Biochemical characterization of RGS14: RGS14 activity towards G-protein α subunits is independent of its binding to Rap2A

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RGS (regulators of G-protein signalling) modulate signalling by acting as GAPs (GTPase-activating proteins) for α subunits of heterotrimeric G-proteins. RGS14 accelerates GTP hydrolysis by $G_{i\alpha}$ family members through its RGS domain and suppresses guanine nucleotide dissociation from $G_{i\alpha1}$ and $G_{i\alpha3}$ subunits through its C-terminal GoLoco domain. Additionally, RGS14 binds the activated forms of the small GTPases Rap1 and Rap2 by virtue of tandem RBDs (Raf-like Ras/Rap binding domains). RGS14 was identified in a screen for Rap2 effectors [Traver, Spingard, Gaudriault and De Gunzburg (2004) *Biochem. J.* 379, 627–632]. In the present study, we tested whether Rap binding regulates RGS14's biochemical activities. We found that RGS14 activity towards heterotrimeric G-proteins, as either a GAP or a GDI (guanine nucleotide dissociation inhibitor), was unaffected by Rap

binding. Extending our biochemical characterization of RGS14, we also examined whether RGS14 can suppress guanine nucleotide exchange on $G_{i\alpha1}$ in the context of the heterotrimer. We found that a heterotrimer composed of N-myristoylated $G_{i\alpha1}$ and prenylated $G_{\beta\gamma}$ is resistant to the GDI activity of the GoLoco domain of RGS14. This is consistent with models of GoLoco domain action on free G_{α} and suggests that RGS14 alone cannot induce subunit dissociation to promote receptor-independent activation of $G_{\beta\gamma}$ -mediated signalling pathways.

Key words: G-protein α subunit, GTPase-activating protein, guanine nucleotide dissociation inhibitor (GDI), lipid modification, Rap2A, regulators of G-protein signalling 14 (RGS14).

INTRODUCTION

Heterotrimeric G-proteins convey signals from heptahelical cell surface receptors to intracellular effectors [1]. Activated receptors act as GEFs (guanine nucleotide-exchange factors), facilitating the exchange of GDP for GTP on G_{α} . Binding of GTP induces a conformational change in G_{α} , dissociating it from $G_{\beta\gamma}$ and freeing both subunits to interact with effectors. Hydrolysis of GTP to GDP by G_{α} returns the protein to its inactive conformation, which promotes its association with $G_{\beta\gamma}$ subunits. RGS (regulators of G-protein signalling) constitute a large family of proteins that modulate the intensity and duration of cellular responses to G-protein activation [2]. Discovered as inhibitors of G-protein signalling, RGS proteins act as GAPs (GTPase-activating proteins) for G_{α} subunits. A protein domain known as the RGS box harbours GAP activity and is the defining feature of RGS proteins. Outside the RGS box, the presence of other protein domains diversifies the range of protein interactions and regulatory activities of RGS proteins. Studies of the RhoA GEF, p115 RhoGEF (p115), established a paradigm for integration of signalling pathways regulated by heterotrimeric G-proteins with those regulated by Rho and Ras family GTPases [3,4]. p115 is an effector of $G_{13\alpha}$. Binding of $G_{13\alpha}$ to p115 stimulates its GEF activity, resulting in RhoA activation and cytoskeletal rearrangements. The RGS-like domain of p115 acts as a GAP on $G_{13\alpha}$ to terminate signalling. Accordingly, p115 can activate small G-protein signalling while simultaneously down-regulating heterotrimeric G-protein signalling [3,4].

Like p115, RGS14 contains multiple domains that give it the capacity to couple heterotrimeric and monomeric G-protein pathways [5]. In addition to its N-terminal RGS domain, RGS14 also contains a C-terminal GoLoco domain and central tandem RBDs

(Raf-like Ras/Rap binding domains). The RGS domain confers GAP activity for $G_{\alpha\alpha}$ and $G_{i\alpha}$ [5–7]. The GoLoco domain of RGS14 has been shown to function as a GDI (guanine nucleotide dissociation inhibitor) for $G_{i\alpha1}$ and $G_{i\alpha3}$ [8,9]. The RBDs bind the small GTPases Rap1 and Rap2, but not Ras [5]. This interaction was dependent on the activation state of Rap2, and mutations in the putative Rap effector domains abolished the RGS14–Rap2 yeast two-hybrid interaction [5,10], suggesting that RGS14 might be a Rap effector. The presence of three distinct domains allows for the possibility that RGS14 interacts with both heterotrimeric and monomeric G-proteins simultaneously.

Rap proteins are members of the Ras subgroup of monomeric GTPases that regulate cellular proliferation, differentiation, and survival. Rap1 was originally identified as an antagonist of Ras transformation [11], consistent with its ability to bind to C-Raf1 but not to activate it [12]. However, Rap1 can also activate the MAPK (mitogen-activated protein kinase) cascade in a cell-type-specific fashion in mammalian cells [13] and mediates Ras-independent MAPK activation by the receptor tyrosine kinase Torso in *Drosophila* [14]. Rap1 has been well characterized as a regulator of cell adhesion [15]. Its effectors include adaptors that link Rap1 with cell junction proteins or integrins, as well as GEFs and GAPs for monomeric GTPases that regulate actin dynamics. Although Rap2 can also regulate cell adhesion, its function is less well characterized [15]. Several candidate effectors specific for Rap2 have been identified, including MAPK kinase kinase 4 [16] and Traf2- and Nck-interacting kinase [17]. A role for Rap2 in the regulation of the Wnt signalling pathway has recently been reported [18]. RGS14 is expressed in similar tissues to Rap2 and the proteins display a similar *in situ* hybridization pattern in brain slices, lending credence to the hypothesis that

Abbreviations used: AGS, activator of G-protein signalling; DTT, dithiothreitol; ERK, extracellular-signal-regulated kinase; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide-exchange factor; GTP[S], guanosine 5'-[γ -thio]triphosphate; IPTG, isopropyl β -D-thiogalactoside; MAPK, mitogen-activated protein kinase; MBP, maltose-binding protein; myr- $G_{i\alpha1}$, N-myristoylated $G_{i\alpha1}$; nOG, octyl β -D-glucopyranoside; Ni-NTA, Ni²⁺-nitrilotriacetate; RBD, Raf-like Ras/Rap binding domain; RGS, regulators of G-protein signalling; RGS14-GL, RGS14 GoLoco peptide; 2ME, 2-mercaptoethanol; WT, wild-type.

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RGS14 is a Rap2 effector [5]. Since the initial identification of RGS14 as a Rap interactor, much work has been focused on the biochemical activities of RGS14's individual protein interaction domains. However, these studies fail to address the key question of whether or not RGS14 is subject to interdomain regulation. We pursued a biochemical characterization of intact RGS14 to test the hypothesis that Rap binding to the RBDs alters the activity of the RGS and GoLoco domains towards G-protein α subunits. Here, we report that we were unable to find any evidence for interdomain regulation. RGS14 functioned as a GAP for G_{α} and a GDI for $G_{i\alpha1}$ when bound to activated Rap2A.

In the present study, we also examined the issue of whether GoLoco-domain proteins act on free versus heterotrimeric G_{α} . The GoLoco-domain protein AGS3 (activator of G-protein signalling 3) was discovered in a screen for receptor-independent activators of G-protein signalling [19]. An AGS3 peptide corresponding to the GoLoco domain interacted with free G_{α} , leading to the suggestion that the GoLoco domain induces subunit dissociation, freeing $G_{\beta\gamma}$ to activate downstream effectors [19]. The structure of $G_{i\alpha1}$ bound to the RGS14-GL (RGS14 GoLoco peptide) reveals that the GoLoco domain and $G_{\beta\gamma}$ share an overlapping binding surface [20]. Thus G_{α} cannot accommodate both the GoLoco domain and $G_{\beta\gamma}$. Several studies have shown that GoLoco-domain peptides can disrupt the heterotrimer *in vitro* [21–23]. However, the activity of GoLoco peptides on G-protein signalling in cells suggests that free G_{α} , not the heterotrimer, is the target of the GoLoco domain [23]. In the present paper, we report that when both $G_{i\alpha}$ and $G_{\beta\gamma}$ are appropriately lipid-modified, the heterotrimer cannot be disrupted by RGS14, suggesting that only free $G_{i\alpha}$ is a substrate for the GoLoco domain of RGS14 or that RGS14 requires another protein to exert its effects on the heterotrimer.

EXPERIMENTAL

Generation of plasmids for Sf9 expression

Standard molecular biology methods were followed to create all constructs. The sequence of all plasmids was verified. Wild-type and activated (G12V) mutants of Rap2A were expressed as N-terminal His₆ fusion proteins. The full-length human Rap2A sequence was PCR amplified from IMAGEclone #3706244 using primers 5'-CCCCCGGATCCGAACAAAACATCATCTCAG-AAGAGGATCTGATGCGCGAGTACAAAGTG-3' (sense) and 5'-CCCCCAAGCTTCTATTGTATGTTACATGC-3' (antisense). This yields an EcoRI (5')–BamHI (3') fragment with a Myc epitope in frame with the N-terminus of the Rap2A coding sequence. The PCR product was inserted into pBlueBacHis2A (Invitrogen) and designated Rap2A/BlueBacHis.

To generate the plasmid encoding the activated Rap2A G12V mutant, Rap2A/BlueBacHis was used as template in a Quick-Change site-directed mutagenesis (Stratagene) reaction with 5'-GGTGGTGCTGGGCTCGGTCGGGGTAGGCAAATCC-3' (sense) and 5'-GGATTGCTACCCGACCGAGCCAGCA-CCACC-3' (antisense). The resultant product was designated Rap2A(G12V)/BlueBacHis.

The plasmids were transfected into Sf9 cells using linearized viral DNA and CELLfectin reagent (Invitrogen). Recombinant Rap2A baculoviruses were recovered and plaque-purified according to the manufacturer's instructions.

Purification of Rap proteins from Sf9 cells

Sf9 cells (1.5×10^6 cells/ml) were infected with recombinant baculovirus for the expression of Rap2A and Rap2A(G12V). Cells were harvested 60 h post-infection, and pelleted by centrifugation.

Cell pellets were suspended in lysis buffer [50 mM Tris/HCl, pH 8.0, 20 mM 2ME (2-mercaptoethanol), 5 mM MgCl₂ and 10% (v/v) glycerol] supplemented with Complete Protease Inhibitors (Roche). Sf9 cells were lysed using nitrogen cavitation at 500 p.s.i. (1 p.s.i. = 6.9 kPa). Unbroken cells and particulate matter were removed by low-speed centrifugation (1000 g) for 15 min. The resulting supernatant was subjected to high-speed (100 000 g) centrifugation for 30 min to separate the particulate and cytosolic fractions. The cytosolic fraction was applied to Ni-NTA (Ni²⁺-nitrilotriacetate) resin (Qiagen) equilibrated in Column Buffer A (50 mM Tris/HCl, pH 8.0, 20 mM 2ME, 100 mM NaCl, 5 mM MgCl₂ and 10% glycerol). Unbound protein was washed with Column Buffer B (50 mM Tris/HCl, pH 8.0, 20 mM 2ME, 500 mM NaCl, 5 mM MgCl₂, 10 mM imidazole and 10% glycerol). Rap proteins were eluted using a 10–200 mM linear gradient of imidazole in Column Buffer A. Rap-containing fractions were pooled and buffer-exchanged in Q Buffer [50 mM NaHepes (sodium salt of Hepes), pH 8.0, 1 mM EDTA, 1 mM DTT (dithiothreitol) and 10% glycerol] using an Amicon pressure cell apparatus (Millipore). The protein solution was applied to a Mono Q anion exchange column (Amersham) in Q Buffer. The column was washed extensively and Rap protein was eluted using a linear salt gradient (0–500 mM NaCl in Q Buffer). Fractions containing Rap were pooled and exchanged into storage buffer (50 mM NaHepes, pH 8.0, 1 mM EDTA, 100 mM NaCl and 10% glycerol) using an Amicon pressure cell apparatus and stored at –80 °C.

Purification of recombinant RGS14

Plasmid DNA containing mouse RGS14 fused to MBP (maltose-binding protein) was kindly provided by Dr Jean De Gunzburg (Institut Curie, Paris, France). *Escherichia coli* strain BL21(DE3) harbouring MBP-RGS14 was grown to an A_{600} (absorbance) of 0.5 and induced with 0.3 mM IPTG (isopropyl β -D-thiogalactoside; Calbiochem). After 3 h, cells were harvested by low-speed centrifugation, flash frozen and stored at –80 °C. Cell pellets were suspended in TENB (20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 200 mM NaCl and 10 mM 2ME) and disrupted using a French pressure cell at 1000 p.s.i. Unbroken cells and particulate matter were removed by centrifugation at 9000 g for 30 min. The resulting supernatant was diluted 5-fold in TENB and applied to amylose resin (New England Biolabs) equilibrated in TENB. Unbound protein was removed by washing with 10 column volumes of TENB. Bound protein was eluted with 10 mM maltose in TENB. Fractions containing MBP-RGS14 were pooled and dialysed overnight at 4 °C in TED (20 mM Tris/HCl, pH 7.4, 1 mM EDTA and 1 mM DTT). Dialysed protein was applied to Q Sepharose Fast Flow resin (Amersham Biosciences) and eluted with a linear salt gradient (0–500 mM NaCl in TED). Purified MBP-RGS14 was concentrated and buffer-exchanged into 50 mM NaHepes (pH 8.0), 1 mM EDTA, 100 mM NaCl and 1 mM DTT, using an Amicon pressure cell apparatus, and stored at –80 °C.

Expression and purification of G_{α} and $G_{\beta\gamma}$

The construction of the plasmids to express His-tagged $G_{i\alpha1}$ and myristoylated $G_{i\alpha1}$ has been described in [9,24]. His-tagged G_{α} was generated by subcloning an NcoI–HindIII fragment encoding rat G_{α} (A isoform) into the vector NpT7-5 with a double-stranded oligonucleotide linker that appends a His₆ tag to the N-terminus of G_{α} [9,25]. Due to the presence of an internal NcoI site, a partial digest with NcoI was used to obtain the full-length coding sequence. His-tagged G_{α} subunits were purified as described by Lee et al. [26] with minor modifications. *E. coli* strain BL21(DE3)

harbouring G_{α} was grown to an A_{600} of 0.4 and induced with 30 μ M IPTG in the presence of 1 μ g/ml chloramphenicol for approx. 16 h. Cells were harvested by low-speed centrifugation and frozen at -80°C . Cell pellets were suspended in lysis buffer (50 mM Tris/HCl, pH 8.0, 20 mM 2ME, 0.2 mM PMSF and 10 % glycerol) and lysed using a French pressure cell at 1000 p.s.i. Unbroken cells and particulate matter were removed by high-speed (100 000 g) centrifugation for 30 min. The supernatant was applied to an Ni-NTA agarose resin (Qiagen) equilibrated in buffer A (50 mM Tris/HCl, pH 8.0, 20 mM 2ME, 0.2 mM PMSF, 100 mM NaCl, 10 mM imidazole and 10 % glycerol). The column was washed with 50 column volumes of buffer B (50 mM Tris/HCl, pH 8.0, 20 mM 2ME, 0.2 mM PMSF, 500 mM NaCl, 10 mM imidazole and 10 % glycerol) and eluted using a linear gradient of imidazole (10–200 mM) in buffer A. Fractions containing G_{α} were pooled and desalted into Q buffer (50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 2 mM DTT and 10 % glycerol) using an Amicon pressure cell apparatus. Protein was applied to Resource Q resin (Amersham Biosciences) and eluted with a linear salt gradient (0–500 mM NaCl in Q buffer). Purified G_{α} was concentrated and buffer exchanged into 50 mM NaHepes (pH 8.0), 1 mM EDTA, 2 mM DTT, 100 mM NaCl and 10 % glycerol using an Amicon pressure cell apparatus and stored at -80°C .

N-myristoylated G_{α} subunits were produced in *E. coli* BL21-(DE3) by the co-expression of G_{α} and yeast N-myristoyltransferase and purified as described in [24]. All G-protein α subunits were purified to near homogeneity and exhibited a stoichiometry of [^{35}S]GTP[S] (guanosine 5'-[γ - ^{35}S]thio)triphosphate binding ranging from 0.4 to 0.9.

Bovine brain $\beta\gamma$ subunits were a gift from Dr William Singer and Dr Paul Sternweis (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.). Recombinant $\beta 1\gamma 2\text{C}68\text{S}$ was purified from insect cells infected with recombinant $\beta 1$ and $\gamma 2\text{C}68\text{S}$ viruses [27,28].

RGS14–Rap2A complex formation

For some experiments, preformed complexes of MBP-RGS14 and Rap2A-GTP[S] were generated. Rap2A was diluted 1:2 into loading buffer {100 mM NaHepes, pH 7.2, 15 mM EDTA, 20 mM 2ME, 1 mM GTP[S] and 60 mM nOG (octyl β -D-glucopyranoside)} and incubated for 15 min at 37°C to load Rap2A with GTP[S]. One-tenth volume of 150 mM MgCl_2 was added to stop the exchange reaction. Rap2A-GTP[S] was incubated with MBP-RGS14 diluted in binding buffer (50 mM NaHepes, pH 7.2, 150 mM NaCl, 5 mM MgCl_2 , 10 mM 2ME, 20 mM imidazole and 30 mM nOG) at a 2:1 molar ratio and incubated for 45 min at 4°C . The protein mix was then applied to Ni-NTA resin in binding buffer and complexes were allowed to bind the resin for an additional 45 min at 4°C . Unbound protein was removed by washing with binding buffer. RGS14–Rap2A–GTP[S] complexes were eluted with elution buffer (50 mM NaHepes, pH 7.2, 150 mM NaCl, 5 mM MgCl_2 , 10 mM 2ME, 200 mM imidazole and 30 mM nOG). The concentration of the complex was determined by the Bradford protein assay and by SDS/PAGE using BSA as a standard.

GAP assays

His- G_{α} was diluted (100 nM final concentration) into a buffer containing 50 mM NaHepes (pH 8.0), 5 mM EDTA, 1 mM DTT, 0.05 % $\text{C}_{12}\text{E}_{10}$ and 1 μM [γ - ^{32}P]GTP (10 000 c.p.m./pmol) to load the G-protein with radiolabelled nucleotide. G_{α} was incubated for 20 min at 25°C . To initiate GTP hydrolysis, one-tenth volume of 150 mM MgSO_4 and 1.5 mM GTP, MBP-RGS14 (200 nM,

final concentration), MBP-RGS14 (200 nM, final concentration) + Rap2A(G12V)-GTP[S] (200 or 400 nM, final concentration), or Rap2A(G12V)-GTP[S] (400 nM, final concentration) was added to the G_{α} mix on ice. Single aliquots (50 μl) were removed at the indicated times and the reactions were quenched in 750 μl of ice-cold activated charcoal. Quenched reactions were centrifuged for 10 min at 670 g in a Beckman GS-15R tabletop centrifuge. Released [^{32}P]P_i was measured by liquid-scintillation counting. Purified RGS4 was generously provided by Andrew Grillo-Hill of this laboratory.

GDP release assays

$G_{i\alpha 1}$ protein was diluted (50 nM, final concentration) into a buffer containing 50 mM NaHepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.1 % $\text{C}_{12}\text{E}_{10}$, 10 mM MgSO_4 and 1 μM [α - ^{32}P]GDP (4000 c.p.m./pmol) to load the G-protein with radiolabelled nucleotide. $G_{i\alpha 1}$ was incubated for 90 min at 30°C . To initiate GDP release, $G_{i\alpha 1}$ -[α - ^{32}P]GDP was transferred to separate Eppendorf tubes containing unlabelled GDP (100 μM , final concentration), buffer (NaHepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1 % $\text{C}_{12}\text{E}_{10}$ and 10 mM MgSO_4), GDP + MBP-RGS14 (50–200 nM, final concentration), or GDP + MBP-RGS14–Rap2A–GTP[S] complex (150 nM, final concentration) at 30°C . Following a 30 min incubation at 30°C , 50 μl aliquots (in triplicate) were removed and reactions were stopped with ice-cold wash buffer (20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 30 mM MgCl_2 , 20 μM AlCl_3 and 10 mM NaF). Reactions were applied to BA85 nitrocellulose filters (Midwest Scientific) and washed with ice-cold wash buffer. Bound radioactive nucleotide was measured by liquid-scintillation counting.

GTP[S] binding assays

G_{α} subunits or $G_{\alpha\beta\gamma}$ heterotrimers were diluted (50 nM, final concentration) into a buffer containing 50 mM NaHepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.1 % $\text{C}_{12}\text{E}_{10}$, 10 mM MgSO_4 and 2 μM [^{35}S]GTP[S] (2500 c.p.m./pmol). $G_{i\alpha}$ subunits were incubated at 30°C for 60 min. At the end of the incubation period, 50 μl aliquots of protein were removed (in triplicate) and reactions were stopped with ice-cold wash buffer (20 mM Tris, pH 7.4, 100 mM NaCl and 25 mM MgCl_2). Reactions were applied to BA85 nitrocellulose filters and washed with ice-cold wash buffer. Bound radioactive nucleotide was measured by liquid-scintillation counting. To monitor GDI activity, assays were performed as above except that purified MBP-RGS14 was added to G_{α} subunits on ice prior to dilution into the binding cocktail.

Protein homology modelling

Models of mouse RGS14 RBD1 and RBD2 were constructed with the SWISS-MODEL Protein Homology Modeling Server using C-Raf1 as a template [29–31]. Co-ordinates of the human C-Raf1 were extracted from the crystal structure of C-Raf1 bound to Rap1A (PDB code 1C1Y) using chain B [32]. PDB files for the models of RBD1 and RBD2 and C-Raf1 were combined using the Swiss PDB viewer (Deepview) [29–31].

RESULTS

RGS14 binds activated Rap2A via its first RBD

To test the hypothesis that RGS14 is an effector of the Rap proteins, we first sought to confirm that RGS14 is able to bind to Rap proteins *in vitro* using purified components. We chose to focus on the Rap2A isoform because it has been reported that the

Figure 1 RGS14 binding to Rap2A is dependent on RBD1

His₆-tagged Rap2A(G12V)-GTP[S] (20 μ M) was incubated with MBP-RGS14 (WT, R336L, H409L and R336L/H409L) (5 μ M) at 4°C for 45 min in the presence of Ni-NTA agarose. Unbound proteins were removed by washing in a buffer containing 20 mM imidazole. Rap2A(G12V)-MBP-RGS14 complexes were specifically eluted in a buffer containing 200 mM imidazole. An aliquot of the elution fractions (10%) was analysed by immunoblotting (IB) using anti-Xpress (Rap2A) and anti-RGS14 antibodies. Molecular masses are indicated in kDa.

RGS14 RBD has a higher affinity for Rap2A than Rap1A [5]. Additionally, *in situ* hybridization analysis of brain tissue slices suggests that RGS14 and Rap2A mRNA are expressed in similar regions of the brain [5]. Rap2A(G12V) bound to recombinant MBP-RGS14 (Figure 1). This association was dependent on activation of Rap2A(G12V) (results not shown).

RGS14 and its related protein, RGS12, are unique among RGS proteins in that they contain two tandem RBDs. The second RBD of RGS14, which spans from residues 370 to 440, does not contain a critical conserved arginine residue. Arg³³⁶ in RBD1 is replaced with histidine (His⁴⁰⁹) in RBD2. In the crystal structure of C-Raf1 bound to Rap1, this arginine residue forms a direct and water-mediated contact with Rap and mutation of this residue to leucine has been shown to abolish Raf-Ras interactions [32]. Therefore it is possible that although RGS12 and RGS14 contain tandem RBDs, only one is functional. To determine whether both RBDs of RGS14 are indeed functional, we generated point mutants of RGS14 in RBD1 (R336L), RBD2 (H409L), or both (R336L/H409L). As shown in Figure 1, mutation of His⁴⁰⁹ had no discernable effect on binding of MBP-RGS14 to Rap2A, whereas mutation of Arg³³⁶ almost completely eliminated binding. Similarly, the R336L/H409L double mutant, which contains mutations in both RBDs, was unable to interact with Rap2A. These results suggest that the first RBD in RGS14 is necessary and sufficient for interaction with Rap family members.

Binding to Rap2A does not alter RGS14 GDI activity

Although active Rap2A can bind RGS14, the effect on RGS14 activity is unknown. We hypothesized that binding of Rap2A to RGS14 could potentially affect RGS14 biochemical activity at two separate domains: the GoLoco domain or the RGS domain. It has been shown *in vitro* that the RGS14 GoLoco domain has GDI activity [7–9]. The release of GDP from α subunits of heterotrimeric G-proteins in the absence of receptor is the rate-limiting step in the G-protein cycle [33]. Therefore RGS14 GDI activity may result in constitutive suppression of G-protein signalling. Using radiolabelled [α -³²P]GDP to directly measure GDP release from G_{iα1}, we examined the ability of MBP-RGS14 to inhibit GDP release from G_{iα1} when added alone or when bound to wild-type Rap2A-GTP[S]. MBP-RGS14 alone inhibited the release of [α -³²P]GDP from G_{iα1} in a dose-dependent manner, consistent with earlier studies (results not shown) [7,8].

We determined whether Rap2A binding altered RGS14 GDI activity in two ways: (i) incubation with excess Rap2A-GTP[S] or (ii) addition of a preformed MBP-RGS14-Rap2A-GTP[S] complex. In the first set of experiments, excess Rap2A-GTP[S] was

Figure 2 RGS14 GDI activity is independent of Rap2A

(A) [α -³²P]GDP release assay was performed as described in the Experimental section. G_{iα1}-[α -³²P]GDP (50 nM, final concentration) was incubated with a buffer containing GDP (unlabelled 100 μ M, final concentration), GDP + MBP-RGS14 (500 nM, final concentration) or GDP + MBP-RGS14 (500 nM, final concentration) incubated with excess Rap2A-GTP[S] (2 or 4 μ M, final concentration) for 30 min at 30°C. Triplicate aliquots (50 μ l) were removed and bound nucleotide was determined by liquid-scintillation counting. (B) A preformed complex of MBP-RGS14 and Rap2A-GTP[S] was used in an [α -³²P]GDP release assay with G_{iα1} as described in (A). G_{iα1}-[α -³²P]GDP (50 nM, final concentration) was incubated with a buffer containing GDP (unlabelled 100 μ M, final concentration), GDP + MBP-RGS14 (50, 100 and 200 nM, final concentration), or GDP + MBP-RGS14/Rap2A-GTP[S] (150 nM, final concentration).

incubated with MBP-RGS14 on ice prior to the addition of [α -³²P]GDP-bound G_{iα1}. MBP-RGS14 GDI activity was unaffected by addition of excess Rap2A-GTP[S] (Figure 2A). In the second series of experiments, His₆-tagged wild-type Rap2A-GTP[S] was incubated with MBP-RGS14 on Ni-NTA resin to form a complex. The complex was eluted with imidazole and used in an [α -³²P]GDP release assay. Similar to the titration experiments, a complex of MBP-RGS14-Rap2A-GTP[S] was able to inhibit GDP release by G_{iα1} similar to MBP-RGS14 alone (Figure 2B). These results represent the first biochemical examination of the effect of Rap2A binding on the RGS14 GoLoco domain, and suggest that RGS14 GDI activity is independent of Rap binding.

Binding of Rap2A does not alter RGS14 GAP activity

In addition to its function as a GDI, RGS14 functions as a GAP for the G_{iα} family members G_{iα1}, G_{iα2}, G_{iα3} and G_{oα} [5–9]. We determined whether binding of Rap2A affected the ability of MBP-RGS14 to stimulate GTP hydrolysis by G_{oα} using a single-turnover GTPase assay. Incubation of MBP-RGS14 with an activated (G12V) mutant of Rap2A bound to GTP[S] had no effect on MBP-RGS14 GAP activity towards G_{oα} (Figure 3). The slightly accelerated rate of GTP hydrolysis in the plot shown was not reproducible, and we attributed this to a possible protein stabilization effect. This suggests that, similar to the GoLoco domain, the RGS14 RGS domain functions independently of Rap binding.

G_{βγ} inhibits RGS14 GDI activity

Several studies have documented that GoLoco peptides can disrupt the association of G_α subunits with G_{βγ} [21–23], whereas cell-based assays suggest that free G_α is the substrate for GoLoco activity [10,23]. We sought to test the ability of the GoLoco domain of RGS14 to displace G_{βγ} from G_α in the context of the full-length protein. We reasoned that RGS14 GDI activity could be used as a readout for RGS14 displacing G_{βγ} and binding to G_{iα}. In all the experiments described below, we generated preformed heterotrimers using stoichiometric amounts of G_α and G_{βγ}. In the initial set of experiments, we reconstituted heterotrimers with myr-G_{iα1} (N-myristoylated G_{iα1}) and prenylated wild-type G_{β1γ2}

Figure 3 RGS14 GAP activity is independent of Rap2A

Single turnover assays of GTP hydrolysis to measure RGS14 GAP activity were performed as described in the Experimental section. Squares, G_{α} alone; circles, G_{α} + 200 nM MBP-RGS14; diamonds, G_{α} + 200 nM MBP-RGS14/200 nM Rap2A(G12V)-GTP[S]; triangles, G_{α} + 200 nM MBP-RGS14/400 nM Rap2A(G12V)-GTP[S].

Figure 4 RGS14 GDI activity is inhibited by $G_{\beta\gamma}$

[35 S]GTP[S] binding assays were performed as described in the Experimental section. (A) myr- $G_{\alpha 1}$ (50 nM, final concentration), N-myristoylated $G_{\alpha 1\beta\gamma}$ (WT) (50 nM, final concentration), or N-myristoylated $G_{\alpha 1\beta\gamma}$ (C68S) (50 nM, final concentration) was incubated in the presence (grey bars) or absence (black bars) of MBP-RGS14 (500 nM, final concentration) and assayed for [35 S]GTP[S] binding. G_{α} subunits were incubated at 30 °C for 60 min. Maximal [35 S]GTP[S] bound in the absence of MBP-RGS14 was 2.2 ± 0.02 pmol (myr- $G_{\alpha 1}$), 2.0 ± 0.07 pmol [myr- $G_{\alpha 1\beta\gamma}$ (WT)] and 2.2 ± 0.17 pmol [myr- $G_{\alpha 1\beta\gamma}$ (C68S)]. (B) [35 S]GTP[S] binding assays were performed as described in (A), except that His- $G_{\alpha 1}$ (50 nM, final concentration) was used in place of myr- $G_{\alpha 1}$ with $G_{\beta\gamma}$ (WT) or $G_{\beta\gamma}$ (C68S) and incubated in the presence (grey bars) or absence (black bars) of MBP-RGS14 (500 nM, final concentration). Maximal [35 S]GTP[S] bound in the absence of MBP-RGS14 was 2.3 ± 0.34 pmol (His- $G_{\alpha 1}$), 2.2 ± 0.30 pmol [His- $G_{\alpha 1\beta\gamma}$ (WT)] and 2.7 ± 0.48 pmol [His- $G_{\alpha 1\beta\gamma}$ (C68S)].

(WT) or with an unprenylated $G_{\beta 1\gamma 2}$ (C68S) mutant. Loss of prenylation results in a reduction in the affinity of interaction between G_{α} and $G_{\beta\gamma}$ [28]. In the absence of MBP-RGS14, myr- $G_{\alpha 1}$, myr- $G_{\alpha 1\beta\gamma}$ (WT) and myr- $G_{\alpha 1\beta\gamma}$ (C68S) bound [35 S]GTP[S] to a similar extent (Figure 4A, black bars). Surprisingly, MBP-RGS14 was unable to inhibit nucleotide exchange on the wild-type heterotrimer, whereas no such impairment was seen with the $G_{\beta 1\gamma 2}$ (C68S) mutant (Figure 4A, grey bars). We hypothesized that the lipid modifications on both G-protein subunits resulted in a high-affinity complex that was resistant to disruption by a 10-fold molar excess of RGS14. To test the requirement for lipid modification of both subunits, we examined the ability of MBP-RGS14 to inhibit nucleotide exchange on N-terminally His-tagged (unmyristoylated) $G_{\alpha 1}$ alone, or His- $G_{\alpha 1}$ bound to $G_{\beta\gamma}$ (WT) or $G_{\beta\gamma}$ (C68S). As expected, MBP-RGS14 was able to inhibit nucleotide exchange in all three instances, confirming the necessity for lipid modification on both G-protein subunits for high-affinity association and resistance to GDI activity (Figure 4B). G-protein

subunit interactions are modulated by Mg^{2+} . The experiments shown were done in the presence of 10 mM $MgSO_4$. Under these conditions, $G_{\beta\gamma}$ does not suppress nucleotide exchange by G_{α} subunits [34]. However, similar results were obtained over a range of Mg^{2+} concentrations (0.5–10 mM; results not shown).

DISCUSSION

In the present paper, we report the ability of RGS14 to bind Rap2A *in vitro* and that this binding was dependent on an intact RBD1. The presence of protein interaction domains for both monomeric and heterotrimeric G-proteins led to the hypothesis that RGS14 may function as an effector of Rap2A [5]. We found that although RGS14 was able to interact with and form a stable complex with Rap2A, this association did not alter the ability of RGS14 to exert its biochemical function on G_{α} subunits, either as a GAP or as a GDI.

The finding that the second RBD of RGS14 was dispensable for interaction with Rap2A, while surprising, is consistent with recent results presented by Traver et al. [10]. Using a yeast two-hybrid analysis, the authors found that RGS14 with the R336L mutation in the first RBD was unable to interact with Rap2A [10]. We examined the potential loss of interaction further by modelling the individual RBDs of RGS14 on the known crystal structure of C-Raf1 bound to Rap1-p[NH]ppG (guanosine 5'-[β,γ -imido]triphosphate). This analysis revealed that His⁴⁰⁹ in RBD2 maintained the appropriate charge, but was unable to make significant contact with Rap1 owing to its shorter side chain. What role this second RBD plays in signalling is unclear. It is possible that it binds to some other protein partner *in vivo*.

While we were unsuccessful in modulating the activity of RGS14 as either a GDI or a GAP through binding of Rap2A, the possibility remains that RGS14 functions in a pathway that intersects with the Rap proteins in an *in vivo* setting. One possible limitation of our analysis of Rap2A–RGS14 interactions is that the assays were performed with proteins that lacked appropriate post-translational modifications. Rap2A is farnesylated and palmitoylated at its C-terminus [35,36]. We purified Rap2A from the soluble fraction of insect cells because we were unable to obtain sufficient quantities of the protein from membranes. We have inferred that the soluble Rap2A is prenylated based on a more rapid electrophoretic mobility compared with Rap2A purified from bacteria (results not shown). However, additional post-translational processing occurs on membranes: CaaX proteolysis, carboxymethylation and palmitoylation. Although lipid modifications are not required *in vitro* for RGS14–Rap2A association, these modifications might be needed for Rap2A binding to induce a change in RGS14 activity.

The role of Rap2A in regulating RGS14 may be independent of RGS14 function as a GAP or GDI. A potential mode of regulation is Rap-mediated localization of RGS14. RGS14 is found in the cytoplasm, nucleus and at centrosomes [37,38]. An intact RBD1 is required for RGS14 to localize to centrosomes [38]. However, this appears to be independent of its ability to bind Rap proteins. The R336L mutant, which we have shown no longer binds Rap2A, displays the same subcellular distribution as the wild-type protein [38]. Thus the centrosomal localization of RGS14 does not appear to be mediated by its association with Rap. However, RGS14 is likely to function at other sites in the cell, and activation of Rap could result in the recruitment of cytoplasmic RGS14 to membranes. A recent study by Traver et al. [10] supports such a possibility. The authors examined the ability of RGS14 to inhibit M_2 -muscarinic acetylcholine receptor-mediated activation of ERK (extracellular-signal-regulated kinase), a $G_{i\alpha}$ -coupled pathway. Expression of either the isolated RGS or GoLoco domains

decreased the levels of phosphorylated ERK in response to agonist. Interestingly, inhibition by the GoLoco domain was potentiated by either addition of a membrane targeting signal or inclusion of the RBDs, suggesting a need for proper targeting of the GoLoco domain for its maximal activity and consistent with a role for Rap association in recruitment of RGS14 to receptor-mediated G-protein signalling at the plasma membrane.

The second goal of our study was to determine whether the RGS14 GoLoco domain acts on free G_{α} versus the heterotrimer. We found that the heterotrimer is not a substrate for the RGS14-GL. The resistance of the heterotrimer to the GDI activity of RGS14 was dependent on the presence of lipid modifications on both $G_{i\alpha}$ and $G_{\beta\gamma}$. This is particularly interesting because the sites of lipid modification on the G_{α} and G_{γ} subunits are distal from the common surface on G_{α} that binds $G_{\beta\gamma}$ and RGS14-GL [20,27,39]. Previous work has shown that myristoylation of G_{α} and prenylation of G_{γ} are required for high-affinity interactions of G_{α} and $G_{\beta\gamma}$ [28,40]. Facilitation of subunit interactions by lipid modification is thought to be indirect, rather than mediated by lipid-binding sites on the protein surfaces. In studies of transducin (T) association with detergent micelles or phospholipid vesicles, the T_{α} acyl chain appears to bind co-operatively with the $T_{\beta\gamma}$ farnesyl chain in the membrane lipids [41]. It is proposed that the lipid-lipid interactions contribute to subunit association by favouring the orientation of protein interaction surfaces and promoting their interaction. This may be especially true for the myristoylated N-terminal helix of G_{α} , which makes direct contact with G_{β} on a surface bounded by the lipid bilayer [1]. In the absence of the lipid-lipid interactions, the affinity of G_{α} for $G_{\beta\gamma}$ is reduced sufficiently that RGS14-GL is able to compete with $G_{\beta\gamma}$.

Our finding that RGS14 is unable to inhibit nucleotide exchange on a heterotrimer of $G_{\alpha\beta\gamma}$ is in contrast with reports that show that GoLoco peptides are able to physically disrupt a complex of G_{α} and $G_{\beta\gamma}$ [21–23]. Bernard et al. [21] studied the effects of a peptide derived from AGS3 on heterotrimer association using a heterotrimer reconstituted with non-myristoylated $G_{i\alpha}$. Webb et al. [23] showed that a peptide corresponding to the RGS14 GoLoco domain prevents heterotrimer formation by non-lipidated $G_{i\alpha}$ and $G_{\beta\gamma}$ subunits. The discrepancy between these results and our own may be explained by the lack of lipid modifications on the G-proteins used in the experiments. In a third study using lipidated G-protein subunits, a peptide derived from AGS3 was able to induce subunit dissociation. However, the peptide was in 20 000-fold excess over the G-protein, which is not likely to reflect physiological concentrations [22]. Finally, all three studies utilized small peptides, which might lack additional restraints imposed on the GoLoco domain in the context of a full-length protein.

Recent studies of GoLoco regulation of G-protein-regulated inwardly rectifying K^{+} (GIRK) currents in AtT20 cells [23] are consistent with our finding that the heterotrimer is not subject to RGS14 GDI activity. Initial injection of AGS3 or RGS12 GoLoco peptides had no effect on channel opening either in the absence of or in response to agonist, suggesting that the presence of the peptide did not result in free $G_{\beta\gamma}$ activating the channel. Importantly, however, subsequent rounds of stimulation result in lowered responses [23]. The results of these cell-based studies suggest that AGS3 and RGS12 GoLoco domains act only after the initial round of GTP hydrolysis, removing free G_{α} -GDP from the receptor–G-protein–channel complex. Taken together with our data that the heterotrimer cannot be dissociated by RGS14-GL, it seems likely that the GoLoco domains of RGS12, RGS14, and AGS3 exert their activities through interactions with free G_{α} subunits, rather than the heterotrimer. This argues against a role for GoLoco domains in activating $G_{\beta\gamma}$ -mediated signalling pathways

and demonstrates an inhibitory role in classical G-protein signalling.

This conclusion is further supported by the demonstration that both the isolated RGS and GoLoco domains of RGS14 inhibit receptor-stimulated ERK activity [10]. The level of inhibition achieved by the full-length protein suggested that the RGS domain and RGS14GL co-operate to inhibit signalling. This interdomain regulation may involve more than the RGS domain and the GoLoco motif. A recent study reports that RGS14 lacking its RGS domain (R14-RBD/GL) contains an additional binding site for $G_{\alpha\alpha}$ -GDP and $G_{i\alpha}$ -GDP that is distinct from the GoLoco domain [42]. R14-RBD/GL potentiates the GAP activity of RGS4 in membranes by increasing the apparent affinity of RGS4 for $G_{i\alpha}$ and $G_{\alpha\alpha}$. This raises the possibility that RGS14 displays similar co-operative interactions intramolecularly.

Although RGS14 may function in classical receptor-mediated G-protein pathways, its best-characterized function to date is in the regulation of mitotic spindle assembly [37]. RGS14 is essential for the first mitosis in the mouse zygote and normal cellular proliferation in tissue culture cells. The RGS and GoLoco motifs are required for RGS14 function in cell division, indicating the importance of RGS14 regulation of the GTP/GDP cycle. The individual contributions of the RGS and GoLoco domains to the regulation of microtubule dynamics have not been elucidated. Our results suggest that the RGS14 GoLoco domain will exert its effects once $G_{i\alpha}$ has been dissociated from $G_{\beta\gamma}$. The identity of the protein that promotes subunit dissociation in this pathway is among the many unanswered questions in this new area of research for heterotrimeric G-proteins.

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