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Modulation of subfamily B/R4 RGS protein function by 14-3-3 proteins

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Abstract

Regulator of G protein signalling (RGS) proteins are primarily known for their ability to act as GTPase activating proteins (GAPs) and thus attenuate G protein function within G protein-coupled receptor (GPCR) signalling pathways. However, RGS proteins have been found to interact with additional binding partners, and this has introduced more complexity to our understanding of their potential role in vivo. Here, we identify a novel interaction between RGS proteins (RGS4, RGS5, RGS16) and the multifunctional protein 14-3-3. Two isoforms, 14-3-3β and 14-3-3ε, directly interact with all three purified RGS proteins and data from in vitro steady state GTP hydrolysis assays show that 14-3-3 inhibits the GTPase activity of RGS4 and RGS16, but has limited effects on RGS5 under comparable conditions. Moreover in a competitive pull-down experiment, 14-3-3ε competes with Gαo for RGS4, but not for RGS5. This mechanism is further reinforced in living cells, where 14-3-3ε sequesters RGS4 in the cytoplasm and impedes its recruitment to the plasma membrane by Gα protein. Thus, 14-3-3 might act as a molecular chelator, preventing RGS proteins from interacting with Gα, and ultimately prolonging the signal transduction pathway. In conclusion, our findings suggest that 14-3-3 proteins may indirectly promote GPCR signalling via their inhibitory effects on RGS GAP function.

Keywords: 14-3-3; G protein; G protein-coupled receptor signalling; Regulator of G protein signalling (RGS) protein; GTPase activating protein (GAP); Molecular chelator

1. Introduction

G protein-coupled receptors (GPCRs) are among the most diverse types of cell surface proteins and are involved in a wide array of important physiological functions [1,2]. 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 due to their function as GTPase activating proteins (GAPs) for the α subunit of heterotrimeric G proteins [3]. However, the roles of RGS proteins appear to be more varied and complex in vivo than previously thought. RGS proteins thus can be viewed as multifunctional signalling regulators based on their ability to interact with proteins other than G proteins and in many cases, this involves regions distinct from the RGS domain [3–5]. This is further supported by the observation that several RGS proteins are found in locations other than the plasma membrane, including in the nucleus [6,7]. 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 can be modulated through various mechanisms including the regulation of their subcellular localization, post-translational modifications, and interactions with protein binding partners [8–13]. For example, phosphorylation of RGS proteins affects their intracellular localization, as is observed with RGS4 [14], RGS19 [15] and RGS10 [16].

14-3-3 proteins are small dimeric proteins (monomeric mass 27–32 kDa), with seven highly conserved isoforms (β, γ, ζ, σ, ε, η and τ,) in mammals, the functions of which appear to be largely similar [17–20]. 14-3-3 proteins were initially thought to bind to either of two specific phosphorylated motifs (RSXpSXP and RXY/FXpSSXP) [21], however many binding partners have been

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identified that lack these particular motifs. It is now recognized that there are more than 200 binding partners, some of whose interactions with 14-3-3 occur in a phosphorylation-independent manner [22,23]. 14-3-3 proteins bind to a number of regulatory proteins and integral components of signal transduction, including GPCRs [$GABA_B$, [24], α_2-adrenergic [25] and parathyroid hormone receptors [26]], tyrosine kinase receptors [27–29], kinases [30–34], phosphatases [35], apoptosis-related proteins [36,37] and protooncogene products [38]. Despite having no detectable catalytic or functional domains [17], 14-3-3 proteins do appear to be regulators of key signalling components and function primarily as chaperones, adaptors and scaffolds [22,39–41].

RGS proteins can bind to, and be negatively modulated by, 14-3-3 proteins [42] 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. [43,44] identified a putative 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 found evidence that RGS3 contains 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 [45,46]. Despite differences in the apparent 14-3-3-binding sites on RGS3 and RGS7, similar conclusions have been drawn, i.e. that 14-3-3 may interfere with RGS–Gα protein interactions. It has been suggested, but not unequivocally shown, that the effects of 14-3-3 may be promoted by RGS phosphorylation [43,45,46]. Irrespective of this, 14-3-3 proteins may modulate intracellular GAP activity without altering RGS protein expression.

Recent reports [46,47] 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 (members of the RGS protein B/R4 subfamily) and two 14-3-3 isoforms (14-3-3β and 14-3-3ε). The main objectives of this study were (1) to characterize the direct interaction of RGS proteins with these two 14-3-3 isoforms in different experimental systems, (2) to establish the functional significance of the protein–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 motif within the RGS domain that is postulated to serve as the binding site for 14-3-3. Overall, the present findings 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. Materials and methods

2.1. Constructs

Human 14-3-3β and 14-3-3ε, cloned into the bacterial/mammalian expression vector pTriEX4 (hexahistidine (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 (Glutathione-*S*-Transferase (GST)-tagged). Briefly, 14-3-3 fragments were cut with *Sma*I and *Not*I from pTriEx4 vector and inserted into pGEX-4T1 vector at blunted-*Eco*RI and *Not*I 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 HIS-tagged rat RGS4 (QE-60-RGS4), mouse RGS16 (pET20b-RGS16) and HIS-tagged Gαi1 and Gαo 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 [48] and was generously provided by Dr. DP Siderovski (Department of Pharmacology, The University of North Carolina, USA). Mouse RGS5 was cut with *Bam*HI and *Xba*I 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). Human GFP-tagged RGS5 was a gift from John H Kehrl (National Institute of Health, Maryland, USA) and rat pEGFP-C2-RGS4 was previously described [9]. Human pDNA3.1(+)-Gαo was supplied by the Guthrie cDNA Resource Center (Sayre, Pennsylvania). In all cases, HIS and GST tags within the RGS and 14-3-3 fusion proteins are located on the N-terminus. Constructs were sequenced and verified by the DNA Sequencing Facility at 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 *PfuTurbo* DNA polymerase (Stratagene). 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. Protein purification

HIS-tagged RGS, 14-3-3 and Gα proteins were purified from *Escherichia coli* (*E. coli*) strain BL21/DE3 essentially as described previously [49,50]. LB media containing ampicillin (final concentration: 100 μg/ml) were inoculated with transformed cells that had been incubated overnight at 37 °C, and were grown to an $OD_{600} \geq 0.5$. Expression of the HIS-tagged proteins was induced by the addition of isopropyl-β, D-thiogalacto pyranoside (IPTG) to a final concentration of 150 μM for 3 h before harvesting the bacteria by centrifugation. Bacteria were resuspended in buffer A (final concentrations: 20 mM Tris–HCl, pH 7.5, 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. 25 μg/ml DNase and 0.5 mM $MgCl_2$ were further incubated on ice for 30 min. After centrifugation, the volume of supernatant was increased to 50 ml/L culture with buffer B (final concentrations: 50 mM Hepes, pH 8.0, 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). A 50% slurry of Ni-NTA affinity resin (Qiagen) equilibrated in buffer B, along with imidazole (final concentration: 20 mM) was added to the supernatant, and the mixture was incubated at 4 °C for 1.5 h 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, pH 8.0, 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, pH 8.0, 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, pH 8.0, 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. This procedure yielded proteins that were >95% pure as determined by Coomassie Blue staining. A maximum of three 500 μl protein samples eluted from the Ni-NTA column were loaded on and eluted from a Superdex 75 HR20/30 column (Pharmacia). Peak fractions were pooled, and stored in aliquots at −80 °C.

For the purified GST and GST-fusion proteins, *E. coli* strain BL21/DE3 was transformed with pGEX-RGS or pGEX-14-3-3 constructs, induced with 200 μM IPTG (4 h, 37 °C). Cells 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 × 15 s bursts, allowing 5 s for cooling between bursts). Triton X-100 (final, 1%) was added (30 min on ice) and the insoluble fraction of material was removed by centrifugation. The supernatant was collected and incubated with glutathione-Sepharose 4B beads (equilibrated in PBS) for 30 min at room temperature, rotating end-over-end (Amersham Pharmacia Biotech). After centrifugation, 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, pH 8.0).

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 manufacturer's instructions.

2.3. Mammalian cell transfection

Human embryonic kidney (HEK)-293 cells were seeded onto 10 cm dishes (7×10^5 cells/plate) the day before transfection and at 50–70% confluency, the cells were transiently transfected with pcDNA HIS-tagged 14-3-3 (β and ε isoforms) or HA-tagged RGS constructs (RGS4, RGS5, RGS16), using calcium phosphate precipitation. Control cells were mock-transfected.

2.4. Cell lysate pull-down experiments

20 to 48 h after transfection, the attached cells were rinsed twice with PBS, treated with Trypsin-EDTA (Gibco), collected by centrifugation, resuspended in buffer F (final concentrations: 50 mM Tris–HCl, pH 7.6, 1 mM EDTA, pH 8.0, 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, subjected to centrifugation and the supernatants were transferred to new microfuge tubes. 500 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 and transferred into fresh tubes. Pre-cleared cell lysates (500 μl) 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 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, pH 7.6, 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). Proteins were released from the glutathione-Sepharose 4B beads by heating at 99 °C for 5 min, subsequent to the addition of 25 μl of loading buffer (final concentrations: 60 mM Tris–HCl, pH 6.8, 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.

2.5. Pull-down experiments with purified proteins

For the purified protein pull-down experiments, HIS-14-3-3ε or HIS-14-3-3β proteins (final concentration: 0.5 μM) were diluted in 500 μl buffer G to which 5 μg of GST (final concentration: 0.4 μM) or GST-RGS4, GST-RGS5 or GST-RGS16 (final concentration: 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 subsequently GST-tagged proteins were added as described above. For G protein pull-down experiments, HIS-Gαo or HIS-Gαi1 proteins (final concentration: 0.2 μM) in the presence or absence of AMF, were combined with purified GST (final concentration: 0.4 μM) or GST-RGS5, GST-14-3-3β or GST-14-3-3ε (final concentration: 0.2 μM) in 500 μl buffer F. For protein loading controls, 1 μg of Gαi1 or 0.1 μg of Gαo were diluted in loading buffer. For competitive pull-down experiments, GST-RGS4 and GST-RGS5 (final concentration: 0.2 μM) were incubated with AMF-activated HIS-Gαo proteins (final concentration: 0.01 μM) or HIS-14-3-3ε (final concentration: 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 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) after adding 25 μl of loading buffer.

2.6. Immunoblotting

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, pH 6.8, 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 each figure, all lanes shown were taken from a single membrane, however in some cases inapposite lanes have been cropped.

2.7. Densitometry

Densitometry of unsaturated immunoblot images was carried out using AlphaEaseFC software (FluorChem 8000 Advanced Chemiluminescence and Visible Light Imaging, Alpha Innotech Corporation). Statistical differences in protein binding were determined by a two-tailed unpaired Student's *t* test. Values of $p < 0.05$ were considered significant.

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, Gβ1 and Gγ2, and membranes from these cells were prepared as described previously [49].

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 essentially as described previously [49] in the presence or absence of HIS-RGS and/or HIS-14-3-3 proteins. Each reaction tube contained a 50 μl mixture, made up of 20 mM HEPES (pH 7.5), 1 mM EDTA, 2 mM $MgCl_2$, (0.5 mM free Mg^{2+}), 1 mM DTT, 0.1 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin. This was incubated at 30 °C for 5 min with 1 μM GTP, 500 μM ATP, [γ^{32}P]GTP (1×10^6 cpm/assay), either 100 μM carbachol (agonist) or 10 μM tropicamide (inverse agonist), and membranes (5 μg/assay) [49]. 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 non-specific membrane GTPase signal was estimated by adding 1 mM unlabeled GTP to one set of reaction tubes. 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.

2.10. Confocal microscopy

Live HEK293 cells were grown in 0.75 cm flasks and seeded onto 10 cm dishes (7×10^5 cells/plate) the day before transfection. At 50–70% confluency, the cells were transiently transfected with either GFP-tagged RGS4 or RGS5 (5 μg/plate) alone or in the presence of Gαi2 (5 μg/plate), HIS-14-3-3ε (20 μg/plate) or both, using calcium phosphate precipitation. The cells were transferred after 16 h to 0.35 cm glass bottom microwell dishes and 24 h after that were visualized through a Zeiss LSM 410 confocal microscope equipped with a Krypton/Argon laser. GFP fluorescence was viewed under a fluorescein isothiocyanate (FITC) filter and a 63× oil immersion lens (488 nm excitation wavelength and emission at 515 nm). The images were scanned every 4 s and the fluorescence patterns represent the majority (>60%) of cells inspected.

3. Results

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

In this study, we first examined the physical interactions between 14-3-3 proteins and RGS proteins belonging to the B/

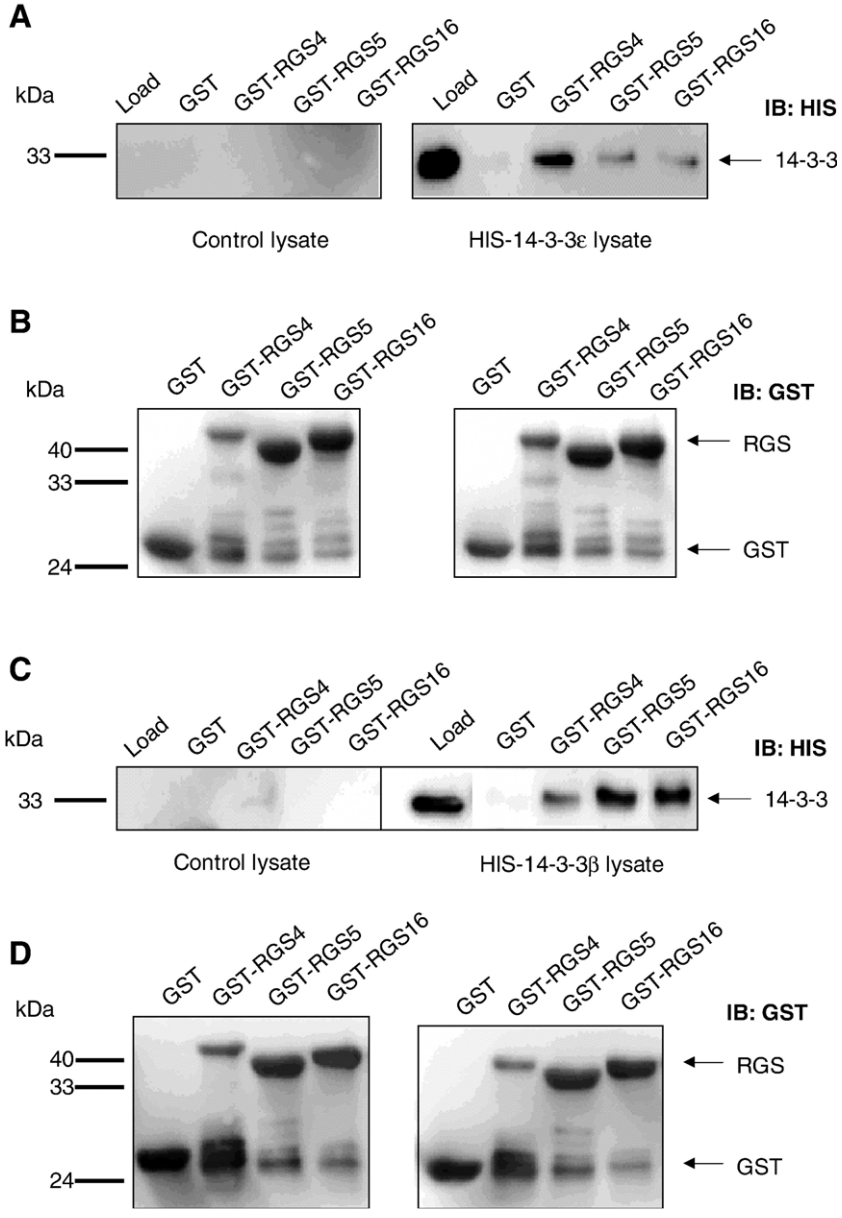


Fig. 1. 14-3-3 in cytosolic extracts interacts with purified RGS4, RGS5 and RGS16. HEK293 cells were transiently transfected with plasmids encoding HIS-14-3-3ε or 14-3-3β or mock-transfected (control lysate). Cell lysates were incubated with purified GST (800 nM) or GST-RGS4, GST-RGS5 or GST-RGS16 (400 nM) for 4 h, followed by an overnight incubation with a slurry of glutathione-Sepharose beads at 4 °C. The samples were eluted and separated by SDS-PAGE and transferred to PVDF membranes. Blots were probed with anti-HIS antibody (A, C), after which they were stripped and reprobed with anti-GST antibody (B, D). The immunoblots were visualized by chemiluminescence. Each lane shown is representative of 3–5 independent experiments carried out under the same conditions.

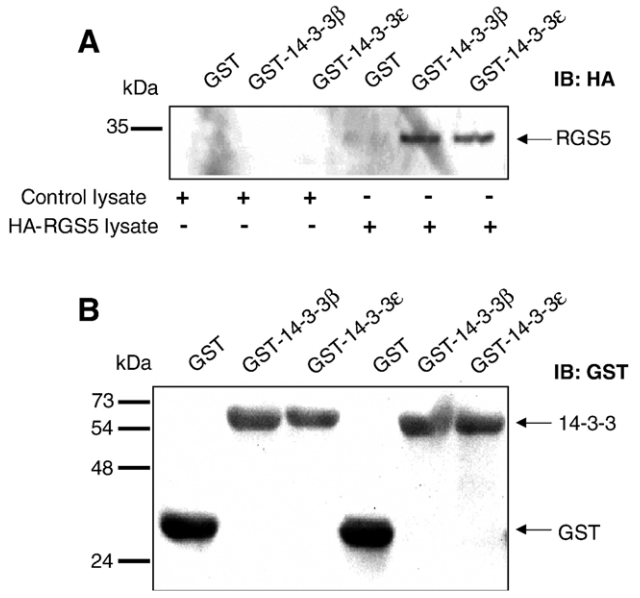


Fig. 2. RGS5 in cytosolic extracts binds to purified 14-3-3 proteins. HEK293 cells were transfected with cDNA encoding HA-RGS5 or mock-transfected (control lysate). The cells were lysed and incubated with GST (800 nM) or GST-14-3-3β or GST-14-3-3ε (360 nM), and then mixed with glutathione-Sepharose beads, followed by overnight incubation. Proteins eluted from the beads were separated by SDS-PAGE and transferred to a PVDF membrane. The blot was probed with anti-HA antibody (A), after which the membrane was stripped and reprobed with anti-GST antibody (B). These results shown are representative of 4 independent experiments. Densitometric analyses were performed on all 4 experiments, and average ratios (GST-14-3-3/GST, ±S.E.M.) for 14-3-3β were 0.8±0.1 with control lysates and 13±6 with lysates from cells transfected with HA-RGS5 and correspondingly for 14-3-3ε, these values were 0.9±0.1 and 44±22, respectively.

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 Fig. 1, we found that cytosolic 14-3-3β and 14-3-3ε both bound to all three purified RGS proteins, whereas no binding to 14-3-3 was observed with GST control protein.

3.2. Purified 14-3-3 proteins bind to RGS5 expressed in HEK293 cells

To further investigate the binding of 14-3-3 and RGS proteins observed in Fig. 1, we carried out an analogous series of cell lysate pull-down experiments complementary to those described above, wherein we assayed the interaction between transiently transfected HA-RGS proteins and purified GST-fusion proteins of both 14-3-3 isoforms. Consistent with the results shown in Fig. 1, RGS5 in cytosolic extracts bound to purified 14-3-3β and 14-3-3ε (Fig. 2). Unfortunately, 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 beads. Taken together, the results shown in Figs. 1 and 2 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.

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 intracellular factors. To address this question, a pull-down experiment was carried out combining purified GST-RGS with purified HIS-14-3-3 proteins in solution, and then incubating this mixture with glutathione-Sepharose beads. Under these conditions, any observed interactions presumably would reflect direct binding between the two proteins and furthermore would be independent of any post-translational modifications that may occur within mammalian cells. Examples of these experiments are shown in Figs. 3 and 4, where RGS4, RGS5 and RGS16 were found to directly bind to both 14-3-3ε and 14-3-3β. Moreover, the two 14-3-3 isoforms bound similarly to all three RGS proteins tested, however binding to these RGS proteins may have been greater with 14-3-3ε than with 14-3-3β. This was consistent between two separate batches of purified 14-3-3β as compared to three separate batches of purified 14-3-3ε proteins; thus the trend probably does not reflect differences in the quality or activity of individual batches of 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.

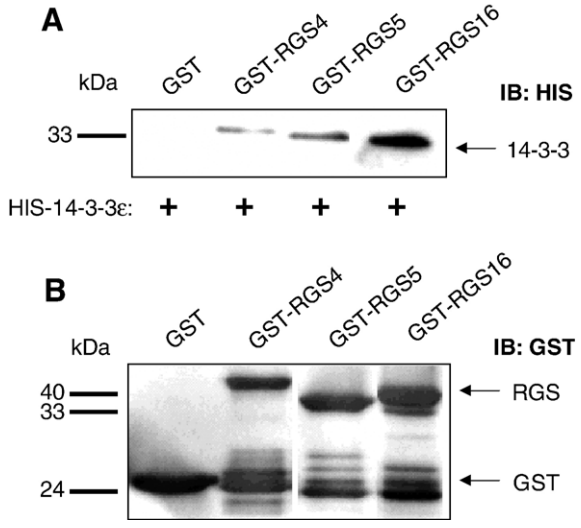


Fig. 3. Direct interaction between purified 14-3-3ε and RGS proteins. Purified HIS-14-3-3ε (500 nM) was mixed together with purified GST (400 nM) or GST-RGS4, GST-RGS5 or GST-RGS16 (200 nM), and incubated together with glutathione-Sepharose beads. Proteins were eluted from the beads and separated by SDS-PAGE, and then transferred to a PVDF membrane. 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 shown are representative of 3–5 individual experiments.

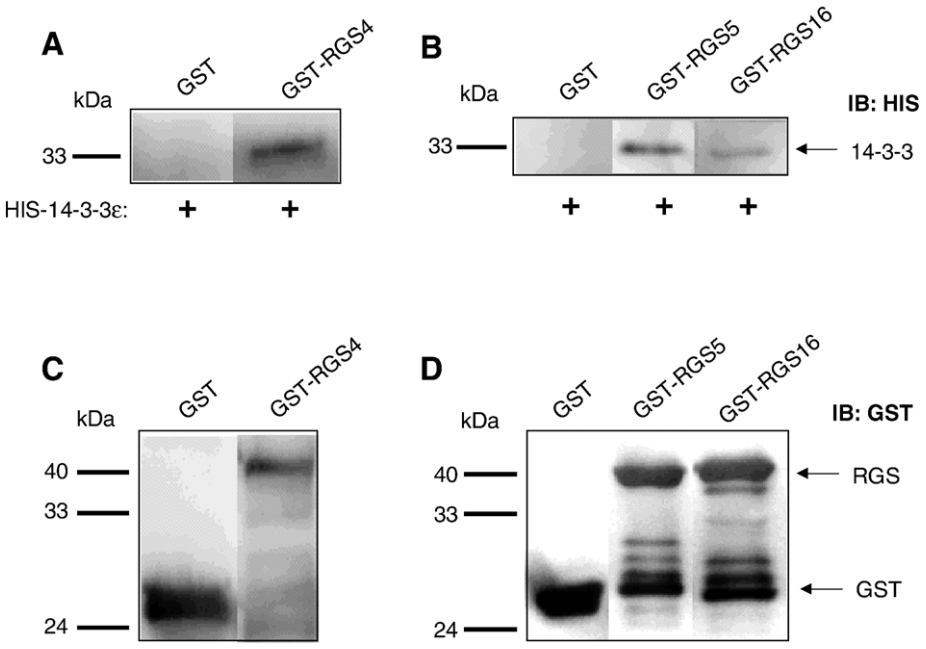


Fig. 4. Direct interaction between RGS proteins and 14-3-3β. Purified HIS-14-3-3β (500 nM) was mixed together with purified GST (400 nM) or GST-RGS4, GST-RGS5 or GST-RGS16 (200 nM), and incubated together with glutathione-Sepharose beads. Proteins were eluted from the beads and separated by SDS-PAGE, and then transferred to a PVDF membrane. The blot was probed with anti-HIS antibody to detect the 14-3-3 proteins (A,B), after which the membrane was stripped and reprobed with anti-GST antibody (C,D). The results shown are representative of 3 independent experiments.

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 follows that 14-3-3 has the potential to interfere with the GAP effects of the RGS proteins examined on their target G proteins. We therefore investigated whether such a regulatory mechanism might exist, using a steady state in vitro assay in which RGS proteins promote receptor-dependent GTPase activity. This assay utilizes the agonist carbachol to stimulate [γ-^{32}P]GTP hydrolysis in membranes derived from Sf9 insect cells expressing the M_2 muscarinic receptor plus Gαo, Gβ1 and Gγ2. Tropicamide, an inverse agonist, inhibits the intrinsic activity of this receptor and therefore was used in the assay to define the receptor-independent signal (Fig. 5). 14-3-3 alone had no apparent effect on receptor-dependent GTPase activity in the absence of RGS protein (Fig. 5). The GAP activities of both RGS4 and RGS16 were significantly inhibited by both 14-3-3

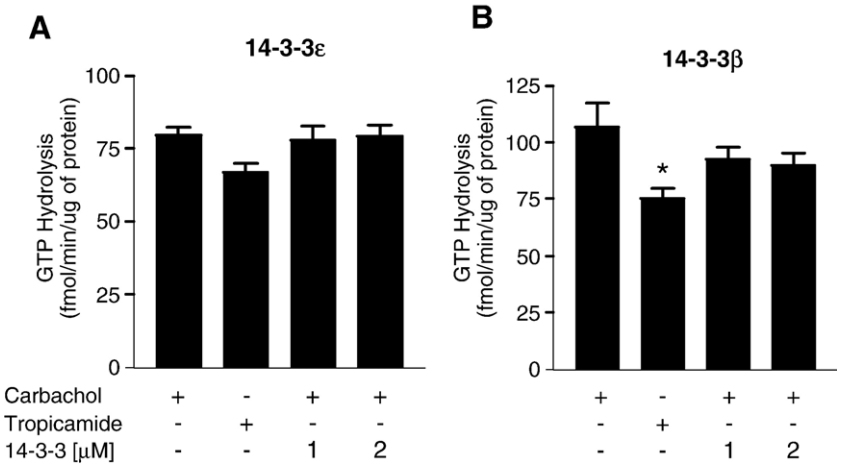


Fig. 5. 14-3-3 has no effect on agonist- and receptor-dependent Go GTPase activity in the absence of RGS proteins. Membranes derived from Sf9 cells coexpressing the M2 muscarinic acetylcholine receptor plus heterotrimeric Go were assayed for GTPase activity with the agonist carbachol (100 μM) or the inverse agonist tropicamide (10 μM), in the absence or presence of 14-3-3ε (A) or 14-3-3β (B), as indicated. Data shown represent the means ± S.E.M. taken from 5 (A) or 8 (B) independent experiments carried out in triplicate. Statistical significance was assessed by the use of a one-way ANOVA, followed by a Tukey's Multiple Comparison Test. *$p < 0.05$.

isoforms. This inhibition appeared more pronounced with 14-3-3β than with 14-3-3ε. Surprisingly, 14-3-3 had little or no effect on RGS5 GAP activity (Fig. 6) notwithstanding the observed binding of RGS5 to 14-3-3. Moreover, increasing 14-3-3 to 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 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.

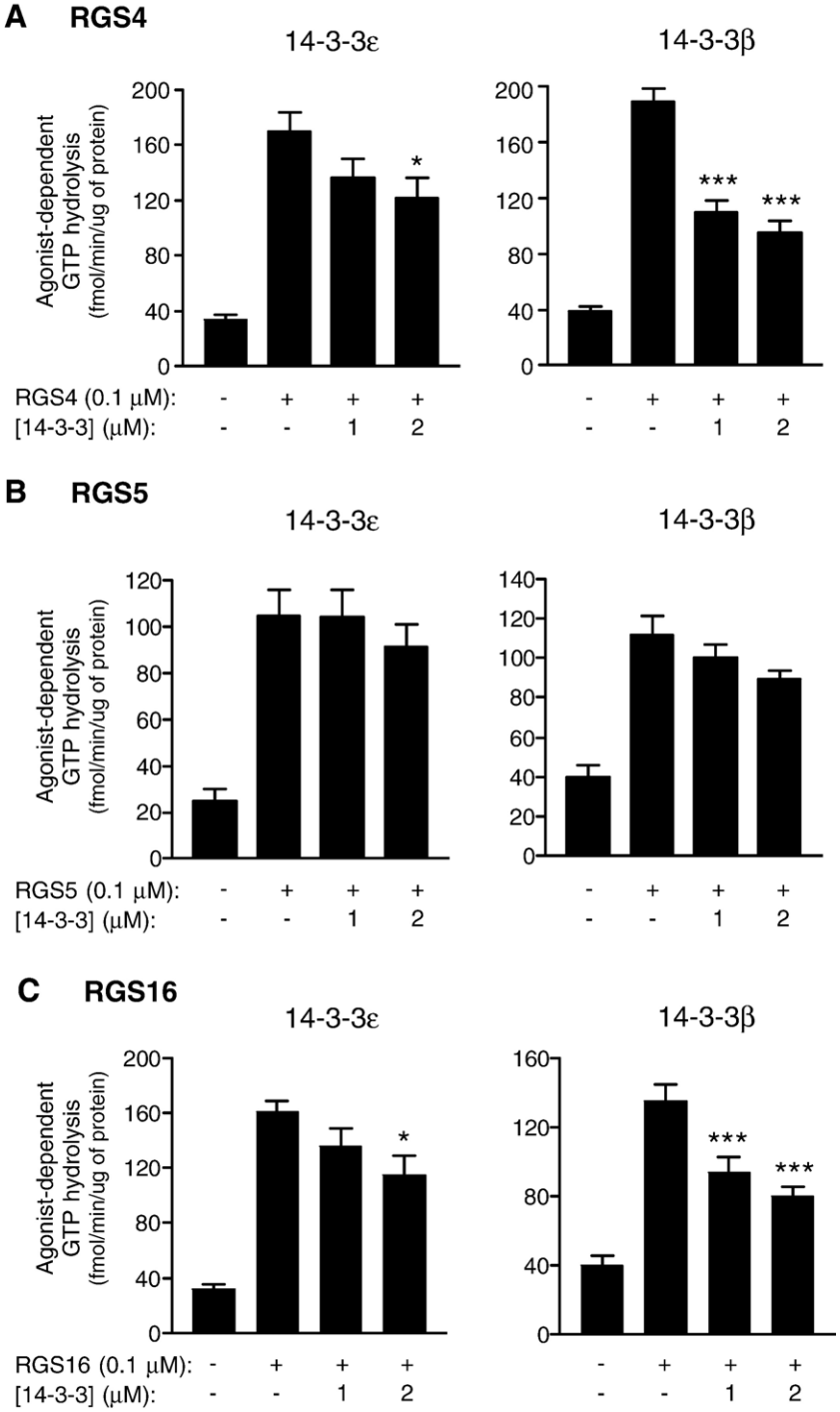


Fig. 6. 14-3-3 inhibits the GAP activity of RGS proteins. Membranes derived from Sf9 cells coexpressing the M2 muscarinic acetylcholine receptor plus heterotrimeric Go were assayed with the agonist carbachol (100 μM) either alone or in the presence of RGS proteins with or without 14-3-3 as indicated. Non-specific signal was defined as that observed in the presence of the inverse agonist tropicamide (10 μM) but without RGS or 14-3-3, and this was subtracted out to yield the values indicated. Each condition was performed in triplicate and data represent means±S.E.M. of at least 4 independent experiments. 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.

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 approach, we examined 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^-) believed to mimic the transition state of the Gα subunit bound to the gamma phosphate of GTP, i.e. activated Gα protein [51]. These effects have long been established with RGS4 [52] and RGS16 [53].

The present data clearly show that RGS5 has a higher affinity for the GDP-AlF_4^- activated form of Gα proteins (Fig. 7), and complement a study by Zhou et al. [54], 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 trend that RGS proteins facilitate the GTP hydrolysis reaction by favouring the Gα protein transition state [55]. In contrast, we were unable to detect any binding of 14-3-3 proteins to either form of Gαo and Gαi1 proteins (data not shown). This implies that the observed effects of 14-3-3 on GTPase activity do not derive from a direct effect on G protein, and is consistent with a mechanism wherein 14-3-3 binds to and inhibits the RGS protein from acting on the Gα protein.

Since RGS proteins bind to both 14-3-3 proteins and AMF-activated Gα, we next studied the effect of 14-3-3 proteins on the binding of RGS to Gα. In a competitive pull-down experiment using glutathione-Sepharose beads, either GST-RGS4 or GST-RGS5 was mixed in solution together with HIS-Gαo, HIS-14-3-3ε, or both. We used 14-3-3ε as this isoform produced a consistently robust signal, thus facilitating the measurement of potential decreases in RGS–14-3-3 binding. As demonstrated in Fig. 8, 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 little or no affect on RGS5 (Fig. 6). Thus, the present results imply that 14-3-3 might selectively impede the GAP effects of certain RGS proteins, based on the relative affinity of the RGS protein for Gα versus 14-3-3.

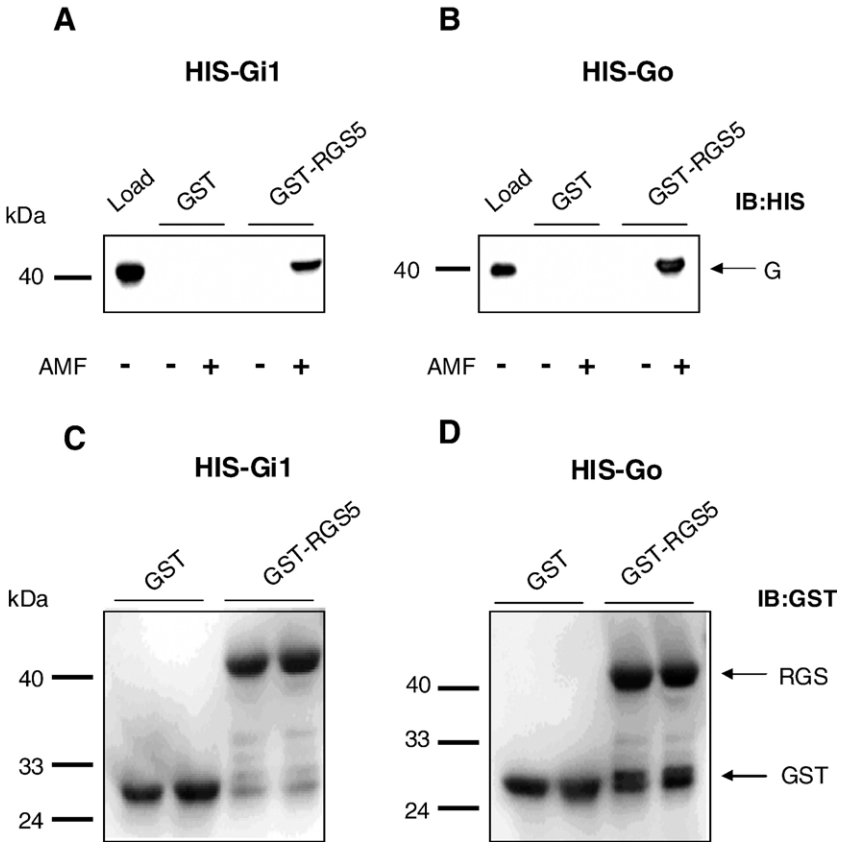


Fig. 7. RGS5 binds to activated Gαo and Gαi1 proteins. Purified G proteins (250 nM) were incubated with GST (400 nM) or GST-RGS5 (200 nM) in the absence and presence of AMF ($MgCl_2$, NaF, $AlCl_3$) and glutathione-Sepharose beads as indicated in Materials and methods. Proteins were eluted from beads, separated by SDS-PAGE, transferred to PVDF membranes, and then assessed by immunoblotting. An anti-HIS antibody was used to detect HIS-Gαi1 (A) and HIS-Gαo (B), and then membranes were stripped and reprobed with anti-GST antibody (C, D). Data represent one of the three independent experiments.

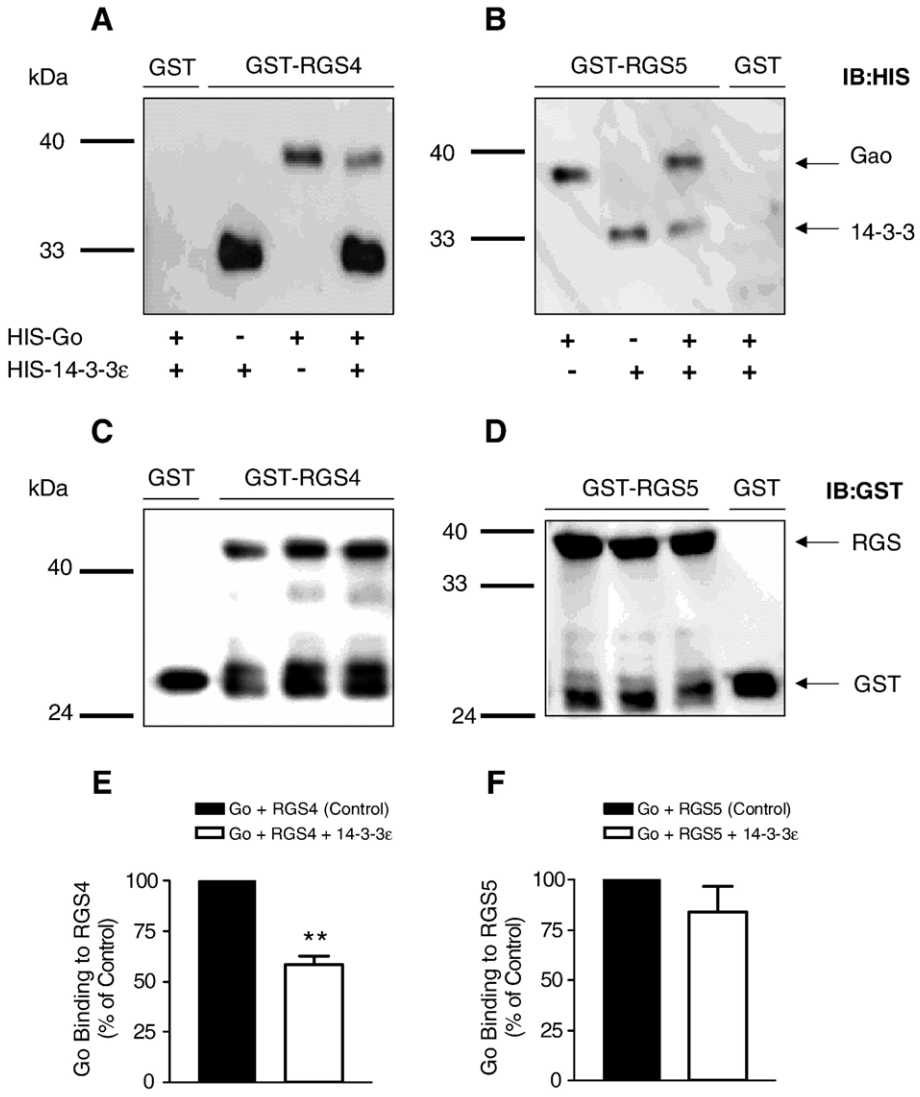


Fig. 8. 14-3-3ε competes with activated Gαo for RGS4, but not RGS5. Purified HIS-14-3-3ε (500 nM) and/or HIS-Gαo (10 nM) were incubated together with GST (400 nM) or GST-RGS4 (200 nM) (A) or GST-RGS5 (200 nM) (B) in the presence of AMF, as described in Materials and methods, followed by further incubation with glutathione-Sepharose beads. Eluted proteins were separated by SDS-PAGE and transferred to PVDF membranes, followed by immunoblotting with anti-HIS antibody (A, B), after which membranes were stripped and reprobed with anti-GST antibody (C, D). The blots shown are representative of three independent experiments, and densitometry was carried out on each of these to determine Gαo binding to either RGS4 (E) or RGS5 (F) in the absence and presence of 14-3-3ε. The data shown represent the average ratio between G protein isolated 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. $^{**}p<0.01$.

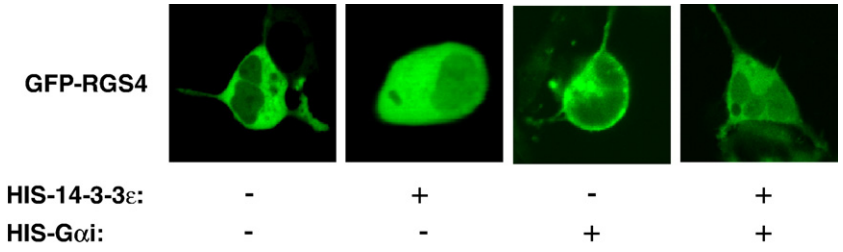


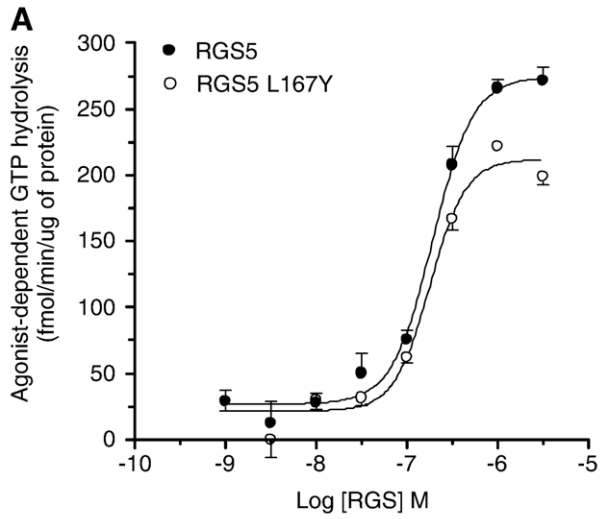
Fig. 9. 14-3-3 affects the intracellular localization of RGS4 in living cells. HEK293 cells were transiently transfected with GFP-RGS4 either alone or in the presence of HIS-Gαi2 and/or HIS-14-3-3ε, as described in Materials and methods. Cells were visualized through a confocal microscope and the images shown are representative of at least 30 living cells.

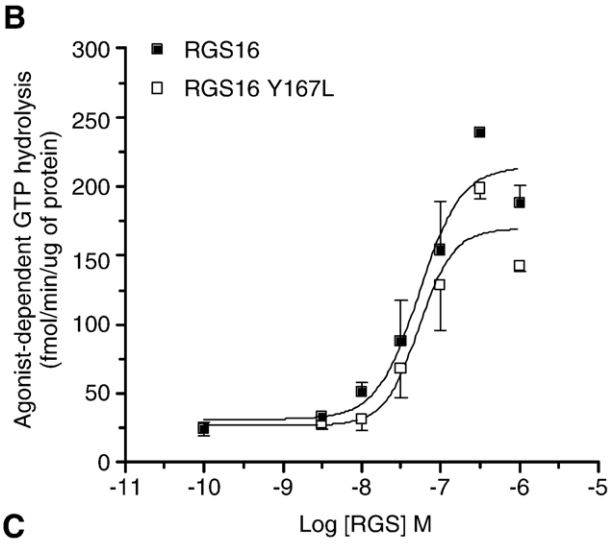
3.6. 14-3-3 affects the intracellular localization of RGS4 in living cells

Since 14-3-3 proteins are primarily cytosolic and have been shown to affect the intracellular localization of several of their protein binding partners [56,57], we addressed the possibility that the functional inhibition of RGS GAP activity, which we observed in the steady state GTP hydrolysis assay (Fig. 6), may reflect the ability of 14-3-3 to sequester RGS proteins away from the plasma membrane. Our lab has previously shown that in living cells, RGS proteins co-localize with Gα subunits and receptors at the plasma membrane [9]. To investigate whether 14-3-3 can alter the intracellular localization of RGS proteins, we transiently co-transfected HEK293 cells with GFP-RGS4 alone or together with Gαi2 and/or HIS-14-3-3ε, and visualized live cells using confocal microscopy (Fig. 9). In the absence of exogenous G protein and 14-3-3ε, GFP-RGS4 was predominantly cytosolic. 14-3-3ε had no apparent effect on the subcellular localization of GFP-RGS4. As observed previously [9], the co-expression of Gαi2 resulted in the recruitment of GFP-RGS4 to the plasma membrane. When 14-3-3ε was additionally expressed together with GFP-RGS4 and Gαi2, the translocation of GFP-RGS4 to the plasma membrane was not observed, suggesting competition for the latter between the cytosolic 14-3-3 protein and the membrane-associated G protein. These data reinforce the results obtained with in vitro GAP assays (Fig. 6) and competitive pull-down experiments (Fig. 8). Corroborating evidence could not be obtained with GFP-RGS5. As shown previously, its expression appeared uniform throughout the cytosol and nucleus with little or no plasma membrane association [58], however no changes in cellular GFP-RGS5 could be detected upon the co-expression of Gαi2 and/or 14-3-3ε (data not shown).

3.7. 14-3-3 proteins do not distinguish between leucine and tyrosine residues within the SxP motif of the RGS domain

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 RGS5 contains a unique point substitution where leucine 167 takes the place of tyrosine in the SYP motif within the hypothesized 14-3-3 binding region. Using site-directed mutagenesis, we constructed the reciprocal RGS protein mutants RGS5 L167Y and RGS16 Y167L, and used them to examine whether this substitution could account for the lack of inhibition of RGS5 GAP activity by 14-3-3. In the steady state GTP hydrolysis assay, purified RGS5 L167Y and RGS16 Y167L proteins both retained full or nearly full GAP activities and exhibited similar potencies relative to their wild-type counterparts (Fig. 10). 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 either isoform of 14-3-3 (Fig. 11). Thus, the tyrosine/leucine





C

	-logEC$_{50}$(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

Fig. 10. RGS5 L167Y and RGS16 Y167L mutants have similar GAP activities to wild-type RGS proteins. Membranes derived from Sf9 cells coexpressing the M2 muscarinic acetylcholine receptor plus heterotrimeric Go were assayed with the agonist carbachol (100 M) at the concentrations of RGS proteins indicated on the abscissae. Non-specific GTPase signal was defined as that observed in the presence of the inverse agonist tropicamide (10 M) but without RGS protein, and this was subtracted out to yield the values indicated. The data shown represent mean values±S.E.M. from triplicate measurements from either two independent experiments with RGS5 and RGS5 L167Y (A) or three independent experiments with RGS16 and RGS16 Y167L (B). The averaged data were fitted by non-linear regression to a single sigmoidal function with a variable slope factor (GraphPad Prism) to yield the lines shown in (A) and (B) as well as the numbers indicated in the table (C). The errors shown were generated during the fitting procedure and provide an estimate of the uncertainty of the fitted parameters.

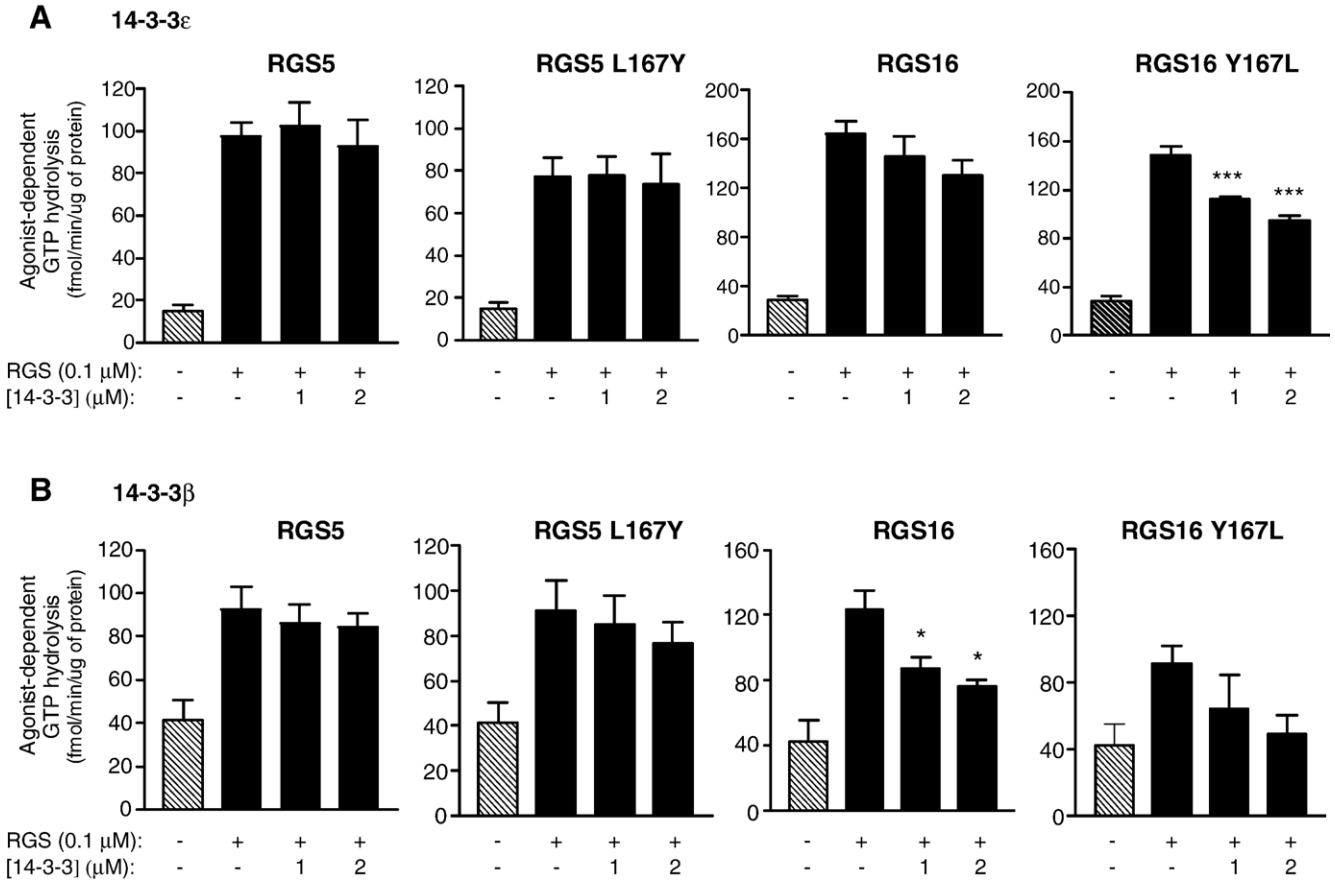


Fig. 11. Effects of 14-3-3 on GAP activities of RGS5 WT, RGS5 L167Y, RGS16 WT and RGS16 Y167L. Membranes derived from Sf9 cells coexpressing the M2 muscarinic acetylcholine receptor plus heterotrimeric Go were assayed with the agonist carbachol (100 μM) either alone or in the presence of RGS proteins with or without 14-3-3ε (A) or 14-3-3β (B) as indicated. Non-specific signal was defined as that observed in the presence of the inverse agonist tropicamide (10 μM) but without RGS or 14-3-3, and this was subtracted out to yield the values indicated. Each assay was carried out in triplicate and data represent means±S.E.M. of 3–4 independent experiments. 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.

substitution within the SxP motif does not account for the functional difference observed between RGS5 and RGS16 in the presence of 14-3-3.

4. Discussion

In the present study, we have identified novel protein–protein interactions between members of the B/R4 subfamily of RGS proteins (RGS4, RGS5, RGS16) and two 14-3-3 isoforms, 14-3-3β and 14-3-3ε (Figs. 1 and 2). Notably, such interactions were detectable in solution-based pull-down assays using 14-3-3 and RGS proteins that had been expressed in and purified from *E. coli*, thus indicating that the association between these proteins is direct and moreover does not require any additional cellular components or processes (Figs. 3 and 4). In at least some cases, this binding has functional consequences and in our hands, these 14-3-3 proteins inhibit the GAP activity of RGS4 and RGS16, with little or no effect on RGS5 activity (Fig. 6). Moreover, 14-3-3ε appears to compete with Gαo for RGS4, but not for RGS5 (Fig. 8), and correspondingly in living cells, 14-3-3ε inhibits the recruitment of RGS4 to the plasma membrane by Gαi2 (Fig. 9). These data imply that 14-3-3 might selectively impede RGS proteins from interacting with Gα, and it follows that 14-3-3 has the potential to prolong or enhance particular G protein-mediated signals. The reasons underlying the inability of 14-3-3 to inhibit RGS5 GAP activity or block its binding to Gα are unclear, but one possibility considered was that this could reflect the unique Y→L substitution in the conserved SYP motif in RGS5. However, this hypothesis could not be substantiated, as reciprocal point substitutions between RGS5 and RGS16 did not affect the sensitivity of either protein to 14-3-3 in the steady state GTP hydrolysis assay (Fig. 11).

The impetus for the current study was our original identification in a yeast 2-hybrid screen of a mouse brain cDNA library, of 14-3-3ε as a novel RGS4 binding partner. The present results confirm that this interaction exists at the protein–protein level and our study would appear to be the first to report that RGS4 interacts with 14-3-3β and 14-3-3ε. Indeed, these findings differ from those of Benzing et al. [43,44], who observed binding of 14-3-3τ to RGS3 and RGS7 but not to wild-type RGS4 in HEK293 cell lysates. It is possible that their experimental conditions were not sufficiently sensitive to detect 14-3-3 binding to RGS4 or that 14-3-3τ differs from the isoforms tested here in its RGS selectivity. However, different 14-3-3 isoforms are typically found to overlap

with respect to their substrate selectivities [59,60] and to date, there is no clear evidence for the binding of a particular RGS protein to one 14-3-3 isoform, but not to another. In addition to the interactions identified in our laboratory (i.e., the binding of RGS4, RGS5, RGS16 to 14-3-3β and 14-3-3ε) and by Benzing et al. (i.e., the binding of RGS3 and RGS7 to 14-3-3τ), Niu et al. [45] demonstrated that transiently expressed RGS3 interacts with endogenous 14-3-3 in CHO cells, Ward and Milligan [46] have shown RGS3 and RGS16 to be binding partners of 14-3-3τ and 14-3-3ζ, and Garzon et al. [47] found that 14-3-3 co-immunoprecipitates with RGS9-2 in solubilized extracts of mouse brain. The latter finding implies that 14-3-3 and RGS proteins can bind to one another in vivo, an interpretation that is further supported by the observed co-immunoprecipitation of 14-3-3 and RGS7 in mouse brain extracts [44].

The binding of 14-3-3 to RGS proteins is expected to limit RGS GAP effects on G proteins [3], and the present results show that this can indeed occur. Similarly, in one previous study, the inhibition of RGS7 GAP activity by 14-3-3τ was observed in a single turnover GTPase assay [43]. In contrast to these findings, Ward and Milligan [46] were unable to detect inhibition of RGS (RGS3, RGS7, RGS16) GAP activity by 14-3-3 proteins in steady state GTP hydrolysis assays using membranes from HEK293 cells expressing an α2A-adrenergic receptor-Gαo1 fusion protein, despite the fact that those authors clearly showed binding between RGS proteins (RGS3, RGS16) and 14-3-3τ.

While the study of Ward and Milligan [46] seemingly calls into question the ability of 14-3-3 to inhibit RGS GAP effects on GPCR-activated G proteins, our data clearly show that 14-3-3 proteins inhibit the GAP activities of RGS4 and RGS16. It is possible that the discrepancy between these two studies reflects differences in the model systems used. Unlike our method, the study by Ward and Milligan [46] employed GPCR-G protein fusion proteins, and it is conceivable 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. A further potential complication is that 14-3-3 can bind to the third intracellular loop of the α2-adrenergic receptor [25] used in that study, which consequently may affect interactions between 14-3-3 and RGS proteins. Other differences exist between the present study and that of Ward and Milligan [46], including the use of pertussis toxin and the identities of the cell lines, expression systems, RGS proteins, and 14-3-3 isoforms tested. Apart from their effects on GTPase activity, it has been shown that RGS proteins in some systems can inhibit G protein signalling by other as yet undefined mechanisms [3], and it follows that 14-3-3 may regulate these GTPase-independent processes as well. Overall it appears that interactions between RGS proteins and their binding partners can be complex, and thus the factors determining whether the binding of 14-3-3 to an RGS protein will inhibit its effects on G protein signalling, clearly require further study.

Since the binding of 14-3-3 to RGS proteins can impede their effects on G proteins, it follows that signals that are negatively regulated by RGS proteins might be enhanced by 14-3-3. Consistent with this possibility, 14-3-3τ was found to abolish the inhibitory effect of RGS3 on the carbachol-mediated activation of MAP kinase in HEK293 cells [43]. Subsequently, Schreiber and co-workers [42] observed that 14-3-3τ suppressed the cystic fibrosis transmembrane conductance regulator (CFTR), specifically 14-3-3τ inhibited the ability of RGS3 to block the negative effects of Gαi2 on CFTR. Finally, the fast RGS7-mediated deactivation kinetics of G protein-coupled inwardly rectifying K^+ channels (GIRKs) were slowed by 14-3-3τ [44].

Overall, the simplest plausible mechanism for the observed inhibitory effects of 14-3-3 on RGS activities is that 14-3-3 acts as a molecular chelator and thus prevents RGS proteins from interacting with their target G proteins, thereby prolonging or enhancing GPCR signalling. Previous reports have 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 may bind predominantly to one or the other [44,45]. Data from the present study are the first to show direct competition between purified 14-3-3 and Gα for an RGS protein (Fig. 8), and such competition would appear to underlie the ability of 14-3-3ε to inhibit both RGS4 GAP activity (Fig. 6) and Gα-dependent localization of RGS4 to the plasma membrane (Fig. 9). In contrast, RGS5 binding to Gαo appeared insensitive to 14-3-3ε (Fig. 8), consistent with the observed lack of effect of 14-3-3 on RGS5 GAP activity (Figs. 6 and 11). Thus, there may be selectivity regarding the effects of 14-3-3 on RGS protein function, and it follows that RGS–14-3-3 binding does not necessarily imply inhibition of RGS GAP activity. Although it remains to be tested explicitly, 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.

One factor that has been postulated to regulate the ability of 14-3-3 to distinguish between different RGS proteins is the phosphorylation state of the latter [46]. 14-3-3 generally binds with greater affinity to phosphorylated target proteins, however there are conflicting data regarding the ability of 14-3-3 to distinguish between phosphorylated and non-phosphorylated RGS proteins. On the one hand, some findings suggest that phosphorylation of the RGS protein may be required for 14-3-3 binding. In the case of RGS3 and RGS7, 14-3-3 binding to these 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 [43,44]. On the other hand, the present results and others [46] indicate that the 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 [46]. 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. Notwithstanding the uncertainty regarding the role of RGS phosphorylation or the lack thereof, it is 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 [43]. In fact, this motif appears to be conserved in half of all mammalian RGS protein 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 [51]. 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 [43,44]. 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 [46]. 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. Thus, it seems that the 14-3-3 sensitive phosphorylation of serine 434 may be unique to RGS7 and that the phosphorylation of other sites on different RGS proteins might alter its association with 14-3-3 proteins.

It still remains unclear whether the conserved SYP motif is the primary 14-3-3 binding site on most RGS proteins, and the present results neither confirm nor refute this notion. Another putative 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 [45]. Similarly, Ward and Milligan [46] concluded 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 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 [17,61,62].

5. Conclusions

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.

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