Biochem. J. (2004) **379**, 627–632 (Printed in Great Britain)

The RGS (regulator of G-protein signalling) and GoLoco domains of RGS14 co-operate to regulate G_i-mediated signalling

Sabine TRAVER, Anne SPLINGARD, Georges GAUDRIAULT and Jean DE GUNZBURG[1]

INSERM U-528, Institut Curie-Section de Recherche, 26 rue d'Ulm, 75248 Paris Cedex 05, France

RGS (regulator of G-protein signalling) proteins stimulate the intrinsic GTPase activity of the α subunits of heterotrimeric G-proteins, and thereby negatively regulate G-protein-coupled receptor signalling. RGS14 has been shown previously to stimulate the GTPase activities of $G\alpha_o$ and $G\alpha_i$ subunits through its N-terminal RGS domain, and to down-modulate signalling from receptors coupled to G_i. It also contains a central domain that binds active Rap proteins, as well as a C-terminal GoLoco/G-protein regulatory motif that has been shown to act *in vitro* as a GDP-dissociation inhibitor for $G\alpha_i$. In order to elucidate the respective contributions of the three functional domains of RGS14 to its ability to regulate G_i signalling, we generated RGS14 mutants invalidated in each of its domains, as well as truncated molecules, and assessed their effects on G_i signalling via the $\beta\gamma$ pathway in a stable cell line ectopically expressing the G_i-coupled M_2 muscarinic acetylcholine receptor (HEK-m2). We show that the RGS and GoLoco domains of RGS14 are independently able to inhibit signalling downstream of G_i. Targeting of the isolated GoLoco domain to membranes, by myristoylation/palmitoylation or Rap binding, enhances its inhibitory activity on G_i signalling. Finally, in the context of the full RGS14 molecule, the RGS and GoLoco domains co-operate to confer maximal activity on RGS14. We therefore propose that RGS14 combines the inhibition of G_i activation or coupling to receptors via its GoLoco domain with stimulation of the GTPase activity of $G\alpha_i$–GTP via its RGS domain to negatively regulate signalling downstream of G_i.

Key words: extracellular-signal-regulated kinase (ERK), G_i, GoLoco domain, GTPase, Rap, regulator of G-protein signalling (RGS).

INTRODUCTION

Numerous receptors with seven transmembrane domains are coupled to heterotrimeric G-proteins, through which they exert their physiological effects. These proteins are composed of a GTP-binding $G\alpha$ subunit and a $G\beta\gamma$ dimer; activated receptors act as GEFs (guanine nucleotide exchange factors) that promote GTP binding to the α subunit and dissociation of the $\beta\gamma$ dimer, both of which are then free to interact with their respective downstream effectors [1,2]. Signal termination is ensured by the hydrolysis of GTP, thereby returning the protein to its basal GDP-bound state; $\beta\gamma$ dimers rapidly re-associate with inactive GDP-bound $G\alpha$ subunits, which also terminates signalling downstream of $\beta\gamma$ [3,4]. $G\alpha$ subunits carry an intrinsic GTPase activity that is stimulated by proteins of the recently discovered RGS (regulator of G-protein signalling) family, which comprises more than 20 members (see [5] for a review). The catalytic core of these proteins consists of a conserved domain of 120 residues (RGS domain) that acts by stabilizing the most favourable conformation of the $G\alpha$ subunits for GTPase activity [6].

RGS14 is a multidomain protein that is able to stimulate the GTPase activity of $G\alpha_i$ and $G\alpha_o$ *in vitro* [7], and to down-regulate signalling from G-protein-coupled receptors acting through G_i [8]. It interacts with the GTP-bound form of the Ras-related Rap1 and Rap2 proteins, through a central RID (Rap interaction domain) that shares a high degree of similarity with the Ras-binding domain of Raf kinases [7]; however, no modulation of RGS14 activity by Rap proteins has yet been shown. In its C-terminal region, RGS14 carries another G_i-interacting motif, termed the GoLoco or G-protein regulatory motif, similar to those found in proteins such as AGS3 (activator of G-protein signalling 3), the AGS3-related protein Pins from *Drosophila melanogaster*, RGS12, LGN, Pcp2 and Rap1GAP (GTPase-activating protein) II [9]. Previous studies have shown that the GoLoco motifs of AGS3 and RGS14 bind to $G\alpha_i$–GDP complexes and are able to inhibit the dissociation of GDP [GDI (GDP dissociation inhibitor) activity] from $G\alpha_i$ subunits *in vitro* [10–15]; however, their functional role in G_i signalling has not yet been elucidated. In the present paper, we show that the RGS and GoLoco domains of RGS14 are independently able to inhibit signalling downstream of G_i, and that they co-operate in the intact RGS14 protein to maximally down-regulate G_i-controlled pathways.

MATERIALS AND METHODS

Expression constructs

An expression vector encoding a (Myc_6)–ERK2 (extracellular-signal-regulated kinase 2) reporter protein was generated by cloning the mouse ERK2 coding sequence fused to six Myc epitopes in the pCS2 + MT vector. The sequences encoding wild-type $G\alpha_o$, $G\alpha_{i1}$ and $G\alpha_{i2}$, the $G\alpha_{i2}$ [Q204L] mutant, H-Ras, Rap1A and residues 1–168 of Rap2A were amplified by PCR and cloned into the yeast two-hybrid bait vector pGBT10.

A synopsis of the RGS14 constructs used in the present study is shown in Figure 1(A). Fragments encoding the following regions were excised with the indicated restriction enzymes from pGAD-RGS14 and cloned into the pRK5myc vector for expression in mammalian cells: sequences encoding residues 427–547 (excised with *Nco*I and *Xho*I) contain the GoLoco domain (GoLoco), those encoding residues 300–547 (excised with *Ecl*XI and *Xho*I) contain the RID and the GoLoco domain (RID-GoLoco), those encoding residues 1–300 (excised with *Bam*HI and *Ecl*XI) contain the RGS

Abbreviations used: AGS, activator of G-protein signalling; βARK, β-adrenergic receptor kinase; ERK, extracellular-signal-regulated kinase; GEF, guanine nucleotide exchange factor; mAChR, muscarinic acetylcholine receptor; RGS, regulator of G-protein signalling; RID, Rap interaction domain.

[1] To whom correspondence should be addressed (e-mail jgunzburg@curie.fr).

© 2004 Biochemical Society

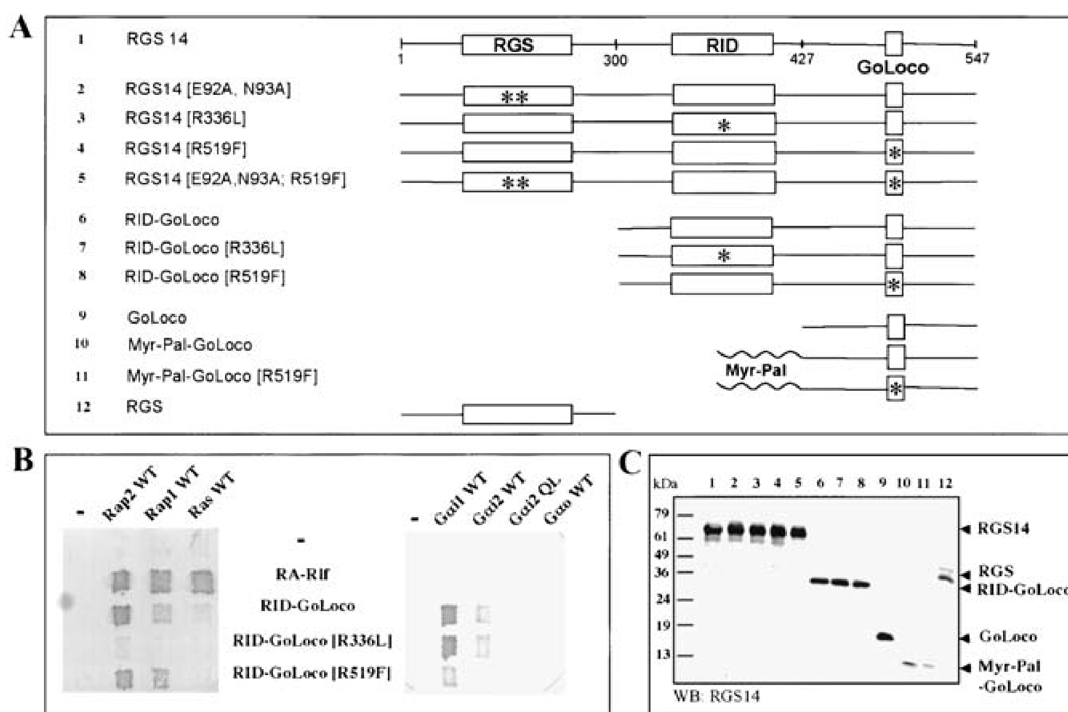

Figure 1 Generation of RGS14 mutant proteins

(**A**) Diagram of the different RGS14 mutants; mutations are indicated by asterisks. The Myr/Pal-GoLoco construct expresses the GoLoco domain fused to the C-terminus of the sequence directing the myristoylation and palmitoylation of the N-terminus of Gα_{i2}. (**B**) Hf7c yeast transformed by empty vector pGBT10 (−), or vectors containing the coding sequences of Rap2, Rap1, Ras, wild-type (WT) Gα_{i1}, Gα_{i2} or Gα_0 or mutant Gα_{i2} [Q204L] (QL) were mated with Y190 yeast transformed with empty pGAD-GE (−) or vector containing the Ras-binding (RA) domain of Rlf, or the RID and GoLoco domains of RGS14 mutated or not within the domains. Interactions were visualized using the β-galactosidase reporter; similar results were obtained by monitoring growth in the absence of histidine. (**C**) HEK-m2 cells were transfected with the different RGS14 constructs depicted in (**A**), and the expression of RGS14 proteins was monitored by Western blotting (WB).

domain (RGS), and those encoding the entire RGS14 protein were excised with *Bam*HI and *Xho*I.

Site-directed mutagenesis of RGS14 at codons 92, 93, 336 and 519 was performed by overlapping PCR, using *Pfu* polymerase (Stratagene). For the [E92A,N93A] mutation of the RGS domain, GAG AAC codons were replaced by GCG GCC. For the [R336L] mutation of the RID, AGA was replaced by CTG, and for the [R519F] mutation of the GoLoco motif, AGA was replaced by TTC. Mutated fragments were subcloned into pRK5-myc2 for mammalian expression, as well as into pGAD-GE [16] for yeast two-hybrid assays. The sequence that directs the myristoylation and palmitoylation of the N-terminus of Gα_{i2} (ATG GGC TGC ACC CTG AGC GCC) was added to the N-terminus of a fragment encoding residues 427–547 of RGS14 by PCR (Myr-Pal-GoLoco) and cloned, without the addition of an epitope tag, into the mammalian expression vector pRK5. The integrity of all constructs, as well as the presence of the indicated mutations, was verified by DNA sequencing.

Two-hybrid assays

Bait constructs in pGBT10 expressing Ras/Rap or Gα subunits, and prey constructs expressing the Ras-binding domain of Rlf or RGS14-derived domains, were respectively transformed into Hf7c and Y190 yeast. Two-hybrid assays were performed by mating as described in [16].

Cell line, transfection and ERK2 phosphorylation assay

HEK-293 cells stably expressing the M_2 mAChR (muscarinic acetylcholine receptor) (HEK-m2 cells) were kindly provided by Professor Marlene Hosey (Northwestern University Medical School, Chicago, IL, U.S.A.) [17]. They were seeded at 600 cells/mm^2 and transfected the next day with 0.1 μg of (Myc)$_6$–ERK2 reporter and 5 μg of the various RGS14 expression constructs or pRK-βARK1-(495–689) (where βARK1 is β-adrenergic receptor kinase 1) [18] by a calcium phosphate precipitation method. At 24 h after transfection, cells were serum-starved for 16 h in Dulbecco's modified Eagle's medium containing 0.2 % (w/v) BSA; 500 ng/ml pertussis toxin (Sigma) was included in the starvation medium when indicated. Cells were stimulated with vehicle, 100 μM carbachol (Sigma) or 10 nM epidermal growth factor (Oncogene Research Products) for 5 min, washed rapidly with ice-cold PBS and solubilized in SDS/PAGE sample buffer containing 5 % (v/v) β-mercaptoethanol, 1 mM sodium orthovanadate and 1 μM okadaic acid. Expression and dual phosphorylation of the (Myc)$_6$–ERK2 reporter protein were assessed by Western blotting using anti-ERK1/2 antibodies (Upstate Biotechnology) and phospho-specific ERK1/2 antibodies (Cellular Signaling) respectively. The expression of RGS14 proteins was monitored using a rabbit polyclonal antibody [7]. Western blots were visualized using a CCD camera (Fuji) and quantified with the Image Gauge software (Fuji). Statistical analysis of differences between the effects of wild-type and mutant RGS14 molecules on ERK phosphorylation was performed using Student's *t* test; $P < 0.02$ was considered statistically significant.

Trapping assay for activated Rap1 and Rap2

The levels of active Rap1–GTP and Rap2–GTP complexes were determined as described previously using RalGDS-RA, the

© 2004 Biochemical Society

Ras-binding domain of RalGDS (Ral guanine nucleotide dissociation stimulator) as an activation-specific probe [19]. HEK-m2 cells were serum-starved, stimulated with vehicle or 100 μM carbachol as described above, and lysed in trapping lysis buffer [50 mM Tris/HCl, pH 7.5, 15 mM NaCl, 20 mM $MgCl_2$, 5 mM EGTA, 1 % Triton X-100, 1 % n-octyl glucoside, 100 μM 4-(2-aminoethyl)benzenesulphonyl fluoride, 10 μM leupeptin and 10 μM pepstatin A]. Lysates were centrifuged for 15 min at 10 000 ***g***, and supernatants were incubated with 10 μg of (glutathione S-transferase)–RalGDS-RA bound to 30 μl of glutathione–Sepharose for 2 h at 4 °C, and washed three times in trapping lysis buffer. The levels of activated Rap1 and Rap2 bound to the beads, as well as total Rap1 and Rap2 in the lysates, were determined by Western blotting using a rabbit polyclonal antibody against Rap1 [20] and a mouse monoclonal antibody against Rap2 (Transduction Laboratories).

RESULTS

Generation of RGS14 mutant proteins invalidated in each functional domain

The individual biochemical activities of the RGS domain, RID and GoLoco domain of RGS14 have been extensively characterized [7,8,13–15]; however, their respective contributions to the regulation of G_i signalling by RGS14 remain to be established. To this end, we generated expression constructs encoding full-length as well as truncated RGS14 proteins carrying amino acid substitutions at critical positions in each of its functional domains (see Figure 1A).

The RGS domain was inactivated by mutating two highly conserved residues, Glu-92 and Asn-93, to alanines, changes that have been shown previously to abolish the capacity of RGS14 to down-regulate G_i-dependent signalling [8].

The RID, which is responsible for the interaction of RGS14 with Rap proteins, is highly similar to the region of Raf kinases involved in Ras binding [7]. Arg-336, corresponding to Arg-89 in Raf-1, a residue critical for its interaction with Ras and Rap1 [21,22], was mutated to leucine. As shown in the yeast two-hybrid assay depicted in Figure 1(B), this mutation abolished the ability of a fragment of RGS14 containing the RID and GoLoco domains (RID-GoLoco) to interact with Rap1 and Rap2.

Arg-519 of RGS14 is a highly conserved residue among GoLoco domains [10], and structural analysis has shown that this residue of RGS14 exerts an important contact with the GDP-binding pocket of $G\alpha_{i1}$ [23]. Arg-519 was mutated to phenylalanine, a substitution that has been shown to abolish the interaction of the GoLoco domains of AGS3 with $G\alpha_{i1}$ [10]. In a yeast two-hybrid assay, the GoLoco domain of RGS14 was able to interact with the wild-type form of $G\alpha_{i1}$, and to a lesser extent with $G\alpha_{i2}$; however, as expected from biochemical experiments (S. Traver and J. de Gunzburg, unpublished work; [14,15,23]), it interacted neither with the active $G\alpha_{i2}$ [Q204L] mutant, which is mainly in its GTP-bound form *in vivo*, nor with $G\alpha_o$ (Figure 1B). Mutation of the conserved Arg-519 to phenylalanine totally abolished its ability to interact with $G\alpha_{i2}$ and considerably reduced its interaction with $G\alpha_{i1}$ (Figure 1B).

The sequences encoding these mutants were introduced into mammalian expression vectors, and their ectopic expression was assessed by Western blotting (Figure 1C). Mutants of the full-length protein and the RID-GoLoco fragment were expressed at levels similar to the corresponding unmutated molecules. The protein expressed from the Myr/Pal-GoLoco construct (expressing the GoLoco domain fused to the C-terminus of the sequence directing the myristoylation and palmitoylation of the N-terminus of $G\alpha_{i2}$) migrated faster in SDS/PAGE than the one devoid of lipidation sequences, attesting that it had indeed been post-translationally modified; however, it was expressed at a lower level than the unlipidated molecule, and the expression of the R519F mutated fragment was slightly reduced as compared with its unmutated equivalent.

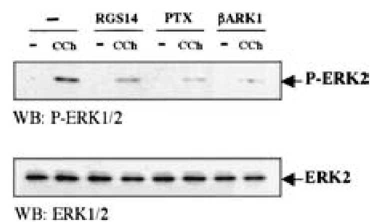

Figure 2 ERK is phosphorylated downstream of G_i in HEK-m2 cells

HEK-m2 cells were co-transfected with the $(Myc)_6$–ERK2 reporter construct together with a control plasmid, or vectors expressing full-length RGS14 or the C-terminal domain of βARK1. Serum-starved cells, preincubated in the absence or presence of pertussis toxin (PTX), were stimulated by vehicle (−) or 100 μM carbachol (CCh) for 5 min. Cells were lysed in SDS/PAGE sample buffer; expression and phosphorylation of the $(Myc)_6$–ERK2 reporter were monitored by Western blotting (WB).

RGS14 down-regulates G_i-mediated signalling from the M_2 mAChR

The M_2 mAChR subtype is specifically coupled to heterotrimeric G_i proteins; its activation by the acetylcholine analogue, carbachol, causes the activation of ERK1 and ERK2 mitogen-activated protein kinases via a signalling pathway involving the $\beta\gamma$ dimer and Ras [24]. We used HEK-m2, a previously characterized stable cell line derived from HEK-293 cells overexpressing the M_2 mAChR [17], and monitored signalling downstream of G_i by measuring the phosphorylation of a $(Myc)_6$–ERK2 reporter expressed by transient transfection as described in the Materials and methods section. As shown in Figure 2, carbachol triggered potent phosphorylation of the ERK2 reporter protein. This response was strongly inhibited by the pretreatment of cells with pertussis toxin, as well as by expression of the C-terminal domain of βARK1, attesting that the M_2 mAChR indeed signalled to the ERK2 reporter via a pathway involving the $\beta\gamma$ dimer of a receptor-coupled G_i. As expected, expression of RGS14 strongly depressed the response to carbachol. Hence measuring the level of ERK phosphorylation in HEK-m2 cells in response to their stimulation by carbachol constitutes a valid biological model in which to study the regulation of a G_i-dependent pathway by RGS14.

Rap proteins do not modulate the inhibition of G_i signalling by RGS14

Since RGS14 binds Rap1 and Rap2 in their active GTP-bound state, we investigated whether these proteins could modulate the regulatory activity of RGS14 on G_i signalling. Using a pull-down assay to measure the level of active Rap proteins (Figure 3A), we established that stimulation of HEK-m2 cells with carbachol led to a rapid and substantial activation of Rap1 (approx. 9-fold within 5 min). In contrast, the basal level of Rap2–GTP was already high, as reported previously for certain cell types [25], and was only modestly increased by stimulating the cells with carbachol. Introduction of an R336L mutation in RGS14, which invalidated the ability of its RID to interact with active Rap proteins, had no effect on the capacity of RGS14 to inhibit G_i signalling (Figure 3); this lack of effect was observed over a range of RGS14 expression levels (results not shown).

© 2004 Biochemical Society

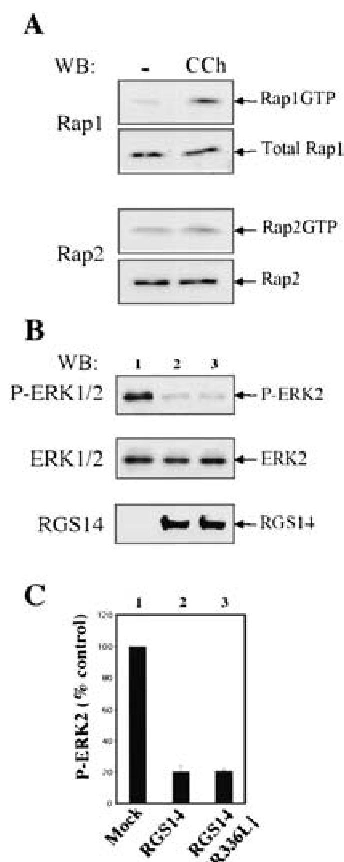

Figure 3 Rap proteins do not contribute to the inhibition of G_i signalling mediated by RGS14 in HEK-m2 cells

(**A**) Serum-starved HEK-m2 cells were stimulated for 5 min with vehicle (−) or 100 μM carbachol (CCh). The levels of active Rap1–GTP and Rap2–GTP complexes were determined by a trapping assay (upper panels); total levels of Rap1 and Rap2 were determined in parallel by Western blotting (WB; lower panels). (**B**, **C**) HEK-m2 cells were co-transfected with the $(Myc)_6$–ERK2 reporter construct together with control plasmid (lane 1) or expression vectors for wild-type RGS14 (lane 2) or mutant RGS14 [R336L] (lane 3). Serum-starved cells were stimulated for 5 min with carbachol, and lysed in SDS/PAGE sample buffer. Phosphorylation and expression of the $(Myc)_6$–ERK2 reporter protein, as well as RGS14 expression, were detected and quantified by Western blotting (WB) as described in the Materials and methods section. (**B**) Results from a typical experiment are shown; (**C**) the quantified data from four independent experiments were normalized relative to the total levels of $(Myc)_6$–ERK2 expressed.

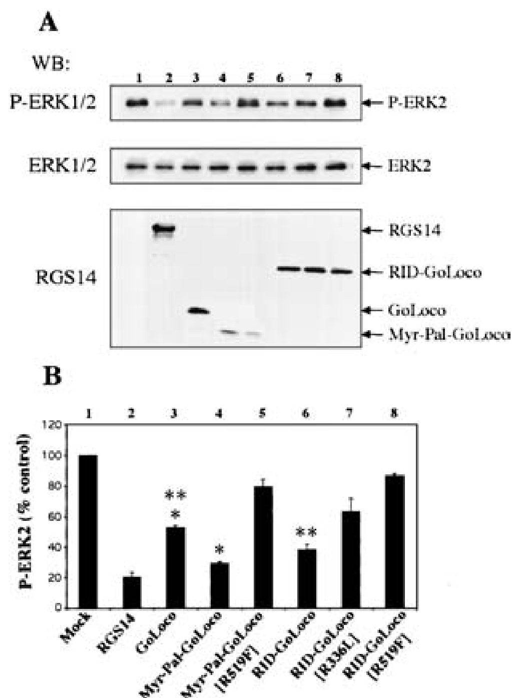

Figure 4 Effect of the GoLoco domain on G_i signalling in HEK-m2 cells

HEK-m2 cells were co-transfected with the $(Myc)_6$–ERK2 reporter construct, together with control plasmid (lane 1) or expression vectors for wild-type RGS14 (lane 2), GoLoco (lane 3), Myr/Pal-GoLoco (lane 4), Myr/Pal-GoLoco [R519F] (lane 5), RID-GoLoco (lane 6), RID-GoLoco [R336L] (lane 7) or RID-GoLoco [R519F] (lane 8). Myr/Pal-GoLoco denotes the GoLoco domain fused to the C-terminus of the sequence directing the myristoylation and palmitoylation of the N-terminus of $G\alpha_i2$. Serum-starved cells were stimulated for 5 min with 100 μM carbachol, and analysed as described in the legend to Figure 3. (**A**) Results from a typical experiment; (**B**) the quantified data from four independent experiments were normalized relative to the total levels of $(Myc)_6$–ERK2 expressed. *Significantly greater inhibition by Myr/Pal-GoLoco than by GoLoco ($P < 0.01$); **significantly greater inhibition by RID-GoLoco than by GoLoco ($P < 0.02$).

The GoLoco domain of RGS14 inhibits G_i signalling in HEK-m2 cells

We then assessed whether the GoLoco domain exerts a regulatory activity on G_i-dependent signalling in HEK-m2 cells. As shown in Figure 4, expression of the isolated GoLoco domain led to a significant (close to 50 %) inhibition of ERK phosphorylation in response to carbachol, and this activity was enhanced further by targeting the GoLoco domain to membranes by the addition to its N-terminus of the myristoylation/palmitoylation signal of $G\alpha_{i2}$. Mutation of the critical residue Arg-519 to Phe in the GoLoco motif substantially reduced its effect; this mutant was expressed at a 1.4-fold lower level than its non-mutated counterpart (as assessed by quantification of the Western blot in Figure 4A), but its ability to inhibit ERK phosphorylation was reduced from 72 % to 20 %, attesting that the observed inhibition of G_i signalling was indeed attributable to the activity of the GoLoco motif. Since we could not measure any regulatory effect of Rap proteins on the activity of the full-length RGS14 protein, we investigated whether they could modulate the activity of the GoLoco domain by interacting with the adjacent RID. Indeed, a protein containing both the RID and the GoLoco domain was more potent at inhibiting G_i-dependent ERK activation than the GoLoco domain alone, although the two proteins were expressed at similar levels. This enhancement was lost upon mutation of the RID, suggesting that Rap proteins, either by recruiting the GoLoco domain to membranes or by relieving interference by the RID with the GoLoco domain, are responsible for this effect.

The RGS and GoLoco domains of RGS14 co-operate to regulate G_i

Finally, we sought to establish the respective contributions of the RGS and GoLoco domains, in the context of the full-length molecule, to the regulation of G_i signalling by RGS14 (Figure 5). Whereas the ectopic expression of intact RGS14 inhibited the phosphorylation of ERK in response to carbachol by approx. 80 %, invalidation of both the RGS and GoLoco domains caused a nearly complete loss of activity of RGS14. As expected, expression of the full-length RGS14 [R519F] mutant molecule, still carrying an intact RGS domain, potently inhibited the phosphorylation of ERK in response to carbachol; however, this effect was only partial (60 % inhibition) and less pronounced than that of the intact molecule (80 % inhibition). Furthermore, expression of the RGS14 [E92A,N93A] mutant, invalidated in its RGS domain, also led to a significant, but not maximal, inhibition

© 2004 Biochemical Society

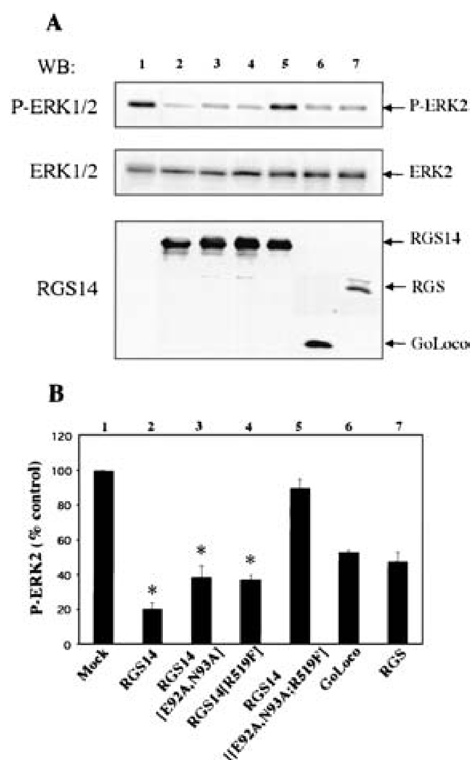

Figure 5 The RGS and GoLoco domains of RGS14 co-operate to down-regulate G_i signalling in HEK-m2 cells

HEK-m2 cells were co-transfected with the $(Myc)_6$–ERK2 reporter construct, together with control plasmid (1) or expression vectors for wild-type RGS14 (lane 2), RGS14 [E92A,N93A] (lane 3), RGS14 [R519F] (lane 4), RGS14 [E92A,N93A,R519F] (lane 5), GoLoco (lane 6) or the isolated RGS domain (lane 7). Serum-starved cells were stimulated for 5 min with 100 μM carbachol, and analysed as in Figure 3. (**A**) Results from a typical experiment; (**B**) the quantified data from four independent experiments were normalized relative to the total levels of $(Myc)_6$–ERK2 expressed. *Significantly greater inhibition by RGS14 compared with RGS14 [E92A,N93A] or RGS14 [R519F] ($P < 0.01$).

of ERK phosphorylation. Further mutation of the GoLoco domain caused a nearly total recovery of ERK phosphorylation. Hence these experiments show that the GoLoco and RGS domains contribute to the inhibitory activity of RGS14 on G_i signalling, and that both domains are required for maximal activity of the protein.

DISCUSSION

Upon activation by ligand binding, serpentine receptors act as GEFs for heterotrimeric G-proteins, stimulating the formation of the active $G\alpha$–GTP complex and concomitant release of the active $\beta\gamma$ dimer. It is thought that RGS proteins, by stimulating GTP hydrolysis, cause re-association of $G\beta\gamma$ with $G\alpha$–GDP, hence terminating the signal. RGS14, identified originally as a novel RGS protein containing a RID [7,26], also contains a GoLoco domain at its C-terminus [9]. GoLoco domains from AGS3 and RGS14 have been shown to inhibit the dissociation of GDP, and hence its exchange for GTP, on isolated $G\alpha_i$ subunits [11,12,14]. In this respect, GoLoco serves a function similar to that of $G\beta\gamma$, although their precise mechanisms of action may differ somewhat [27], a notion supported by structural data showing that a peptide containing the GoLoco domain from RGS14 binds $G\alpha_i$ near the $\beta\gamma$ interface [23]. This led to the proposal that GoLoco peptides could enhance or prolong signalling via the $\beta\gamma$ pathway, by competing with α subunits for rebinding to the $\beta\gamma$ dimer by steric occlusion of the $\beta\gamma$ binding site on α; such competition by the GoLoco domains of AGS3 with $\beta\gamma$ dimers for their binding to $G\alpha$ has indeed been observed *in vitro* [28]. Alternatively, GoLoco peptides could act to activate $G\beta\gamma$ pathways, independently of receptor activation and nucleotide exchange on $G\alpha$ subunits, a hypothesis supported by the observation that peptides containing the GoLoco consensus sequence promote the dissociation of $\beta\gamma$ dimers from $G\alpha$ subunits *in vitro* [29]. However, our data show that the ectopic expression of the GoLoco domain of RGS14, either isolated and targeted to membranes, in tandem with the Rap-binding domain, or in the context of the full-length RGS14 molecule, leads neither to receptor-independent $G\beta\gamma$ signalling nor to the enhancement of $\beta\gamma$-dependent responses; on the contrary, the GoLoco domain from RGS14 reduces the amplitude of the G_i response proceeding via the $\beta\gamma$ pathway downstream of the M_2 mAChR receptor (Figures 4 and 5) without affecting its kinetics (results not shown).

We therefore propose that in cells, where α subunits and $\beta\gamma$ dimers are tethered to the plasma membrane and may be pre-coupled to receptors, the GoLoco domain could somehow interact with $G\alpha_i$–GDP in the intact heterotrimer and inhibit G_i activation induced by the activated receptor. Accordingly, the addition of GoLoco domains from AGS3 to heterotrimeric transducin in the presence of rod outer segment membranes was shown to inhibit binding of guanosine 5′-[γ-thio]triphosphate to transducin in response to illuminated rhodopsin [12]. Several distinct mechanisms, which are not mutually exclusive, could account for the inhibition of G_i signalling by GoLoco. $G\beta\gamma$ subunits have been shown to be essential for the activation of heterotrimeric G-proteins by G-protein-coupled receptors, and may be involved in the process via direct contacts with the activated receptors [30]; it is possible that, through their interaction with $G\alpha$, close to the α–$\beta\gamma$ interface, GoLoco domains could modify the topology of $G\beta\gamma$ in the complex and thereby prevent receptor-induced activation. Another possibility is that the interaction of GoLoco with heterotrimeric G_i could lead to its uncoupling from receptors, as observed with the GoLoco domain from AGS3, which was able to prevent G_i from inducing the high-affinity state of 5-HT_1 receptors for their agonists in membranes from Sf9 cells [10].

Our observations show that the RGS and GoLoco domains of RGS14 contribute independently to the inhibition of $\beta\gamma$-mediated signalling downstream of G_i, and that they act in an additive, rather than synergistic, manner to confer on the intact RGS14 molecule its maximal inhibitory activity. The GoLoco domain prevents G_i molecules from being activated by receptors, while the RGS domain ensures the rapid and efficient return of activated G_i to its resting inactive state. Hence, by combining these two mechanisms, RGS14 could achieve minimum spontaneous G_i activation, thereby minimizing ‘noise’, as well as the transient nature of responses downstream of G_i following receptor stimulation. Further work, using physiologically relevant biological models, will be required to assess the importance of these mechanisms in phenomena such as lymphocyte signalling responses and neural transmission occurring in those tissues where RGS14 is naturally expressed.

We thank Professor Marlene Hosey (University Medical School, Chicago, MI, U.S.A.), Dr John Kehrl (NIH, Bethesda, MD, U.S.A.), Dr Michael Weber (University of Virginia, Charlottesville, VA, U.S.A.) and Dr Anil Bushan (UMR144, Institut Curie, Paris) for providing HEK-m2 cells, cDNAs encoding $G\alpha$ subunits, the cDNA encoding ERK2, and the pCS2 + MT vector respectively. S. T. was the recipient of fellowships from the Ministère Français de l'Education et de la Technologie, the Association pour la Recherche contre le Cancer, and the Fondation Cancer et Solidarité. This work was supported in part by a grant from the Association pour la Recherche contre le Cancer.

© 2004 Biochemical Society

REFERENCES

1 Bourne, H. R. (1997) How receptors talk to trimeric G proteins. Curr. Opin. Cell Biol. **9**, 134–142
2 Hamm, H. E. (1998) The many faces of G protein signaling. J. Biol. Chem. **273**, 669–672
3 Li, Y., Sternweis, P. M., Charnecki, S., Smith, T. F., Gilman, A. G., Neer, E. J. and Kozasa, T. (1998) Sites for Galpha binding on the G protein beta subunit overlap with sites for regulation of phospholipase Cbeta and adenylyl cyclase. J. Biol. Chem. **273**, 16265–16272
4 Ford, C. E., Skiba, N. P., Bae, H., Daaka, Y., Reuveny, E., Shekter, L. R., Rosal, R., Weng, G., Yang, C. S., Iyengar, R. et al. (1998) Molecular basis for interactions of G protein betagamma subunits with effectors. Science **280**, 1271–1274
5 De Vries, L. and Gist Farquhar, M. (1999) RGS proteins: more than just GAPs for heterotrimeric G proteins. Trends Cell Biol. **9**, 138–144
6 Tesmer, J. J., Berman, D. M., Gilman, A. G. and Sprang, S. R. (1997) Structure of RGS4 bound to AlF4-activated G(i alpha1): stabilization of the transition state for GTP hydrolysis. Cell **89**, 251–261
7 Traver, S., Bidot, C., Spassky, N., Baltauss, T., De Tand, M. F., Thomas, J. L., Zalc, B., Janoueix-Lerosey, I. and de Gunzburg, J. (2000) RGS14 is a novel Rap effector that preferentially regulates the GTPase activity of galphao. Biochem. J. **350**, 19–29
8 Cho, H., Kozasa, T., Takekoshi, K., de Gunzburg, J. and Kehrl, J. H. (2000) RGS14, a GTPase-activating protein for Gialpha, attenuates Gialpha- and G13alpha-mediated signaling pathways. Mol. Pharmacol. **58**, 569–576
9 Siderovski, D. P., Diverse-Pierluissi, M. and De Vries, L. (1999) The GoLoco motif: a Galphai/o binding motif and potential guanine-nucleotide exchange factor. Trends Biochem. Sci. **24**, 340–341
10 Peterson, Y. K., Bernard, M. L., Ma, H., Hazard, III, S., Graber, S. G. and Lanier, S. M. (2000) Stabilization of the GDP-bound conformation of Gialpha by a peptide derived from the G-protein regulatory motif of AGS3. J. Biol. Chem. **275**, 33193–33196
11 De Vries, L., Fischer, T., Tronchere, H., Brothers, G. M., Strockbine, B., Siderovski, D. P. and Farquhar, M. G. (2000) Activator of G protein signaling 3 is a guanine dissociation inhibitor for Galpha i subunits. Proc. Natl. Acad. Sci. U.S.A. **97**, 14364–14369
12 Natochin, M., Lester, B., Peterson, Y. K., Bernard, M. L., Lanier, S. M. and Artemyev, N. O. (2000) AGS3 inhibits GDP dissociation from galpha subunits of the Gi family and rhodopsin-dependent activation of transducin. J. Biol. Chem. **275**, 40981–40985
13 Natochin, M., Gasimov, K. G. and Artemyev, N. O. (2001) Inhibition of GDP/GTP exchange on G alpha subunits by proteins containing G-protein regulatory motifs. Biochemistry **40**, 5322–5328
14 Kimple, R. J., De Vries, L., Tronchere, H., Behe, C. I., Morris, R. A., Gist Farquhar, M. and Siderovski, D. P. (2001) RGS12 and RGS14 GoLoco motifs are G alpha(i) interaction sites with guanine nucleotide dissociation inhibitor activity. J. Biol. Chem. **276**, 29275–29281
15 Hollinger, S., Taylor, J. B., Goldman, E. H. and Hepler, J. R. (2001) RGS14 is a bifunctional regulator of Galphai/o activity that exists in multiple populations in brain. J. Neurochem. **79**, 941–949
16 Nancy, V., Wolthuis, R. M., de Tand, M. F., Janoueix-Lerosey, I., Bos, J. L. and de Gunzburg, J. (1999) Identification and characterization of potential effector molecules of the Ras-related GTPase Rap2. J. Biol. Chem. **274**, 8737–8745
17 Pals-Rylaarsdam, R., Xu, Y., Witt-Enderby, P., Benovic, J. L. and Hosey, M. M. (1995) Desensitization and internalization of the m2 muscarinic acetylcholine receptor are directed by independent mechanisms. J. Biol. Chem. **270**, 29004–29011
18 Koch, W. J., Hawes, B. E., Allen, L. F. and Lefkowitz, R. J. (1994) Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by G beta gamma activation of p21ras. Proc. Natl. Acad. Sci. U.S.A. **91**, 12706–12710
19 Franke, B., Akkerman, J. W. and Bos, J. L. (1997) Rapid Ca^{2+}-mediated activation of Rap1 in human platelets. EMBO J. **16**, 252–259
20 Beranger, F., Goud, B., Tavitian, A. and de Gunzburg, J. (1991) Association of the Ras-antagonistic Rap1/Krev-1 proteins with the Golgi complex. Proc. Natl. Acad. Sci. U.S.A. **88**, 1606–1610
21 Nassar, N., Horn, G., Herrmann, C., Block, C., Janknecht, R. and Wittinghofer, A. (1996) Ras/Rap effector specificity determined by charge reversal. Nat. Struct. Biol. **3**, 723–729
22 Block, C., Janknecht, R., Herrmann, C., Nassar, N. and Wittinghofer, A. (1996) Quantitative structure-activity analysis correlating Ras/Raf interaction *in vitro* to Raf activation *in vivo*. Nat. Struct. Biol. **3**, 244–251
23 Kimple, R. J., Kimple, M. E., Betts, L., Sondek, J. and Siderovski, D. P. (2002) Structural determinants for GoLoco-induced inhibition of nucleotide release by Galpha subunits. Nature (London) **416**, 878–881
24 Crespo, P., Xu, N., Simonds, W. F. and Gutkind, J. S. (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. Nature (London) **369**, 418–420
25 Ohba, Y., Mochizuki, N., Matsuo, K., Yamashita, S., Nakaya, M., Hashimoto, Y., Hamaguchi, M., Kurata, T., Nagashima, K. and Matsuda, M. (2000) Rap2 as a slowly responding molecular switch in the Rap1 signaling cascade. Mol. Cell. Biol. **20**, 6074–6083
26 Snow, B. E., Antonio, L., Suggs, S., Gutstein, H. B. and Siderovski, D. P. (1997) Molecular cloning and expression analysis of rat Rgs12 and Rgs14. Biochem. Biophys. Res. Commun. **233**, 770–777
27 Natochin, M., Gasimov, K. G. and Artemyev, N. O. (2002) A GPR-protein interaction surface of Gi(alpha): implications for the mechanism of GDP-release inhibition. Biochemistry **41**, 258–265
28 Bernard, M. L., Peterson, Y. K., Chung, P., Jourdan, J. and Lanier, S. M. (2001) Selective interaction of AGS3 with G-proteins and the influence of AGS3 on the activation state of G-proteins. J. Biol. Chem. **276**, 1585–1593
29 Ghosh, M., Peterson, Y. K., Lanier, S. M. and Smrcka, A. V. (2003) Receptor- and nucleotide exchange-independent mechanisms for promoting G protein subunit dissociation. J. Biol. Chem. **278**, 34747–34750
30 Rondard, P., Iiri, T., Srinivasan, S., Meng, E., Fujita, T. and Bourne, H. R. (2001) Mutant G protein alpha subunit activated by Gbeta gamma: A model for receptor activation? Proc. Natl. Acad. Sci. U.S.A. **98**, 6150–6155

Received 8 December 2003; accepted 3 February 2004
Published as BJ Immediate Publication 3 February 2004, DOI 10.1042/BJ20031889

© 2004 Biochemical Society