligan and White, 2001; Kreienkamp, 2002). Thus, investigating proteins that interact directly or indirectly with GPCRs may not only expand our understanding of their physiological roles, but also offer the possibility to improve treatment of diseases and be important in the development of new classes of drugs that more efficiently target the site of action (Presland, 2004). RGS proteins represent a major class of such accessory proteins and the present study explores a mechanism by which several RGS proteins are themselves modulated by the multifunctional protein 14-3-3.

Here, we have identified novel protein-protein interactions between RGS proteins of the B/R4 subfamily (RGS4, RGS5, RGS16) and two 14-3-3 isoforms (14-3-3β and 14-3-3ε), supporting the following hypothesis: 14-3-3 proteins directly bind to RGS proteins and act as negative modulators of RGS GAP activity by sequestering them away from their G protein targets.

The following is a summary of our main objectives and findings:

(1) to characterize the direct interaction of RGS proteins with two 14-3-3 isoforms in different experimental systems

- 14-3-3 in cytosolic extracts interacts with purified RGS4, RG5 and RGS16
- RGS5 in cytosolic fractions interacts with purified 14-3-3

- Purified RGS proteins directly bind to purified 14-3-3 in a concentration-dependent manner

(2) to establish the functional significance of the protein interactions observed using *in vitro* steady state GTP hydrolysis assays and competitive pull-down experiments

- 14-3-3 has no effect on Gαo protein-coupled M_2 cholinergic receptor signalling pathway in the absence of RGS proteins
- 14-3-3 inhibits the GAP activity of RGS4 and RGS16, but not RGS5 in the *in vitro* steady state GTP hydrolysis assay
- 14-3-3ε competes with activated Gαo proteins for RGS4, but not RGS5 in the competitive pull-down experiment

(3) to investigate the role of the tyrosine residue in the SYP putative 14-3-3-binding motif within the RGS domain

- RGS5 L167Y and RGS16 Y167L mutants demonstrate similar GAP activities as wild-type RGS proteins
- The leucine/tyrosine amino acid substitution is not accountable for the functional difference observed between RGS5 and RGS16 in the presence of 14-3-3 under these experimental conditions

Based on our data, we suggest a mechanism wherein 14-3-3 proteins negatively modulate RGS function and act as molecular chelators that sequester RGS proteins away from both

the G protein and the plasma membrane. Thus, we conclude that 14-3-3 proteins indirectly promote GPCR signalling via their inhibitory effects on RGS proteins.

The observations presented in this thesis and in other studies show that 14-3-3 proteins are important modulators of RGS function *in vitro,* and thus the next step is to confirm the mechanism underlying the RGS-14-3-3 interaction *in vivo*. Based on their intracellular localization and their co-expression in multiple tissues including the brain and the heart (Larminie *et al.*, 2004; Dougherty and Morrison, 2004), it is plausible that RGS proteins are indeed modulated by 14-3-3 and that disruption in their binding may be a contributing factor in the development of certain diseases. For example, several RGS proteins such as RGS4, RGS5 and RGS16 have been shown to play a role in GPCR signaling in the cardiovascular system (Riddle *et al.*, 2005), where alterations in their protein expression may participate in hypertrophy, heart failure and sepsis (Wieland and Mittmann, 2003). Similarily, 14-3-3γ protein expression has been observed to be induced in arterial trauma by cytokines (Autieri, 2004), suggesting that 14-3-3 isoforms may be important in the progression of vascular proliferative diseases (Autieri, 2004).

RGS proteins are not always localized near their target G proteins and the mechanism underlying the translocation of RGS proteins to and from the plasma membrane is important in understanding their regulation and their impact on signalling (Burchett, 2003). One process in which the translocation of some RGS proteins takes place is by their phosphorylation and dephosphorylation. For instance, phosphorylation of RGS3 and RGS4 does not affect their GAP activities but results in their relocation to the plasma

membrane from the cytoplasm (Pedram *et al.*, 2000). As discussed in other chapters of this thesis, it has been established that 14-3-3 proteins are mostly cytosolic and can affect the intracellular localization of a number of their protein binding partners (Lopez-Girona *et al.*, 1999; Zhang *et al.*, 1999; Yaffe, 2002; Kino *et al.*, 2003). In fact, one common 14-3-3 function is to promote cytosolic retention of nuclear proteins (Gorlich and Mattaj, 1996; Sehnke *et al.*, 2002; Kino *et al.*, 2003). The present results and other findings in the literature suggest that the mechanism by which 14-3-3 inhibits the GAP activity of RGS proteins *in vivo* is by sequestering them in the cytoplasm and thus away from their G protein targets. We have addressed this issue by performing competitive pull-down experiments where we observed that 14-3-3ε competes with Gα for RGS4 but not RGS5, however more data still need to be collected to confirm this mechanism under more physiological conditions. Previously, it has been demonstrated that RGS proteins co-localize with the Gα subunit and the receptor at the plasma membrane (Chidiac and Roy, 2003) and based on this observation, further studies in the lab will use confocal microscopy to visualize any changes that 14-3-3 may have on the plasma membrane localization of fluorescent-tagged RGS proteins when co-expressed with G proteins or GPCRs in mammalian cells. From functional data already established, we hypothesize that RGS4 and RGS16, but not RGS5 will be translocated from the plasma membrane and sequestered in the cytoplasm in the presence of 14-3-3 proteins.

Despite the clear interaction observed between 14-3-3 and RGS proteins *in vitro* and *in vivo*, it remains unclear whether the SYP motif is the primary 14-3-3 binding site on most RGS proteins, since several RGS proteins (i.e. RGS4 and RGS9-2) bind to 14-3-3 in the

absence of this site, while others (i.e. RGS3) appear to have multiple putative 14-3-3 binding motifs (Niu *et al.*, 2002; Ward and Milligan, 2005). It therefore seems plausible that not all RGS proteins share an identical 14-3-3 binding motif, but rather they may have numerous low affinity binding sites. Evidence in the present study suggests that RGS5 and RGS4 (which do not possess the SYP motif) may employ additional sites for their interaction with 14-3-3 and as of yet, their identity is unknown. Alternatively, the RGS5 SLP motif and the RGS4 SYR motif may be previously unrecognized 14-3-3 binding sites.

As described in Chapter 1, RGS phosphorylation as a determinant for 14-3-3 binding still remains controversial and in the present study, we have clearly demonstrated that 14-3-3 proteins bind to non-phosphorylated RGS4, RGS5 and RGS16. However, we cannot neglect the possibility that phosphorylation of these particular RGS proteins might affect the protein-protein interaction in question. The most obvious outcome is that this post-translational modification may increase the binding affinity of RGS proteins for 14-3-3 as has been described for many 14-3-3 protein targets (McGonigle *et al.*, 2001; Foschi *et al.*, 2001; McGonigle *et al.*, 2002). Thus RGS protein phosphorylation may represent a mechanism of modulating 14-3-3 binding to different RGS proteins and in our case, 14-3-3 might exhibit an inhibitory effect on phosphorylated RGS5 that was not observed with the non-phosphorylated form. Moreover as illustrated in Figure 3.1., RGS4, RGS5 and

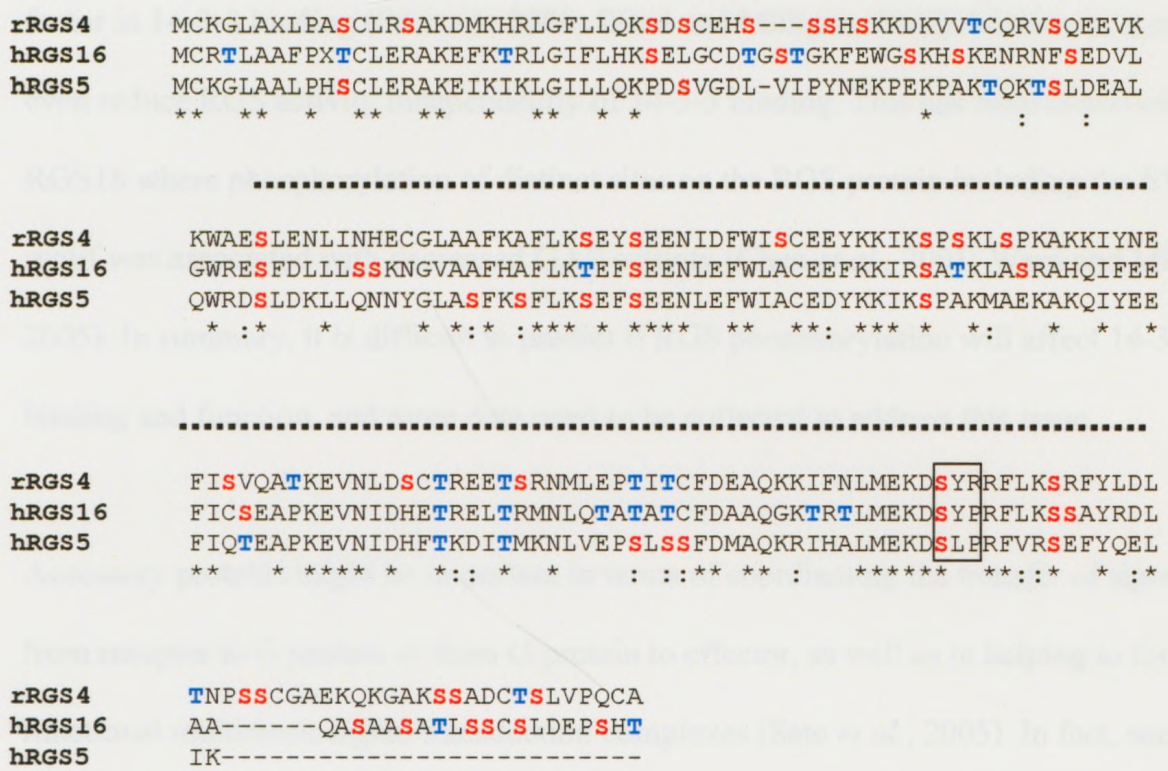

Figure 3.1. *Other putative RGS-14-3-3 binding sites.* Sequence alignment of rat RGS4, human RGS5 and human RGS16 used in pull-down experiments described in Chapter 2. 14-3-3 proteins have been shown to have a high affinity for phosphorylated serine (**S**) and threonine (**T**) residues on some target binding proteins (Yaffe, 2002). The SYP putative 14-3-3 binding motif (in box) is located at the end of the RGS domain (designated by (▪▪▪▪▪). * indicates highly conserved residues within the RGS subfamily and **:** highlights amino acids that are present in RGS4 and RGS16, but not RGS5.

RGS16 possess a number of serine and threonine residues that could potentially be phosphorylated and represent one or more novel putative 14-3-3 binding motif(s). However, it is also possible that RGS phosphorylation may have little or no effect on the 14-3-3 protein interaction, demonstrating that the former is not always a determining factor in 14-3-3 binding (Fu *et al.*, 2000; Ward and Milligan, 2005). Moreover, it might even reduce RGS activity independently of 14-3-3 binding. This has been observed with RGS16 where phosphorylation of distinct sites on the RGS protein including the SYP motif was associated with decreased GAP activity (Chen *et al.*, 2001; Ward and Milligan, 2005). In summary, it is difficult to predict if RGS phosphorylation will affect 14-3-3 binding and function, and more data need to be collected to address this issue.

Accessory proteins might be important in terms of coordinating the transfer of signals from receptor to G protein or from G protein to effector, as well as in helping to form functional multimeric signal transduction complexes (Sato *et al.*, 2005). In fact, some larger RGS proteins themselves have been shown to function as scaffolds, and it has been postulated that they are able to integrate both G protein-dependent and G protein-independent signalling cascades (Hollinger and Hepler, 2002). As described in Chapter 1, 14-3-3 proteins interact with a wide variety of targets inside the cell and have been shown to directly bind to several GPCRs including the cytosolic tail of $GABA_B$ R1 (Couve *et al.*, 2001) and the third intracellular loop of the α2-adrenergic receptor (Prezeau *et al.*, 1999). Thus, one possible function of 14-3-3 in signal transduction might be to act as an adaptor protein, cross-linking the RGS protein to GPCRs or to other receptors, and assisting in the recruitment of additional components for downstream signalling. This may constitute a mechanism by which RGS proteins are selectively targeted to signalling pathways, as is

known to occur with other accessory proteins including R9AP, R7BP, GIPC and spinophilin (De Vries *et al.*, 1998; Keresztes *et al.*, 2004; Martemyanov *et al.*, 2005; Wang *et al.*, 2005). As of yet there is no direct evidence to support this concept (and the present data do not address this possibility), however our results do imply that RGS proteins may be differentially affected by 14-3-3 proteins, particularly in the case of RGS5 where we observed a definite protein interaction with 14-3-3 but failed to detect any inhibition of its GAP activity. Taken together, 14-3-3 proteins may be complex RGS modulators and exhibit multiple functions other than inhibition of RGS protein GAP activity.

3.2. **References**

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APPENDIX A

ADDITIONAL DATA

A. **Introduction**

Many genes for RGS proteins (i.e. RGS5, RGS6, RGS9, RGS12 and RGS20) have been shown to encode other genetic variants and one example of the latter is the presence of splice variants that may or may not show specificity in terms of their tissue and cellular localization, as well as function (Zhang *et al.*, 1999; Chatterjee and Fisher, 2000; Chatterjee *et al.*, 2003; Liang *et al.*, 2005). At a late stage in this study, we found that the human RGS16 construct used exhibited a point mutation (TCT to CCT) in the RGS domain, resulting in an amino acid change at position 174 from a proline to a serine residue. The described RGS16 has not been recorded on the NCBI sequence database and we suspect that this variation maybe a single nucleotide polymorphism (SNP) and not the result of a cloning error. Since these two amino acids have very different chemical properties and the residue switch occurs in the RGS domain, a region responsible for the RGS protein GAP activity, we decided to compare this RGS16 variant with the previously known RGS16, especially with respect to 14-3-3 binding.

B. **Methods**

Constructs

Serine to proline (TCT to CCT) substitution in pET20b-RGS16 (labeled RGS16 S174P) was carried out using the Stratagene QuikChange site-directed mutagenesis method as described previously. The following oligonucleotide, and its reverse complement were used as a primer in the PCR reaction: Sense-CGCTTCCTGAAGTCGCCTGCTTACC GGGACC. The presence of the appropriate mutations was confirmed by sequencing (DNA Sequencing Facility, Robarts Research Institute, University of Western Ontario, Canada).

Detailed protocols describing the protein purification, the pull-down experiments and the data analysis have been described in *Materials and Methods* in Chapter 1.

C. **Results and Discussion**

To investigate the characteristics of the novel human RGS16 splice variant, we designed an RGS16 (labeled RGS16 S174P) that has previously been identified, by switching the serine residue back to the proline residue at position 174. We then compared the two RGS16 proteins by looking at their RGS GAP activities, and their ability to bind to 14-3-3 proteins. Both RGS16 proteins demonstrated similar GAP activities in the *in vitro* steady state GTP hydrolysis assay (data not shown), implying that this amino acid substitution does not interfere with Gαo binding or impair its GAP function. Furthermore, as assessed by cell lysate and purified protein pull-down experiments, there was no

apparent difference in 14-3-3 binding to either RGS16 or RGS16 S174P proteins, and both RGS16 variants readily interacted with 14-3-3ε (Figures A.1 and A.2) and 14-3-3β (data not shown). Taken together, the data suggest that the serine substitution within the RGS domain of RGS16 used in the present study did not have a detectible effect on protein function.

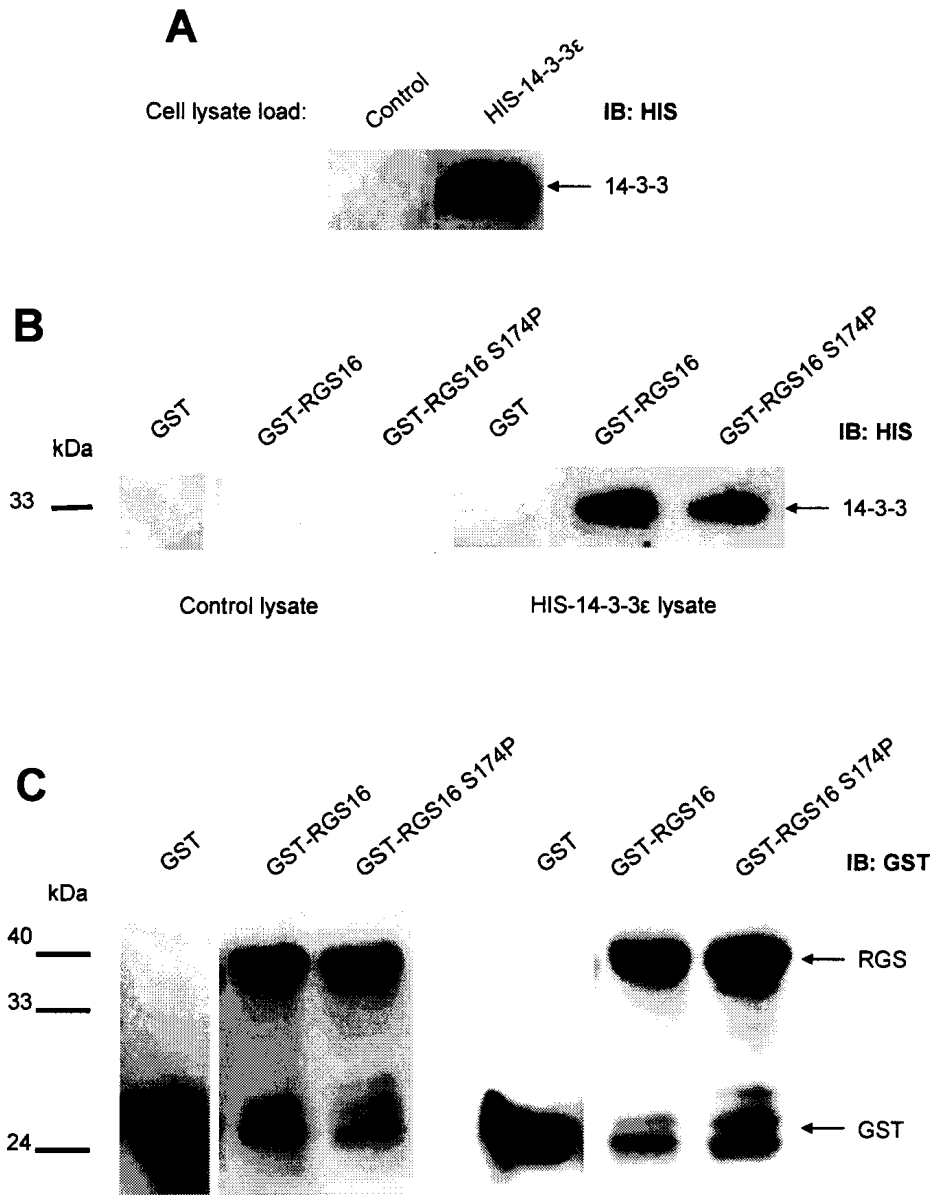

Figure A.1. *14-3-3ε in cytosolic fractions interacts with both purified RGS16 and RGS16 S174P.* Western blot of a pull-down experiment using lysates of HEK293 cells expressing HIS-14-3-3ε or a mock-transfected control. (A) Lysates taken prior to pull-down experiment (transfection control), in which the membrane was probed with anti-HIS antibody to detect 14-3-3 proteins. For the pull-down experiment, cell lysates were incubated with GST [0.8 μM] or GST-RGS16 or GST-RGS16 S174P [0.4 μM]. Glutathione-Sepharose 4B beads were added to the proteins overnight. The blot was probed with anti-HIS antibody (B), after which the membrane was stripped and reprobed with anti-GST antibody (C). Data represent one of two independent experiments. All lanes shown were taken from a single membrane (each probed once with anti-HIS and once with anti-GST).

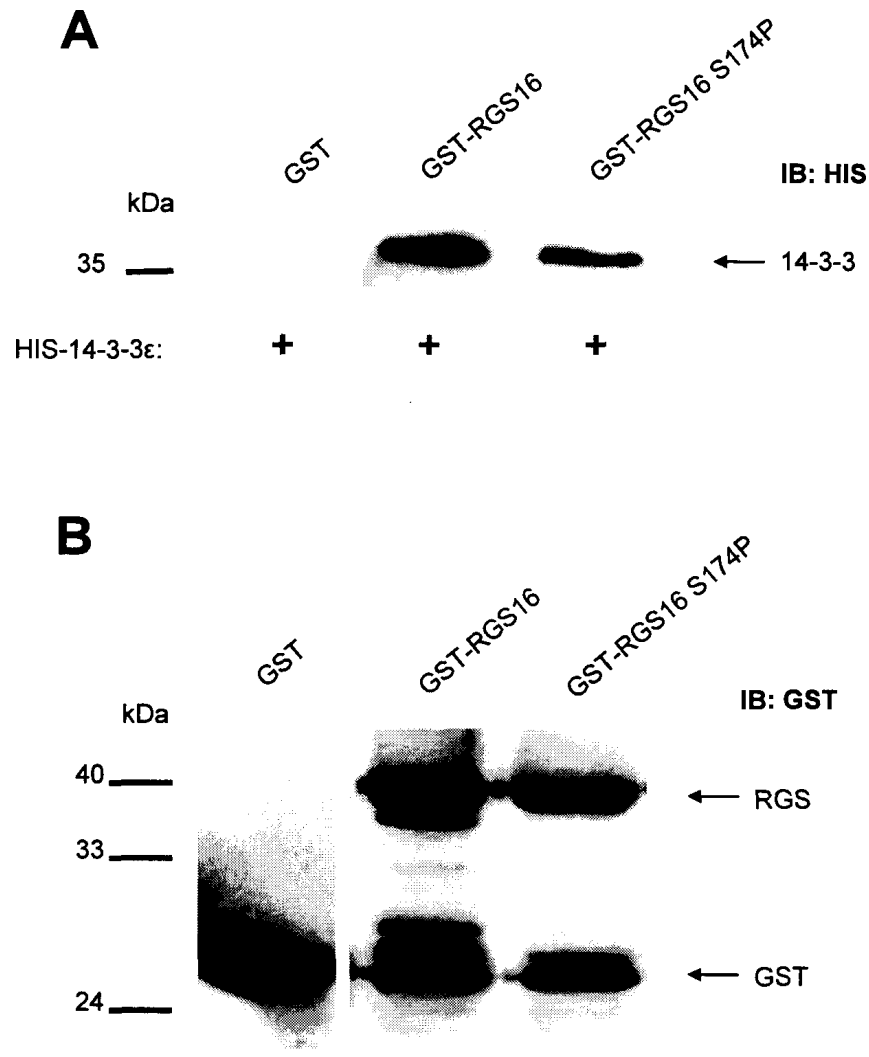

Figure A.2. *Direct interaction between purified RGS16 S174P and purified 14-3-3ε proteins.* Western blot of protein binding experiment between GST [0.4 μM] or GST-RGS16 or GST-RGS16 S174P [0.2 μM], and HIS-14-3-3ε [0.5 μM]. Proteins were incubated with glutathione-Sepharose 4B beads overnight. The blot was probed with anti-HIS antibody to detect 14-3-3 proteins (A), after which the membrane was stripped and reprobed with anti-GST antibody (B). Data represent one of three independent experiments. All lanes shown were taken from a single membrane (each probed once with anti-HIS and once with anti-GST).

D. **References**

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APPENDIX B

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