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Cellular Signalling 18 (2006) 336 – 348
CELLULAR SIGNALLING
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RGS2 interacts with Gs and adenylyl cyclase in living cells

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Received 15 April 2005; accepted 6 May 2005
Available online 10 August 2005

Abstract

Regulator of G Protein Signalling (RGS) proteins impede heterotrimeric G protein signalling. RGS2 decreases cAMP production and appears to interact with both adenylyl cyclase (AC) and its stimulatory G protein Gs. We showed previously that Green Fluorescent Protein-tagged RGS2 (GFP-RGS2) localizes to the nucleus in HEK 293 cells and is recruited to the plasma membrane when co-expressed with Gsα, or the Gs-coupled β_2-adrenergic receptor (β_2AR). Here, using confocal microscopy we show that co-expression of various AC isoforms (ACI, ACII, ACV, ACVI) also leads to GFP-RGS2 recruitment to the plasma membrane. Bioluminescence Resonance Energy Transfer (BRET) was also used to examine physical interactions between RGS2 and components of the Gs-signalling pathway. A BRET signal was detected between fusion constructs of RGS2-Renilla luciferase (energy donor) and Gsα-GFP (energy acceptor) co-expressed in HEK 293 cells. BRET was also observed between GFP-RGS2 and ACII or ACVI fused to *Renilla* luciferase. Additionally, RGS2 was found to interact with the β_2AR. Purified RGS2 selectively bound to the third intracellular loop of the β_2AR in GST pulldown assays, and a BRET signal was observed between GFP-RGS2 and β_2AR fused to *Renilla* luciferase when these two proteins were co-expressed together with either ACIV or ACVI. This interaction was below the limit of detection in the absence of co-expressed AC, suggesting that the effector enzyme stabilized or promoted binding between the receptor and the RGS protein inside the cell. Taken together, these results suggest the possibility that RGS2 might bind to a receptor-G protein-effector signalling complex to regulate Gs-dependent cAMP production.
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Keywords: Regulator of G protein Signalling 2 (RGS2); Adenylyl cyclase; β_2-adrenergic receptor; Gs; Bioluminescence Resonance Energy Transfer (BRET)

1. Introduction

G protein-coupled receptors (GPCRs) activate heterotrimeric G proteins by promoting the dissociation of bound GDP and the association of the activating nucleotide GTP. Both the Gα subunit and the Gβγ dimer of the activated G protein heterotrimer are able to interact with effectors in the plasma membrane, and signalling is terminated by intrinsic hydrolysis of GTP by the Gα subunit. RGS proteins are GTPase-activating proteins (GAPs) that increase the rate of hydrolysis of GTP by Gα subunits. Approximately 20 different RGS proteins, not including splice variants and "RGS-like" proteins, have been identified. However, selectivity for different G protein subfamilies is limited in that most RGS proteins interact with the Gi family of G proteins, with a subset also capable of interacting with Gq [1,2].

Although RGS proteins do not seem to act as GAPs for Gs [3,4], there is evidence for RGS protein/Gs interactions.

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doi:10.1016/j.cellsig.2005.05.004

We have recently demonstrated that RGS2 is recruited from the nucleus to the plasma membrane of HEK 293 cells upon co-expression of Gsα [4]. RGS2 has also been shown to attenuate Gs-stimulated increases in intracellular cAMP levels [4–8]. The C1 domain of AC type V binds purified RGS2, providing a possible mechanism for the inhibitory effects of RGS2 on intracellular cAMP accumulation [6]. However, other evidence suggests that these inhibitory effects may reflect physical interactions between RGS2 and Gs, since RGS2 binds to Gs-GDP in a fluoroaluminate-sensitive manner [9], and similarly has been co-immunoprecipitated with Gs from cells upon the activation of a Gs-coupled receptor [8]. Signalling via the β_2-adrenergic receptor (β_2AR) is also dependent upon the activation of Gs and adenylyl cyclase, and we have recently demonstrated that co-expression of the β_2AR also facilitates GFP-RGS2 recruitment to the plasma membrane of HEK 293 cells [4].

GPCR signalling has historically been described as the result of random collisions between interacting proteins occurring at or near the plasma membrane. The use of in vivo interaction assays based on bioluminescence or fluorescence resonance energy transfer (BRET/FRET) has altered this perception. BRET has been used to screen for physical interactions between GPCRs, G protein subunits and their effectors. A number of studies have demonstrated that interactions between the above mentioned proteins are stable, and persist during signal transduction (reviewed in [10]). For example, the β_2AR has been demonstrated to form stable homo-[11] and heterodimers (with both β_1AR and β_3AR) [12,13], and to stably interact with its effector enzyme adenylyl cyclase [14]. Taken together, these data suggest the possible existence of stable multicomponent Gs-coupled signalling complexes localized to the plasma membrane.

We have used BRET to determine whether direct interactions between RGS2 and Gs, adenylyl cyclase or the β_2AR occur in living cells. We show that RGS2 interacts with both Gsα and several adenylyl cyclase isoforms at the plasma membrane of HEK 293 cells, to regulate and limit Gs-mediated signalling.

2. Materials and methods

2.1. DNA constructs

The construction of pEGFP-C3-mRGS2 has been described by us previously [4]. pcDNA3-β_2AR-EGFP was a gift from J. Benovic (Thomas Jefferson University). pGL3-Basic encoding firefly luciferase was purchased from Promega. pEBG2 encoding Glutathione-S-Transferase (GST), Flag-β_2AR and pRLuc-N2 were generously donated by S. Ferguson (Robarts Research Institute). pcDNA3-mRGS2 was generously donated by D. Siderovski (University of North Carolina). Human Gsα-Long expression vector was purchased from the Guthrie cDNA resource (Sayre, PA). A GFP-Gsα construct was generously donated by M.Rasenick (University of Chicago) [15]. cDNAs encoding bovine brain adenylyl cyclase type I, Flag-rat adenylyl cyclase type II, canine adenylyl cyclase type V, and Flag-rat adenylyl cyclase type VI were generous gifts from R. Feldman (Robarts Research Institute). Plasmids coding for rat type II adenylyl cyclase and β_2AR each with a *R. reniformus* luciferase (Rluc) tag have been described previously [14]. pBluescript (SK)-adenylyl cyclase type IV and pBluescript (KS)-adenylyl cyclase type III were donated by R.V. Rebois (NIH). cDNA for human Gαq-Q209L-G188S was a generous gift from Paul Albert (University of Ottawa, Ottawa, ON, Canada). The Gly-Ser (GS) mutant of hGsαL was constructed using the Stratagene QuikChange site-directed mutagenesis protocol. All constructs were confirmed by DNA sequencing.

Adenylyl cyclase type IV was amplified from pBS(SK) construct with primers encoding *Bam*HI and *Xho*I using High Fidelity (Pfu) Taq Polymerase (Invitrogen), while adenylyl cyclase type III was amplified from pBS (KS) with primers encoding *Eco*RI and *Xho*I, and both products were inserted into the corresponding sites of pcDNA3.1. RGS2 was digested from pEGFP-C3-mRGS2 with *Xho*I and *Sma*I and inserted into pEBG2 at *Sal*I and *Sma*I. Luc-RGS2 was constructed by amplification of RGS2 from pcDNA3-mRGS2. RGS2 was subcloned with RLuc into pcDNA3.1 to express RGS2 tagged at its C-terminus with RLuc. Adenylyl cyclase type I, type III and type IV were amplified from the corresponding constructs available and subcloned with RLuc into pcDNA3.1. RLuc was fused to the COOH-terminus of each adenylyl cyclase isoform (AC-RLuc). Adenylyl cyclase type VI was amplified from the above mentioned construct and subcloned with firefly luciferase into pcDNA3.1. Adenylyl cyclase type VI was also amplified and inserted into the *Hin*dIII site of pRLuc-N2 (Perkin Elmer).

2.2. Cell culture and transfection

HEK-293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, 2 mM L-glutamine in a humidified incubator at 37 °C and 5% CO_2. For transient transfection for BRET assays, cells were grown to 60–80% confluency. cDNA constructs were transfected into cells using LipofectAMINE2000 and Opti-MEM I. The transfection protocol was optimized as recommended by the supplier (Invitrogen). BRET assays were performed 48 h after transfection. For all other experiments, HEK-293 cells were transfected in 10-ml dishes using calcium phosphate precipitation. Cells were replated with fresh medium approximately 18 h after transfection and assayed on the following day. Protein expression was verified by immunoblotting. Within experiments the total concentration of DNA was kept constant by

adding vector pCDNA3.1 or pCDNA3.1-luciferase. Sham transfections were also performed with vectors alone.

2.3. Induction and purification of GST fusion proteins

Plasmids encoding GST-β2i3 [16], GST-M1i3[17], GST-M2i3 [17] or GST alone were transformed into *E. coli* BL21(DE3). For each protein, one-liter cultures were grown in LB/carbenicillin and induced with 500 μM IPTG for 2 h at 37 °C. Cells were pelleted and suspended in 25 ml harvest buffer (10 mM HEPES pH 8, 50 mM NaCl, 5 mM EDTA, 0.5% Triton-X100) supplemented with Complete Mini protease inhibitor cocktail (Roche Diagnostics GmBH) and lysozyme. The pellets were then frozen at −80 °C overnight. Samples were thawed and sonicated, followed by centrifugation at 15,000 ×*g*, 4 °C to collect soluble material. Streptomycin sulphate (30 mg) was added to precipitate remaining nucleic acid material, and the samples were centrifuged an additional time to produce the soluble bacterial lysate. Lysates were incubated with glutathione–Sepharose 4B beads (Amersham) 1 h at 4 °C, rotating end-over-end to bind fusion proteins. Protein-bead complexes were washed 3× with harvest buffer, and stored as a slurry in harvest buffer at −80 °C.

The concentration of GST protein bound to the glutathione resin varied among GST fusion proteins. For each protein used, a single common batch of protein/beads was generated and aliquoted for freezing and storage at −80 °C. Direct quantitation of total bound protein by Bradford assay was unsuccessful due to non-specific interaction of the resin with Bradford reagent. Therefore, the concentration of protein bound was determined by comparison of Coomassie staining intensity of GST fusion proteins against varying amounts of a known protein standard (BSA). For experiments, the volume of beads used for each protein was adjusted to ensure that the same amount of total protein was used for binding interactions. Approximately 2.5 μg of GST fusion protein was used in each pull-down.

2.4. Purified protein pull-down assays

Purified RGS2-His or RGS16-His proteins [17] (0.25 μg) were mixed with GST or GST-i3 bound to glutathione-sepharose beads in pull-down buffer (10 mM HEPES pH 8, 80 mM NaCl, 5 mM EDTA, 0.5% Triton-X100, 30 mM imidazole, 80 mM NaCl). Equal amounts of GST fusion proteins were added as determined by Coomassie staining. The total reaction volume was 250 μl. Reactions were incubated by rotating overnight at 4 °C. Beads were collected by centrifugation at 500 ×*g* for 5 min at 4 °C and washed once with harvest buffer, followed by one wash with harvest buffer minus TritonX-100. Bound proteins were eluted from the beads by the addition of 2X SDS-sample buffer. Bound RGS proteins were detected by immunoblot.

For immunoblots, nitrocellulose membranes were incubated in blocking buffer (Tris buffered-saline with 5% milk, 0.5% Tween-20, 0.02% sodium azide) for 1 h at room temperature (RT) or overnight at 4 °C. Membranes were probed with mouse anti-His (Qiagen) antibodies diluted 1:1000 in blocking buffer for 1–2 h at RT. Membranes were washed 3× with Tris-buffered saline + 0.1% Tween-20, and then probed with horseradish peroxidase (HRP)-conjugated goat anti-mouse (Rockland) diluted 1:20 000 in Tris-buffered saline + 0.1% Tween-20. Protein bands were visualized using chemiluminescence and exposed to film.

2.5. Co-precipitation assays

Prior to harvesting, HEK 293 cells transfected with GST-RGS2 and potential interacting proteins were washed three times with ice-cold phosphate-buffered saline (PBS; pH 7.2) and subsequently collected by scraping in 10 ml of ice-cold PBS and spun at 1000 g for 5 min. Pellets were resuspended in 500 μl lysis buffer (50 Mm Tris–HCl, pH 7.6, 1 mM EDTA, pH 8.0, 0.2 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin plus 0.4 M NaCl, 1% TritonX-100, and 10% glycerol) and incubated at 4 °C for 20 min. Next, the cells were sonicated on ice (3 × 30 s bursts, maximum intensity). The homogenate was spun for 30 min at 100,000 ×*g* and the resulting supernatant, containing GST-RGS2 and other soluble proteins (including proteins solubilized from the plasma membrane), was incubated with glutathione-Sepharose 4B at 4 °C with gentle rocking overnight. The beads were pelleted and washed three times in lysis buffer.

For determination of bound proteins by Western analysis the beads were heated for 5 min at 100 °C in 75 μl of SDS loading buffer, spun quickly at 14,000 ×*g,* and separated by gel electrophoresis using 12% Tris glycine polyacrylamide gels, followed by transfer to polyvinyl difluoride (PVDF) membranes. For immunological detection of material, nonspecific binding to PVDF membranes was blocked by 1 h treatment with 10% nonfat dry milk powder in Tris-buffered saline with 0.05% Tween 20 (TBS-T), followed by overnight incubation with primary antibody at 4 °C (titre 1:500). After incubation with the primary antibody, membranes were washed extensively in TBS-T, and secondary antibody detection was performed using standard protocols using peroxidase-coupled antisera and enhanced chemiluminescence.

For determination of the luciferase activity of proteins bound to the glutathione Sepharose, pelleted beads were resuspended in glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0) and incubated for 10 min at room temp. Competitive elution with reduced glutathione was repeated twice to obtain a total of 150 μL glutathione eluate. Both the immunoprecipitable and non-precipitable material were assayed for luciferase activity (Promega Dual Luciferase assay kit) with a Dynex MLX Microplate Luminometer.

2.6. cAMP assays

Briefly, cells in 12-well tissue culture plates were incubated at 37 °C for 2.5 min in serum-free media containing 0.5 mM 3-isobutyl-1-methylxanthine in the absence or presence of 10 μM isoproterenol or 100 μM forskolin. Cells were assayed for basal and agonist-stimulated levels of cAMP as described previously [4].

2.7. Confocal microscopy

Intracellular localization of GFP-RGS2 was visualized in living HEK293 cells. Microscopy was performed using a Zeiss LSM 410 confocal microscope equipped with a Krypton/Argon laser. EGFP fluorescence was examined using a fluorescein isothiocyanate filter under a 63x oil immersion lens. For each experimental condition, fluorescence distribution patterns similar to the images shown in Figs. 1 and 7 were observed in the majority (60–90%) of cells inspected.

2.8. BRET

Forty-eight hours post-transfection, HEK-293 cells were washed twice in PBS, detached with PBS, and resuspended in PBS containing 0.1% glucose (w/v) and 10^{-4} M ascorbic acid and the protease inhibitor mixture (5 μg/ml leupeptin, 10 μg/ml benzamidine, 5 μg/ml soybean trypsin inhibitor). The cell suspension was assayed for protein concentration using the Bio-Rad protein assay with bovine serum albumin as a standard. Cells were then distributed in 96-well microplates (white Optiplate from Packard Bioscience) at a density of ~100,000 cells (50–100 μg of protein) per well. The expression of enhanced GFP was quantified by assaying fluorescence (excitation filter 475/20 nm, emission filter 515/30 nm) with a Packard Fusion instrument.

The BRET technology uses coelenterazine H (Molecular Probes), which after being oxidized by Renilla luciferase, emits light at a wavelength of 470 nm. GFP is excited by the light released from luciferase and re-emits fluorescence at 515 nm. The BRET signal generated is calculated by the ratio of light emitted by the GFP partner over the light emitted by the RLuc partner. The assay was started by the addition of coelenterazine H to a final concentration of 1 μM. BRET was assessed in these experiments by measuring the light emitted by RLuc-and GFP-fusion proteins using 450/58 and ≥480 nm filters, respectively. Whether or not BRET occurred was determined by calculating the ratio of the light passed by the ≥480 filter to that passed by the 450/58 filter. This ratio is referred to as the BRET ratio.

BRET saturation experiments were performed by increasing the [cDNA] for the GFP-tagged partner transfected while keeping the [cDNA] for the RLuc-tagged partner constant. GFP-fusion protein expression was determined by quantifying total fluorescence (excitation filter 475/20 nm, emission filter 515/30 nm), which was measured as relative fluorescence units (RFU). Upon addition of coelenterazine H, total RLuc-fusion protein expression was determined by the light passed by the 450/58 filter, and was measured in relative luciferase units (RLU). The resulting BRET ratio was also simultaneously measured. For each increase in GFP-fusion protein expression, RFU was calculated as a fraction of RLU to correct for differences in RLuc-fusion protein expression. The RFU/RLU ratio was plotted against the resulting BRET ratios to obtain hyperbolic curves. Nonlinear regression was performed by fitting the resulting curves to the equation for a rectangular hyperbola with GraphPad Prism 4. $BRET_{max}$ and $BRET_{50}$ values reported are the fitted values and standard deviations reported are indicated by the fitting procedure.

3. Results

3.1. Co-expression of adenylyl cyclase alters the intracellular localization of RGS2

GFP-RGS2 was expressed in HEK 293 cells and as expected demonstrated a predominantly nuclear localization [4]. In contrast, when GFP-RGS2 was co-expressed with either adenylyl cyclase type I, II, V or VI, GFP-RGS2 was recruited to the plasma membrane compartment in the

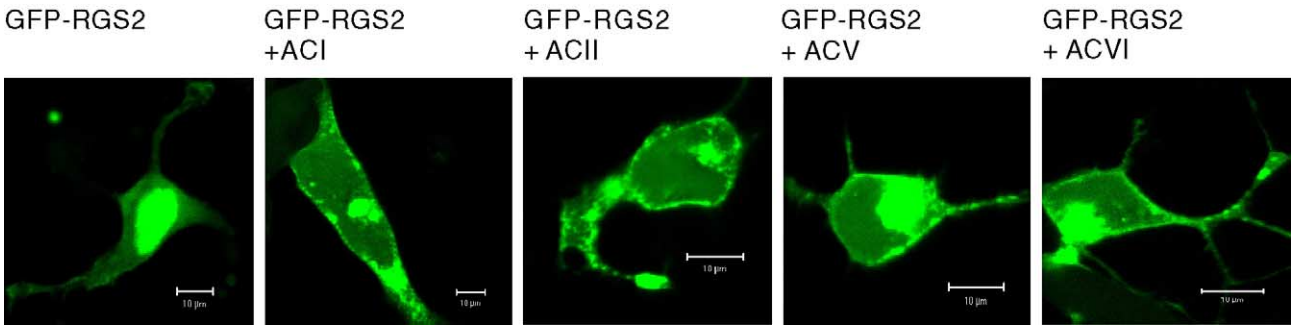

Fig. 1. Effects of Adenylyl Cyclase Expression on the Intracellular Localization of GFP-RGS2. HEK 293 cells were transiently transfected with 10 μg of GFP-RGS2 plus 10 μg of control vector or different adenylyl cyclase isoforms as indicated in Experimental Procedures. Images shown are representative of at least 100 living cells for each condition tested.

majority of cells examined (Fig. 1). One possible explanation for the recruitment of GFP-RGS2 to the plasma membrane by adenylyl cyclase is that the two proteins interact. Further experiments thus were directed towards examining this possibility, focusing on direct physical interactions between RGS2 and adenylyl cyclase types II and VI.

3.2. RGS2 interacts with adenylyl cyclase in cellular extracts

Flag-tagged adenylyl cyclase type II (Flag-ACII) was expressed either alone, or co-expressed with GST-RGS2 or with GST. Cell lysates were incubated with glutathione beads, and associated Flag-ACII was detected in cells expressing GST-RGS2 and not GST, indicating either a direct physical interaction between these two proteins or co-purification as part of a complex. Based on comparisons between immunoblots of cell lysates (data not shown) and protein recovered from glutathione beads (Fig. 2a), it is estimated that approximately 1% of the total Flag-ACII bound to GST-RGS2 in these experiments.

In a similar series of experiments, GST-RGS2 was co-expressed with adenylyl cyclase type VI fused to firefly luciferase at its C-terminus (ACVI-FLuc). The eluate from glutathione-conjugated beads that were incubated overnight with lysates from cells transfected with GST-RGS2 and ACVI-FLuc was measured for luciferase activity (Fig. 2b). The luciferase activity in these samples was clearly elevated above control levels, indicating that ACVI-FLuc bound to GST-RGS2. Despite the specific binding of ACVI-FLuc to GST-RGS2, the luciferase activity of the precipitates of the glutathione beads was less than 10% of the total luciferase activity measured from the cellular lysates. This weak interaction between GST-RGS2 and adenylyl cyclase may be due to the absence of other proteins required for formation of more stable complexes between RGS2 and adenylyl cyclase, a low affinity of GST-RGS2 for adenylyl cyclase, or the disruption of the RGS2-adenylyl cyclase complex by detergents required for extraction from the plasma membrane.

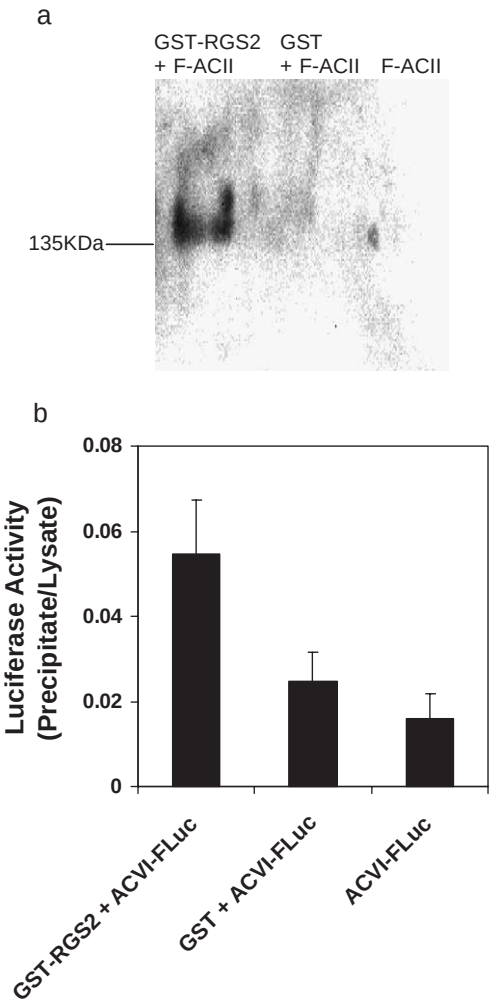

Fig. 2. Co-Precipitation of GST-RGS2 and Adenylyl Cyclase Types II and VI. (a) HEK 293 cells were co-transfected with NH_2-terminal FLAG-AC type II (8 μg) plus either NH_2-terminal GST-RGS2 (8 μg), GST, or empty vector (pcDNA3.1+). Protein precipitates eluted from glutathione-Sepharose beads were separated by SDS-PAGE and membranes were probed with Anti-Flag antibody. The figure is representative of at least three separate experiments. (b) HEK 293 cells were co-transfected with COOH-terminal firefly luciferase–conjugated ACVI (ACVI-Fluc) (8 μg) and either NH2-terminal GST-RGS2 (8 μg), GST, or empty vector (pcDNA3.1+). Luciferase activity of both the precipitates and remaining cellular lysates was calculated as a fraction of total protein in the measured samples. The amount of precipitated ACVI-FLuc activity was then calculated as a fraction of ACVI-FLuc activity remaining in solution to account for differences in ACVI-FLuc expression. All data shown are representative of four independent experiments.

3.3. BRET provides evidence for direct association between RGS2 and adenylyl cyclase in living cells

The BRET technique was employed here to determine if a stable protein/protein interaction occurs between RGS2 and various adenylyl cyclase isoforms. To study the interaction between two proteins of interest, one protein is fused to *Renilla* luciferase (as an energy donor) and the other is fused to a fluorescent protein, such as GFP (as an energy acceptor). BRET occurs if the distance between donor (RLuc) and acceptor (GFP) is less than 100 Å, indicating that the two proteins are directly associated with each other or with a minimum of common interacting partners between them [18]. In contrast, the lack of a BRET signal can indicate that either the two proteins do not interact, or that the two proteins interact in such a way that distance or orientation between the energy donor and acceptor is unfavourable for generation of a positive BRET signal (e.g., they may be part of a larger complex detectable by co-purification).

To investigate the binding of RGS2 to adenylyl cyclase in living cells, we co-expressed GFP-RGS2 with different adenylyl cyclase subtypes, tagged at their C-termini with *Renilla* luciferase (AC-Rluc) and measured resulting BRET

Table 1
Activity of adenylyl cyclase-*Renilla* luciferase constructs

cDNA	Intracellular ^{3}H cAMP/total intracellular ^{3} H(×1000)
Gsα	8.6±3.8
Gsα+ACI-RLuc	97.7±4.6
Gsα+ACIII-RLuc	56.8±2.4
Gsα+ACIV-RLuc	27.6±1.3
Gsα+ACVI-RLuc	38.2±2.9

HEK 293 cells were co-transfected with 5 μg of Gsα plus 10 μg of COOH-terminal *Renilla* luciferase–adenylyl cyclase types I, III, IV, and VI (AC-RLuc) or empty vector (pcDNA3.1+). Cells were stimulated with 100 μM forskolin for 2.5 min and cAMP was measured as indicated under Experimental Procedures. ^{3}H-cAMP (dpm) was measured as a fraction of total ^{3}H uptake (dpm). cAMP production in non-forskolin treated, control-transfected cells (pcDNA3.1+) was subtracted from each condition as background cAMP. Activities of AC-RLuc constructs were analysed by paired *t*-tests. The signal from the cells transfected with AC-RLuc plus Gsα differed significantly from cells transfected with Gsα alone ($p<0.05$).

ratios. For meaningful conclusions to be drawn from the BRET technique, the tagged partners must retain their function and thus the activity of the various AC-RLuc fusion proteins was first verified. The activity of adenylyl cyclase type II (ACII-Rluc) has been reported previously [14]. Here, we tested the activity of adenylyl cyclase type I (ACI-RLuc), adenylyl cyclase type III (ACIII-RLuc), adenylyl cyclase type IV (ACIV-RLuc), and adenylyl cyclase type VI (ACVI-RLuc) by co-expressing these constructs with Gsα and measuring their ability to generate cAMP (Table 1). Each of the AC-RLuc constructs tested demonstrated increased cAMP accumulation in response to forskolin treatment compared to when Gs was expressed alone.

The interactions between GFP-RGS2 and various AC-RLuc constructs were then examined using BRET (Fig. 3). Consistent with the results shown in Fig. 2, a BRET signal was observed between GFP-RGS2 and either ACII-RLuc or ACVI-RLuc (Fig. 3), suggesting that RGS2 and these adenylyl cyclase isoforms are directly associated with one another in living cells. We also examined the effect of Gsα expression on RGS2-AC interactions. When Gsα was co-expressed with GFP-RGS2 and the different AC-RLuc isoforms, a significant BRET ratio again was observed with ACII and ACVI, and additionally with ACIII (Fig. 3). In contrast to these results, no interaction could be detected with BRET between GFP-RGS2 and ACI-RLuc or ACIV-RLuc (Fig. 3), whether or not Gsα was present, or with ACIII when Gαs was not co-transfected (Fig. 3).

To establish comparative BRET values for a known interaction, we used GFP-and luciferase-tagged forms of the β_2AR. It has been previously demonstrated that the β_2AR forms oligomers in vivo as measured by BRET that occurs in cells co-expressing β_2AR-YFP and β_2AR-RLuc [11]. We therefore used co-expression of β_2AR-GFP and β_2AR-RLuc as a positive BRET control for our experiments. In contrast, to establish control values for two non-interacting proteins, we used the RLuc fusion proteins with soluble GFP. The BRET ratio was the same for cells co-expressing soluble GFP and either AC-RLuc or β_2AR-RLuc fusion proteins, or simply the RLuc fusion proteins alone, confirming the specificity of the assay and showing that BRET did not occur between donor and acceptor if the proteins did not associate physically (Fig. 3).

To further characterize the specificity of the interaction between GFP-RGS2 and AC-RLuc, BRET saturation experiments were also carried out. According to Kenworthy and Edidin [19] and Mercier et al. [20], if two proteins specifically interact to form a stable complex, then the BRET signal will be sensitive to the ratio of the donor (AC-RLuc) to acceptor (GFP-RGS2). Indeed, as GFP-RGS2 expression was increased, against the background of a constant ACII-RLuc expression level, the BRET increased asymptotically (Fig. 4a). Furthermore, when the amount of plasmid encoding ACII-RLuc was increased, the maximal BRET of the resulting saturation curve decreased (1.0 μg

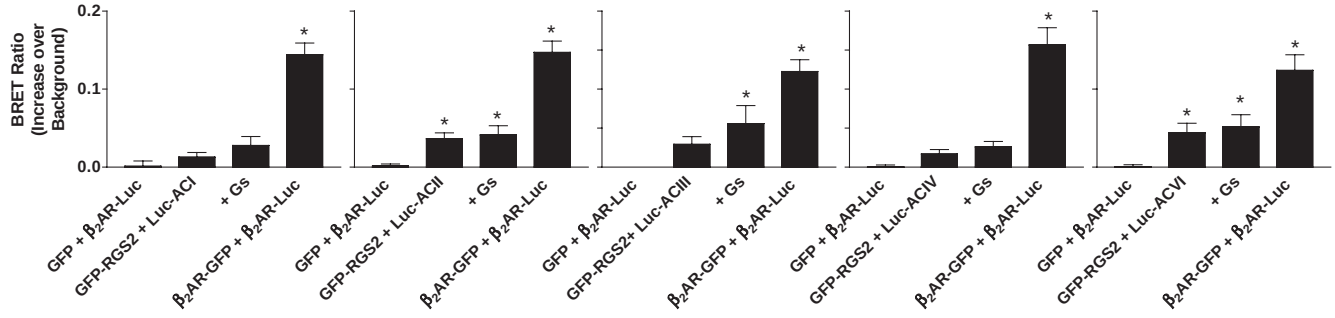

Fig. 3. Monitoring RGS2 Interactions with Adenylyl Cyclase in vivo using BRET. HEK 293 cells were transiently transfected with 2 μg of GFP-RGS2 and 2 μg AC-RLuc (±1 μg Gsα or control vector). Approximately 48 h post-transfection, cells were assayed for BRET as indicated under Experimental Procedures. Plotted BRET ratios are from cells expressing both GFP-RGS2 and AC-RLuc minus the BRET ratio from cells expressing AC-RLuc alone (background). β_2AR have been shown to form homo-oligomers by BRET, thus cells expressing β_2AR-RLuc and β_2AR-GFP were included in experiments as a positive control. Negative controls for each series of experiments were cells expressing β_2AR-RLuc and soluble GFP. Values are expressed as the mean±SEM calculated from more than six independent experiments. Results from each individual experiment were compared by repeated-measures analysis of variance followed by Dunnett's multiple comparisons test to determine if the BRET signal for each condition differed significantly ($p<0.05$) from cells transfected with soluble GFP plus β_2AR-RLuc (negative control). Asterisks denote statistical difference between the indicated conditions and β_2AR-RLuc and GFP alone ($p<0.05$).

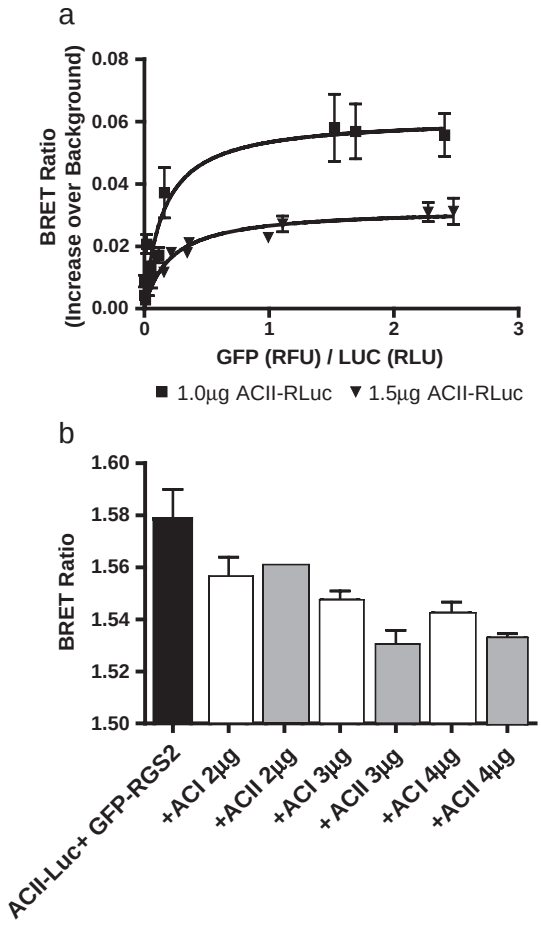

Fig. 4. Specificity of BRET Interactions between RGS2 and Adenylyl Cyclase. (a) Representative BRET Saturation Curves. BRET was measured in living HEK-293T cells co-expressing GFP-RGS2 and ACII-Rluc. Co-transfections were performed with increasing amounts of plasmid DNA for the GFP-RGS2 construct, whereas the ACII-Rluc construct was kept constant. Total plasmid DNA was kept constant with empty vector (pcDNA3.1+). Relative fluorescence unit (RFU) measurements represent increasing expression levels of GFP-RGS2. All samples were also subjected to luminescence analysis and RFU values were plotted as a fraction of relative luciferase units (RLU) to control for changes in adenylyl cyclase expression. All values are expressed as the mean ± SEM calculated from five independent experiments. (b) HEK 293 cells were transiently transfected with 2 μg of GFP-RGS2 and 1 μg ACII-RLuc plus up to 4 μg of untagged AC (or control vector or both). Plotted BRET ratios are from cells expressing both GFP-RGS2 and ACII-RLuc. Values are expressed as the mean ± SD calculated from 4 independent experiments.

ACII-RLuc, $BRET_{max}=0.061\pm0.005$ vs. 1.5 μg ACII-RLuc, $BRET_{max}=0.032\pm0.002$) demonstrating that the BRET signal was sensitive to the donor (ACII-RLuc) concentration and suggesting an increased signal to noise ratio at the lower expression level of ACII-RLuc. However, the relative affinities between RGS2 and ACII remained constant ($BRET_{50}=0.15\pm0.05$ and $BRET_{50}=0.21\pm0.04$, respectively). These results indicate that the interaction between GFP-RGS2 and ACII-Rluc is specific, presumably due to the direct binding of RGS2 to adenylyl cyclase.

Although GFP-RGS2 is recruited to the plasma membrane upon co-expression with ACI, GFP-RGS2 did not interact with ACI-RLuc in the BRET assay. This suggests the possibility that the two proteins are in close proximity to one another but that the donor and acceptor motifs may not be properly aligned between GFP-RGS2 and ACI-RLuc to facilitate resonance energy transfer. To test for this possibility, competition assays were performed to test the binding of ACI to GFP-RGS2. As demonstrated in Fig. 4b, co-expression of increasing concentrations of ACI inhibited the BRET between GFP-RGS2 and ACII-RLuc, suggesting that ACI competes with ACII-Rluc for binding to GFP-RGS2. Untagged ACII was also used as a positive control, to demonstrate that the inhibition of BRET involves competition for specific binding to RGS2.

Taken together, the BRET results presented in Figs. 3 and 4 strongly suggest that RGS2 binds to ACII and ACVI, and furthermore indicate that RGS2 may also interact with ACI and ACIII as well.

3.4. Intracellular interaction between RGS2 and Gs revealed by BRET

We have previously established that GFP-RGS2 is recruited to the plasma membrane upon co-expression of Gsα [4]. Others have also demonstrated that RGS2 protein can be immunoprecipitated with purified Gsα using anti-Gsα antibodies [8], and that purified Gsα specifically binds to purified GST-RGS2 [9]. We therefore used BRET to determine whether RGS2 and Gs directly associate with each other in living cells. BRET was observed between GFP-Gsα and RGS2-RLuc (Fig. 5a). A basal interaction between GFP-Gsα and RGS2-RLuc was detectable suggesting that they may be constitutively associated. Further, co-expression of untagged adenylyl cyclase isoforms did not compete against the BRET between Gs-GFP and RGS2-RLuc suggesting that the sites of interaction between RGS2 and AC and RGS2 and Gs do not overlap.

The specificity of the interaction between GFP-Gs and RGS2-RLuc was also confirmed using BRET saturation experiments with increasing expression levels of GFP-Gs (Fig. 5b). Here, when the amount of RGS2-RLuc was increased, the maximal BRET again decreased (0.5 μg RGS2-RLuc, $BRET_{max}=0.035\pm0.005$ vs. 1.0 μg RGS2-RLuc, $BRET_{max}=0.027\pm0.002$), while the relative affinity remained constant ($BRET_{50}=0.36\pm0.15$ and $BRET_{50}=0.34\pm0.19$, respectively). These results imply that the interaction between RGS2-RLuc and Gs-GFP is also dependent upon donor (RGS2-RLuc) concentration, and is therefore specific.

3.5. Effect of Gs and Gq on the intracellular localization of GFP-RGS2

We have previously found that GFP-RGS2 is recruited to the plasma membrane compartment of HEK 293 cells upon co-expression of either Gsα or Gqα [4]. In contrast, GFP-RGS2 did not associate with the plasma membrane upon co-

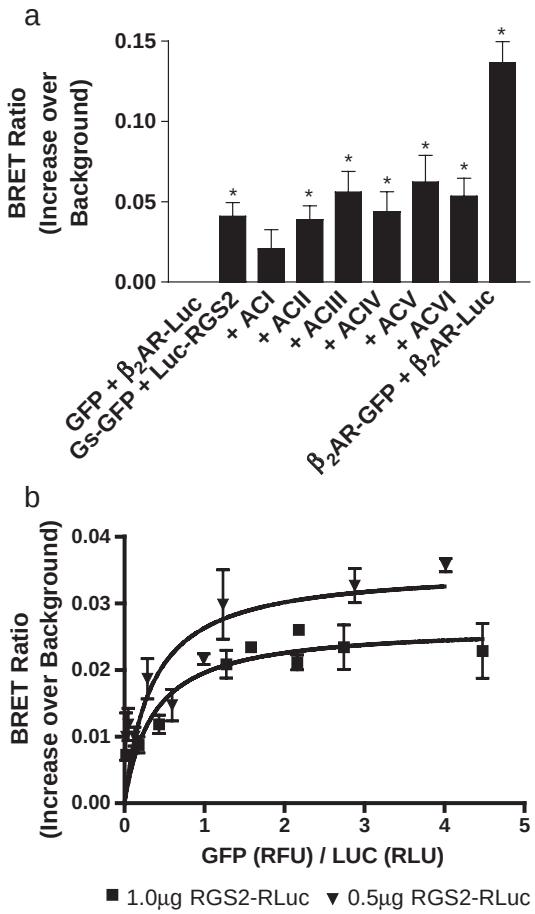

Fig. 5. Monitoring RGS2 Interactions with Gs in vivo using BRET. (a) HEK 293 cells were transiently transfected with 2 μg of GFP-Gs and 2 μg RGS2-RLuc (±1 μg AC or control vector). Approximately 48 h post-transfection, cells were assayed for BRET. Plotted BRET ratios are from cells expressing both GFP-Gs and RGS2-RLuc minus the BRET ratio from cells expressing RGS2-RLuc alone (background). Values are expressed as the mean±SEM calculated from five independent experiments. Results from each individual experiment were compared by repeated-measures analysis of variance followed by Dunnett's multiple comparisons test to determine if the BRET signal for each condition differed significantly ($p<0.05$) from cells transfected with soluble GFP plus β_2AR-RLuc (negative control). Asterisks denote statistical difference between the indicated conditions and β_2AR-RLuc and GFP alone ($p<0.05$). (b) BRET was measured in cells co-expressing GFP-Gsα and RGS2-RLuc. Co-transfections were performed with increasing amounts of plasmid DNA for the GFP-RGS2 construct, whereas the ACII-Rluc construct was kept constant. Total plasmid DNA was kept constant with empty vector (pcDNA3.1+). RFU represents increasing expression levels of GFP-Gs. All samples were also subjected to luminescence analysis and RFU values were plotted as a fraction of RLU to control for changes in RGS2 expression. All values are expressed as the mean±SEM calculated from four independent experiments.

expression with a constitutively activated Gqα mutant containing an additional point substitution that substantially reduces RGS protein affinity (Gαq-Q209L/G188S; Fig. 6) [4], suggesting that sustained localization of GFP-RGS2 at the plasma membrane involves direct binding to Gqα. In the present study, we tested an analogous double point mutant of Gsα (Gsα-Q227L/G209S) for its effects on the intracellular localization of GFP-RGS2 (Fig. 6). In contrast to the corresponding Gαq mutant, Gsα-Q227L/G209S retained its ability to recruit GFP-RGS2 to the plasma membrane. This points to a distinct mechanism for the inhibition of Gs signalling by RGS2 compared to its inhibition of Gq signalling, and suggests the possibility that RGS proteins may bind to Gsα at an interface that is not analogous to those identified on Gqα and Giα.

3.6. Evidence for an interaction between the β_2-adrenergic receptor and RGS2

We have recently demonstrated that GFP-RGS2 can also be recruited to the plasma membrane upon co-expression of the β_2AR [4]. Neither agonists nor inverse agonists altered receptor-promoted RGS2 association with the plasma membrane, implying that the interaction between GFP-RGS2 with the β_2AR signalling complex is relatively stable and insensitive to the activation state of either Gs or the receptor. BRET was again employed here, to determine if the effect of β_2AR-dependent recruitment of RGS2 to the plasma membrane is due to a direct association between these two proteins at the membrane. No specific BRET signal was detected when β_2AR-GFP was co-expressed with RGS2-RLuc (data not shown), nor when GFP-RGS2 was co-expressed with β_2AR-RLuc (Fig. 7a). The co-expression of either Gsα or various adenylyl cyclase isoforms tended to increase the BRET between GFP-RGS2 and β_2AR-RLuc above control values, and this tendency was statistically significant for adenylyl cyclase types IV and VI (Fig. 7a). These data raise the possibility that weak interactions (e.g., detectable in the context of a larger signalling complex) may exist between RGS2 and the β_2AR, which might be stabilized or enhanced by the presence of Gs or adenylyl cyclase, or alternatively that the binding of these proteins to RGS2 and/or the receptor results in conformational changes which favour a BRET signal.

To distinguish among these possibilities, we used GST fusion proteins of receptor third intracellular loops in an in vitro pull-down assay. We have previously demonstrated that RGS2 selectively binds to the third intracellular (i3) loop of the Gq-coupled M1 muscarinic cholinergic receptor (M1-AchR) (M1i3) [17]. This interaction was selective since the closely related protein RGS16 did not bind M1i3, and neither RGS2 nor RGS16 bound to the G(i/o)-coupled M2i3 loop [17]. Fig. 7b shows strong binding of purified His-tagged RGS2 to the i3 loops of β_2AR and M1-AchR (positive control), but no detectable binding of RGS2 to the M2-ACh i3 loop (negative control) or GST alone. These data suggest that in vitro, RGS2 can selectively and directly interact with the β_2AR, which further supports our hypothesis that RGS2 and the β_2AR may interact directly with one another in vivo and that these interactions may be modulated by other members of a signalling complex (Fig. 7b).

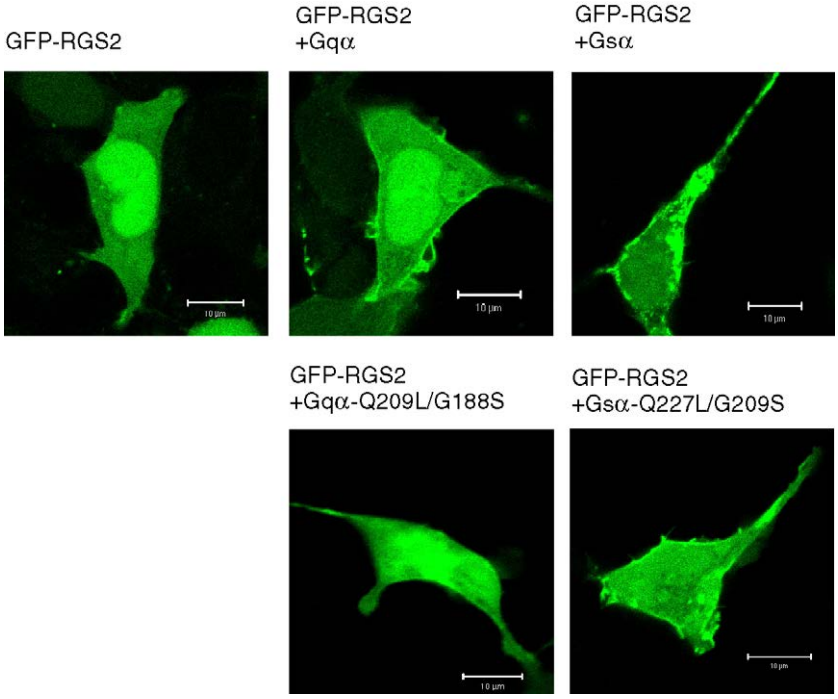

Fig. 6. Effect of Gs and Gq on the Intracellular Localization of GFP-RGS2. HEK 293 cells were transfected with 10 μg of GFP-RGS2 plus 10 μg of control vector or G protein mutants as indicated in Experimental Procedures. Acquired confocal images shown are representative of at least 100 living cells for each condition tested.

3.7. Functional significance of the interaction between RGS2 and the Gs-signalling complex

In a further attempt to characterize the selectivity of interactions between RGS2 and other proteins involved in the Gs-signalling complex, cAMP accumulation assays were performed. RGS2 was able to inhibit the basal accumulation of intracellular cAMP in cells transfected with Gsα plus various adenylyl cyclase isoforms (Fig. 8a). The different adenylyl cyclase isoforms tested displayed varying levels of basal activity, and were therefore normalized to determine if RGS2 produced proportionally different effects among the various isoforms. RGS2 reduced the production of intracellular cAMP by 20–50% for each of the conditions tested, clearly demonstrating that there is inhibition of cAMP production via Gs and adenylyl cyclase.

To address the question of whether the inhibitory effect of RGS2 on cAMP accumulation in HEK 293 cells possibly involves multiple proteins in the Gs-signalling pathway, we tested the ability of RGS2 to inhibit receptor-promoted cAMP production. Indeed, a 20% inhibition of isoproterenol-stimulated intracellular cAMP in cells transfected with the β_2AR was observed (Fig. 8b), demonstrating that RGS2 inhibits agonist-activated Gs-coupled signalling, in addition to basal activity of the Gs-signalling complex.

Overall, these results show that RGS2 inhibits the Gs-mediated activation of multiple isoforms of adenylyl cyclase. However, it remains difficult to distinguish whether this inhibition arises principally from an effect on the G protein, the effector or both.

4. Discussion

We sought to determine which potential binding partners might be responsible for plasma membrane recruitment of GFP-RGS2 by components of the β-adrenergic signalling system. GFP-RGS2 was recruited from the nucleus to the plasma membrane upon co-expression of all adenylyl cyclase subtypes tested. To investigate the possibility that RGS2 might interact with adenylyl cyclase, HEK 293 cells were co-transfected with GFP-RGS2 and various adenylyl cyclase isoforms. Our results indicate that RGS2 binds to at least some isoforms of adenylyl cyclase. The clearest picture emerged with ACII and ACVI, as each of these recruited RGS2 to the plasma membrane, yielded a BRET signal with RGS2, and co-purified with RGS2. Additionally, ACVI appeared to promote an interaction between RGS2 and the β_2AR. Other adenylyl cyclase isoforms, including ACI, ACIII, ACIV and ACV, all yielded data suggesting binding to RGS2 in some but not all assays.

To further study the possible mechanisms underlying this intracellular relocalization, BRET was used to screen for binding partners for RGS2 among proteins within the Gs signalling pathway. Our results suggest that the recruitment of RGS2 to the plasma membrane by Gsα and adenylyl cyclase may reflect its direct binding to either or both of those proteins. Whether or not this occurs in situ at the plasma membrane or during trafficking of the various signalling partners following biosynthesis remains unclear. Although we could not detect a constitutive interaction between RGS2 and the β_2AR, co-expression of some forms

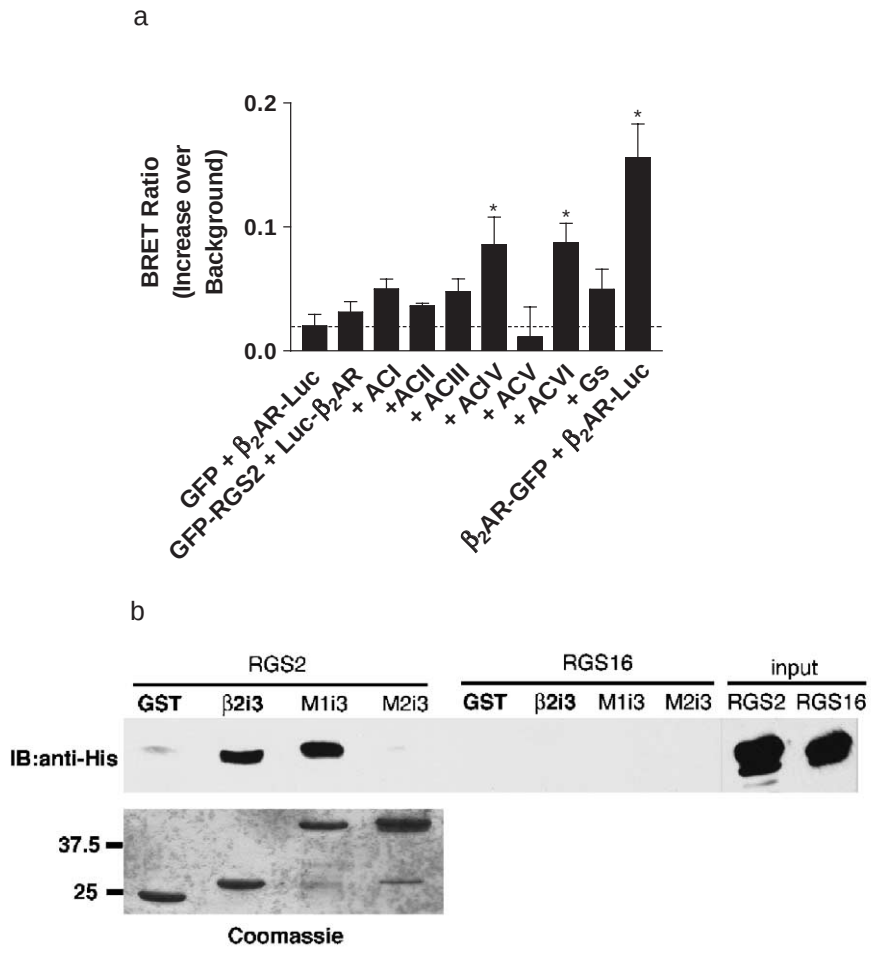

Fig. 7. Monitoring RGS2 Interactions with β_2AR. (a) HEK 293 cells were transiently transfected with 2 μg of GFP-RGS2 and 2 μg Luc-β_2AR (±1 μg AC or control vector). Approximately 48 h post-transfection, cells were assayed for BRET. Plotted BRET ratios are from cells expressing GFP-RGS2 and Luc-β_2AR minus the BRET ratio from cells expressing Luc-β_2AR alone (background). Values are expressed as the mean±SEM calculated from four independent experiments. Results from each individual experiment were compared by repeated-measures analysis of variance followed by Dunnett's multiple comparisons test to determine if the BRET signal for each condition differed significantly ($p<0.05$) from cells transfected with soluble GFP plus β_2AR-RLuc (negative control). Asterisks denote statistical difference between the indicated conditions and β_2AR-RLuc and GFP alone ($p<0.05$). (b) Purified RGS2-His (top panel, left) or RGS16-His (top panel, middle), 0.25 μg, were incubated with 2.5 μg of GST-i3 or GST alone bound to glutathione-Sepharose (bottom panel, Coomassie). Protein loads are shown in top panel, right. Following centrifugation, recovered beads were examined for bound RGS proteins by immunoblot using anti-His antibody. Bottom panel indicates relative amounts of each protein used in the experiment.

of adenylyl cyclase did facilitate a BRET signal between the receptor and the RGS protein, suggesting the existence of weak interactions in vivo that may be promoted by adenylyl cyclase. A series of weak or competing interactions at multiple sites between multiple partners within a stable signalling complex would allow a great deal of conformational flexibility while preserving a certain specificity. Individual interactions might be broken and reformed as required while the entire complex remained relatively stable. A combination of unique and overlapping binding sites between different partners would allow a metastable complex to have many different conformations depending on the arrangements of these individual interactions [21]. Measures of the affinity for these individual interactions may be quite different when compared in vivo and in vitro. This interpretation is further supported by our results demonstrating the selective and direct binding of RGS2 to the third intracellular (i3) loop of the β_2AR in vitro. These latter interactions appear to be of much greater affinity then detected with the intact proteins expressed in living cells where multiple competing interactions might be expected within a stable signalling complex.

BRET has been used previously to detect protein–protein interactions between GPCR monomers within receptor dimers [11,22], GPCRs and effectors [23,14], GPCRs and G proteins [24] and between GPCRs and proteins involved in receptor desensitization [25,26]. To our knowledge, no prior BRET analysis of RGS protein interactions has been performed, although FRET has been employed to study interactions between $G\beta_5$ and the Gγ subunit-like domains of RGS7 and RGS11 [27,28]. Recently, FRET was used to demonstrate that stable physical interactions can be observed between either RGS7 or RGS8 and Goα and between RGS8 and Gγ2

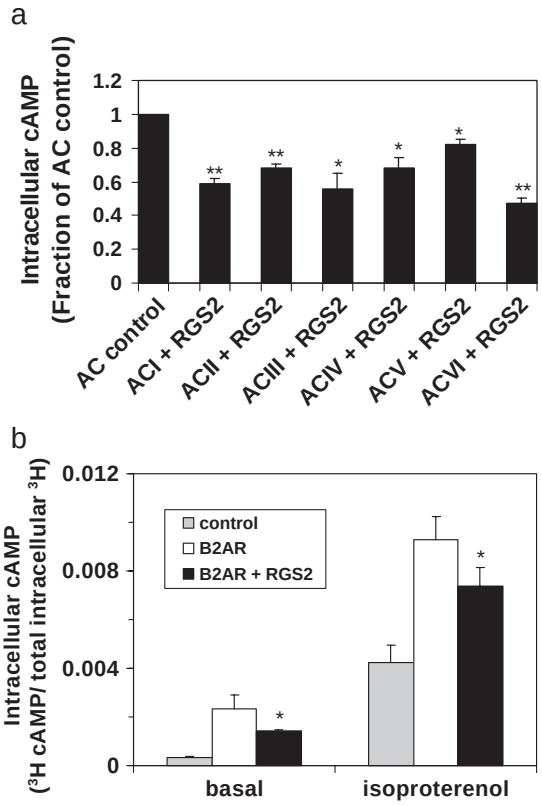

Fig. 8. Effects of RGS2 on cAMP Production. (a) HEK 293 cells were transiently transfected with 5 μg of Gsα and 8 μg of the indicated isoform of adenylyl cyclase plus 8 μg of RGS2 (or control vector). For each isoform of adenylyl cyclase tested, activity in the absence of RGS2 was normalized to 100% and activity in the presence of RGS2 was calculated as a fraction of control ($n \geq 4$ for each isoform of adenylyl cyclase tested). Effects of RGS2 on adenylyl cyclase activity were analysed by paired *t*-tests. The signal from the RGS2-transfected cells differed significantly from cells transfected with Gsα and adenylyl cyclase only. Double asterisks indicate $p < 0.01$, asterisk indicates $p < 0.05$. (b) HEK 293 cells were transiently transfected with 8 μg of β_2AR plus 8 μg of RGS2 (or control vector). Cells were stimulated with 10 μM isoproterenol (or control buffer), and recovered cAMP was calculated as a fraction of total intracellular ^{3}H. Effects of RGS2 on cAMP accumulation was analysed by paired *t*-tests. The signal from the RGS2-transfected cells differed significantly from cells transfected with β_2AR only. Asterisk indicates $p < 0.05$.

[29]. Our study provides novel evidence characterising the interaction between an RGS protein and different members of a G protein signalling cascade. Other groups have demonstrated that purified protein extracts of Gsα directly bind to RGS2 in co-immunoprecipitation assays [8,9]. However, in this study we have been able to demonstrate that RGS2 binds to Gsα in living cells, confirming that RGS2 directly interacts with Gsα using the BRET technique.

Several studies have shown that RGS2 can impede the formation of cAMP by adenylyl cyclase [4–8,30], however the precise mechanism via which this inhibition occurs has yet to be identified. Inhibition is atypical in that it does not appear to involve a GAP effect on Gs, since increased GTPase activity due to RGS2 has not been observed with either free Gsα-GTP [3] or receptor-activated heterotrimeric Gs [4]. The recruitment of GFP-RGS2 to the plasma membrane by Gsα-Q227L/G209S in the present study suggests that the Gsα-RGS2 binding interface may also be atypical, since analogous double Gly–Ser (GS) mutations in Gαi and Gαq fail to recruit RGS2 and RGS4 to the plasma membrane [4] and have greatly reduced RGS affinity [31]. The idea of an alternate protein–protein interface is consistent with evidence that "typical" RGS protein binding to the switch regions of Gsα is blocked due to the presence of an Asp residue at a key contact point (where a conserved Ser is found in RGS-sensitive G proteins) [32]. Perhaps in an analogous fashion, the atypical binding of the RGS-like domain of GRK2 to Gqα is also characterized by a poor GAP effect [33].

The protein–protein interactions identified in the present study extend and confirm previous observations by ourselves and others, and point to a possible mechanism of signalling inhibition. Consistent with the recruitment of GFP-RGS2 to the plasma membrane by ACV (Fig. 1) and the inhibition of cAMP accumulation in cells co-transfected with this AC isoform plus RGS2 (Fig. 8a), Kehrl and co-workers have shown that RGS2 binds to the C_1 domain of ACV [6] and inhibits the production of cAMP by this domain in concert with the ACV C_2 domain in solution [5]. Similar to our findings, these authors showed inhibition by RGS2 of ACIII and ACVI activities (in whole cells and membranes, respectively) [5], and here we further show inhibition of ACI and ACII. In addition, our BRET results indicate that RGS2 can bind directly to Gsα, ACII and ACVI, and that the G protein does not block RGS2-AC interactions, and in at least one case (ACIII) seems to enhance it. Overall, the composite results suggest that RGS2 binds to both Gsα and at least some subtypes of AC to attenuate the ability of the activated G protein to modulate AC, thereby decreasing cAMP production. This inhibition appears to be independent of any change in G protein GTPase activity, and thus may be steric or conformational in nature.

Increasing evidence shows that RGS proteins can interact directly with GPCRs (reviewed in [34]). In a previous study, we reported that the β_2AR recruits GFP-RGS2 to the plasma membrane in HEK-293 cells in a manner independent of the activation state of the receptor (or that of Gs) [4]. More recently, we showed that RGS2 binds to the third intracellular loop of the M1 muscarinic receptor [17]. This latter finding led us to investigate whether RGS2 might bind directly to the β_2AR as well, a possibility borne out by the results shown in Fig. 7. We had difficulty in measuring BRET between GFP-RGS2 and β_2AR-Luc (possibly due to an unusually high background signal in these experiments), however a clear signal between these two proteins did emerge in the presence of either ACIV or ACVI. The co-expression of an untagged protein that associates with both the energy donor and energy acceptor could increase the resulting BRET by

stabilizing or promoting the formation of a multiprotein complex, and such an effect may account for the increased signal between β_2AR-Rluc and GFP-RGS2 in the presence of adenylyl cyclase. This interpretation is reinforced by our previous finding that the β_2AR interacts physically with adenylyl cyclase II [14]. Apart from a postulated scaffolding effect [35], the functional significance of RGS protein binding to GPCRs is unclear. RGS2 does not appear to affect the ability of the β_2AR to promote nucleotide exchange on Gs, since it neither increased nor decreased steady-state GTPase activity in either the presence or absence of the β_2AR agonist isoproterenol [4].

The apparent ability of RGS2 to bind to Gsα, adenylyl cyclase and β_2AR in the present study suggests the possibility that RGS proteins may regulate the activity of receptor-G protein-effector complexes. There is now an extensive literature supporting the existence of such complexes [10]), as well as mounting evidence that RGS proteins can bind to many of their components. For example, RGS4 has been demonstrated to directly bind to Gβγ and to effector proteins such as phospholipase C-β1 [36] or heteromeric Kir3.1/Kir3.4 channels [37]. Similarly, RGS12 has been shown to form a regulated complex with the α_{1B} subunit of the G protein-regulated N-type calcium channel [38]. The present results would appear to suggest that RGS proteins can inhibit signalling without necessarily causing complexes to dissociate, although it is not clear whether this idea can be generalized to other combinations of receptor, G protein and effector.

Previous investigators have postulated that the inhibition of Gs-stimulated cAMP production occurs through a direct RGS effect on either the G protein [8,9] or the effector protein [5,6], or possibly via a novel mechanism [32]. In contrast to the mechanism proposed by Kehrl and Sinnarajah [39], we did not detect any obvious correlation among the AC subtypes tested with respect to inhibition of cAMP production by RGS2 versus the tendency to interact physically with RGS2. While we cannot rule out the possibility that direct effects of RGS2 on both Gsα and AC contributed to the observed decreases in intracellular cAMP, the simplest explanation may be that RGS2 primarily causes this inhibition by binding to both proteins simultaneously.

In comparing the BRET between GFP-RGS2 and AC-RLuc, RGS2 seems not to interact identically with the various isoforms of adenylyl cyclase tested. Furthermore, there seems to be some selectivity for which isoforms of adenylyl cyclase increase the interaction between RGS2 and the β_2AR. Due to differences in the protein stability, protein expression, transfection efficiencies, or intrinsic activity of the different adenylyl cyclase isoforms, it is difficult to directly compare the interaction between GFP-RGS2 and the different AC-RLuc tested in these experiments. However, further studies examining the selectivity of RGS2 interactions with different adenylyl cyclase isoforms and GPCRs would be of interest.

This study is a first step towards examining the interactions between RGS2 and the Gs-signalling pathway in living cells. RGS2 is a predominantly nuclear protein that is recruited to the plasma membrane when co-expressed with the β_2AR, Gsα or adenylyl cyclase. It would be useful to know what the precise requirements are for this, and what percentage of RGS2 in the cell binds to Gs or adenylyl cyclase at the plasma membrane under different conditions. These data would further reveal the mechanism(s) by which RGS2 inhibits the Gs-signalling pathway.

Further BRET studies may also characterize the effects of associations between different Gs protein subunits, Gs-coupled receptors, and adenylyl cyclase isoforms, on the plasma membrane recruitment and binding of RGS2. In preliminary experiments, treatment of cells with βAR agonists did not increase BRET between RGS2 and either β_2AR, Gsα or adenylyl cyclase (A. Roy, unpublished). However, βAR agonist effects may also require the presence of complete signalling complexes at the plasma membrane in stoichiometric amounts. Therefore it would be of importance to determine what effects agonist activation may have on the association between RGS2 and Gsα or adenylyl cyclase after co-expression of complete heterotrimeric Gs proteins along with their cognate receptors.

5. Conclusions

Previous work has suggested that RGS2 may produce its inhibitory effects on Gs-mediated adenylyl cyclase activity via binding to Gsα or adenylyl cyclase. The present results would appear to resolve this discrepancy, as we found that RGS2 can bind to both of these proteins. Since the G protein and the effector protein did not appear to compete for binding to RGS2, it follows that RGS2 may attenuate cAMP production by binding to both of these proteins within a heteromeric signalling complex. Previous studies have shown the existence of signalling complexes containing both β_2AR and AC [40,14], and presumably the Gs heterotrimer as well [41,10], and thus it appears that RGS2 may be targeted intracellularly to such complexes.

Acknowledgements

This work was supported by grants from the Canadian Institutes of Health Research to P.C. and T.E.H. and Heart and Stroke Foundation of Quebec to T.E.H. T.E.H. is a MacDonald Scholar of the Heart and Stroke Foundation of Canada. A.A.R. is supported by the Natural Sciences and Engineering Research Council of Canada and the Heart and Stroke Foundation of Canada. A.B. holds a Government of Canada award in conjunction with the Government of Italy. We thank R.V. Rebois for helpful discussions, and Hong Ming for technical assistance.

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