RGS3 interacts with 14-3-3 via the N-terminal region distinct from the RGS (regulator of G-protein signalling) domain

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RGS3 belongs to a family of the regulators of G-protein signalling (RGS), which bind and inhibit the $G\alpha$ subunits of heterotrimeric G-proteins via a homologous RGS domain. Increasing evidence suggests that RGS proteins can also interact with targets other than G-proteins. Employing yeast two-hybrid screening of a cDNA library, we identified an interaction between RGS3 and the phosphoserine-binding protein 14-3-3. This interaction was confirmed by *in vitro* binding and co-immunoprecipitation experiments. RGS3-deletion analysis revealed the presence of a single 14-3-3-binding site located outside of the RGS domain. Ser²⁶⁴ was then identified as the 14-3-3-binding site of RGS3. The S²⁶⁴A mutation resulted in the loss of RGS3 binding to 14-3-3, without affecting its ability to bind $G\alpha_q$. Signalling studies showed that the S²⁶⁴A mutant was more potent than the

wild-type RGS3 in inhibition of G-protein-mediated signalling. Binding experiments revealed that RGS3 exists in two separate pools, either 14-3-3-bound or G-protein-bound, and that the 14-3-3-bound RGS3 is unable to interact with G-proteins. These data are consistent with the model wherein 14-3-3 serves as a scavenger of RGS3, regulating the amounts of RGS3 available for binding G-proteins. This study describes a new level in the regulation of G-protein signalling, in which the inhibitors of G-proteins, RGS proteins, can themselves be regulated by phosphorylation and binding 14-3-3.

Key words: G-protein, phosphorylation, regulation, RGS protein, signalling.

INTRODUCTION

RGS3 belongs to a family of proteins which share the core regulator of G-protein signalling (RGS) domain and function as GTPase-activating proteins (GAPs) for the α subunit of heterotrimeric G-proteins [1–4]. The GAP activity of RGS proteins results in a tight control of signalling, which is induced by seven transmembrane receptors and mediated by heterotrimeric G-proteins [5]. Increasing evidence indicates that the non-RGS regions of RGS proteins contain structurally distinct domains, which interact with targets other than heterotrimeric G-proteins [6], suggesting that RGS proteins have multiple functions. For example, the protein kinase A (PKA)-anchoring protein, D-AKAP2, which contains an RGS domain, binds the regulatory subunit of PKA [7]. RGS6, RGS7, RGS9, RGS11 and EGL-10 (encoded by the ‘egg-laying abnormal’ genes in *Caenorhabditis elegans*) directly bind the β_5 subunit of heterotrimeric G-proteins [8,9]. A number of RGS proteins bind the Rho family of small GTPases, functioning as their guanine-nucleotide-exchange factors [10,11]. $G\alpha$ -interacting protein (GAIP) binds the PDZ domain of GAIP-interacting protein C-terminus (GIPC) [12]. On the other hand, a number of RGS proteins themselves possess the PDZ domain, allowing interactions with other targets [13–17]. The N-terminal tail of RGS3 is three times larger than its C-terminal RGS domain; however, the function of this region is poorly understood. In the present study, we identify a 14-3-3-binding site in the N-terminal region of RGS3 outside of its RGS domain.

14-3-3 proteins constitute a family of conserved eukaryotic 28–30 kDa proteins which bind their targets at phosphoserine or phosphothreonine sites and which participate in the regulation of diverse biological processes related to signal transduction and cell-cycle control [18]. Two 14-3-3 recognition motifs have been identified: RSXpSXP (type 1; where pS is phosphoserine) and RX₁₋₂pS₂₋₃pS (type 2) [18]. Among the established functions of 14-3-3 are the regulation of the intracellular localization and enzymic activity of effector proteins implicated in gene expression, cell growth and survival [18–21]. In the present study, we show that 14-3-3 interferes with RGS3’s ability to bind G-proteins, even though the 14-3-3-binding site is distant from the RGS domain of RGS3. We identified Ser²⁶⁴ as a 14-3-3-binding site of RGS3. The S²⁶⁴A mutation of RGS3 resulted in the loss of 14-3-3 binding and in increased potency for both G-protein binding and regulation of G-protein-mediated signalling.

MATERIALS AND METHODS

Yeast two-hybrid screening

A human full-length (FL) RGS3 cDNA was subcloned in-frame with LexA in pEG202 using *EcoRI* and *XhoI* as unique restriction sites. A mouse pre-B-cell cDNA library inserted into the *EcoRI* and *XhoI* sites of PJG4-5 was kindly provided by Dr Frederick Alt (Harvard Medical School, Boston, MA, U.S.A.). Transformation of the yeast strain EGY48 and selection on the appropriate media was performed as described previously [22]. A total of 1.3×10^6 primary transformants were screened, and

Abbreviations used: CHO, Chinese hamster ovary; ET1, endothelin-1; ET_A, type A endothelin receptor; GFP, green fluorescent protein; EGFP, enhanced GFP; FL, full-length; GAP, GTPase-activating protein; MBP, maltose-binding protein; PKA, protein kinase A; RGS, regulator of G-protein signalling; WT, wild-type; GAIP, $G\alpha$ -interacting protein.

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86 colonies were selected, 18 of which were β -galactosidase-positive. After growth control experiments for selectable markers and β -galactosidase assays, the independently isolated plasmids coding for interacting protein sequences were obtained. We isolated six independent clones containing an approx. 1.2 kb insert for 14-3-3 τ and two independent clones with 1.8 kb inserts encoding 14-3-3 ϵ . All clones were in-frame with the GAL4 activation domain. Two plasmids encoding the two isoforms of 14-3-3 proteins were used in control experiments for growth on selective media and β -galactosidase activity in a yeast strain transformed with RGS3.

Cell culture and transient transfection of cDNA

Chinese hamster ovary (CHO) cells were maintained in Ham's F12 medium supplemented with 2 mM glutamine, 100 units/ml streptomycin, 100 units/ml penicillin and 10% fetal bovine serum. For transient expression of proteins, subconfluent cells were transfected with cDNA using LIPOFECTAMINE^{TM} Plus reagent (Gibco-BRL, Gaithersburg, MD, U.S.A.) for 24–48 h, following the manufacturer's protocol.

Immunoprecipitation, pull-down assays and Western blotting

Generally, the lysates from 2×10^6 transfected cells were used for one immunoprecipitation or pull-down experiment. The CHO cells were washed twice with ice-cold PBS and lysed in extraction buffer (300 μ l/ 10^6 cells) containing 150 mM NaCl, 25 mM Hepes (pH 7.4), 1% Triton X-100, 2 mM EDTA, 10 mM NaF, 100 μ M orthovanadate and protease inhibitors (200 μ M PMSF, 2 μ g/ml leupeptin and 2 μ g/ml aprotinin). The lysates were cleared by centrifugation at 20000 *g* for 5 min. The immunoprecipitation employing RGS3 antibodies (see Figures 1 and 2, below) was performed by a 2 h incubation of cleared lysates with 5 μ l of RGS3 antiserum or preimmune serum, followed by a 1 h incubation with 15 μ l of agarose beads conjugated with Protein A. The beads were then washed three times with 1 ml of lysis buffer and boiled in Laemmli buffer.

For immunoprecipitation of Myc-tagged RGS3 and its fragments (see Figures 3, 4, 6 and 7, below), the cell lysates were incubated with 8 μ l of agarose beads conjugated to Myc antibodies for 2 h, followed by three washes with the lysis buffer. In the pull-down experiments (see Figures 2, 3 and 6, below) the lysates were incubated with 20 μ l of Sepharose beads conjugated to maltose-binding protein (MBP) or MBP-14-3-3 ζ for 2 h, followed by three washes with lysis buffer. The samples were then boiled in Laemmli buffer for 5 min, subjected to PAGE (12% gel) and analysed by Western blotting, employing 1 μ g/ml primary antibodies followed by a 1:3000 dilution of horseradish peroxidase-conjugated secondary antibodies.

[³⁵S]methionine labelling of intact cells

The [³⁵S]methionine labelling of intact cells was performed by overnight incubation of cells with 0.1 mCi/ml [³⁵S]methionine in a mixture of complete growth medium and methionine-free medium at a ratio of 1:10. The cells were washed three times, followed by stimulation with desired agonists.

Elk1-luciferase *trans*-reporter assay

The endothelin-1 (ET1)-induced activity of Elk1 was assessed by luciferase PathDetect *trans*-reporter system (Stratagene, La Jolla, CA, U.S.A.) as described previously [23]. CHO cells (0.2×10^6 /well) grown on 24-well plates were transfected with

Figure 1 Co-immunoprecipitation of RGS3 and interacting proteins from [³⁵S]methionine-labelled cell lysates

(A) Schematic diagram of the FL-RGS3 and its N-terminal (1–379) fragment, RGS3N. (B) CHO cells were transfected with the cDNAs for RGS3 or RGS3N and labelled with [³⁵S]methionine overnight. The cells were then lysed in buffer containing 150 mM NaCl, 25 mM Hepes (pH 7.4), 1% Triton X-100, 2 mM EDTA, 10 mM NaF, 100 μ M orthovanadate and protease inhibitors. The cell lysates were subjected to immunoprecipitation (IP) with preimmune serum (–) or with anti-RGS3 serum (+). The proteins in the immune complexes were separated by electrophoresis and analysed by autoradiography.

the following plasmids (per well): 200 ng of pFR-Luciferase (reporter plasmid), 12.5 ng of pFA2-ELK1 (fusion *trans*-activator plasmid), 50 ng of pCMV-LacZ (transfection-efficiency control plasmid), 50 ng of pME18SF-ETA [type A endothelin receptor (ET_A) cDNA] and different amounts of the cDNAs for wild-type (WT) RGS3 or the S²⁶⁴A mutant, balanced with empty pCMV vector to a total of 200 ng. The day before stimulation the cells were serum-starved in 0.2% fetal bovine serum overnight, followed by incubation with 100 nM ET1 for 4 h. The cells were washed twice with PBS, lysed in protein extraction reagent, and the cleared lysates were assayed for luciferase and β -galactosidase activity using the corresponding assay kits (Promega, Madison, WI, U.S.A.). In order to account for differences in transfection efficiency, luciferase activity of each sample was normalized to β -galactosidase activity. The values of control samples were subtracted from the corresponding values of ET1-stimulated samples and expressed as a percentage of the maximal response to ET1.

DNA and reagents

The original human RGS3 cDNA was cloned and described previously [2]. The cDNAs for the Myc-tagged RGS3 and its fragments were obtained by subcloning into the pCMV-tag3 vector (Stratagene) to introduce the Myc tag at the 5' end of each insert. As was shown previously, addition of tags to other RGS proteins (RGS10, RGS4 and GAIP) did not affect their

Figure 2 The N-terminal region of RGS3 mediates the interaction between RGS3 and 14-3-3

CHO cells were transfected with the empty vector, or with the cDNAs for RGS3 or RGS3N as indicated. The cells were lysed as in Figure 1, and the cell extracts were subjected to immunoprecipitation with RGS3 antibodies (A, B), or to pull down with Sepharose beads conjugated to MBP or MBP-14-3-3 ζ (D). The binding complexes (A, B, D) or total cell lysates (C) were analysed by Western blotting with RGS3 antibodies (A, D), or with 14-3-3 pan antibodies (B, C).

RGS function [3,24,25]. The RGS3 mutants were generated by site-directed mutagenesis and confirmed by sequencing. The cDNA for ET_A [26] was kindly provided by Dr Masashi Yanagisawa (University of Texas Southwestern Medical Center at Dallas, Dallas, TX, U.S.A.). The fusion protein of 14-3-3 ζ with MBP conjugated to Sepharose beads was prepared as described previously [27]. Polyclonal anti-RGS3 antibodies against N-terminus of RGS3 were generated in this laboratory and described previously [28]. Antibodies generated against 14-3-3 ϵ , which recognize all isoforms of 14-3-3, were from Upstate Biotechnology (Waltham, MA, U.S.A.). Anti- α_q/α_{11} antibodies and the agarose-conjugated anti-Myc antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-Myc antibodies for Western blotting and protease inhibitors were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). ET1 was from Calbiochem (Cambridge, MA, U.S.A.).

RESULTS

Identification of 14-3-3 as an RGS3-binding protein

To assess whether RGS3 interacts with proteins other than G-proteins via the N-terminal region, we immunoprecipitated FL-RGS3 and the N-terminal (1–379) fragment, RGS3N (Figure 1A), from transiently transfected and [³⁵S]methionine-labelled CHO cells. The immunoprecipitation was performed under conditions that maintained G-proteins in the inactive state. As shown in Figure 1(B), three proteins with approx. molecular masses of 30, 100 and 130 kDa co-precipitated with both FL-RGS3 and RGS3N. This suggests that RGS3 has targets distinct from G-proteins.

To identify the RGS3-interacting proteins, we performed a yeast two-hybrid screening of the mouse pre-B-cell cDNA library, using FL-RGS3 as bait. Six clones containing the coding region of 14-3-3 τ and two clones containing the coding region of 14-3-3 ϵ were isolated. In each case, the coding sequence was in-frame with the GAL4 activation domain. Two plasmids, each encoding one of the two identified isoforms of 14-3-3, underwent re-transformation in control experiments for growth and

β -galactosidase activity in a yeast strain transformed with RGS3. These experiments confirmed the yeast two-hybrid interaction between RGS3 and the two 14-3-3 isotypes (results not shown). Finally, we confirmed the binding of RGS3 and RGS3N to 14-3-3 by co-immunoprecipitation with anti-RGS3 antibodies (Figures 2A–2C) and by the pull down of RGS3 from cell lysates with Sepharose beads conjugated to MBP-14-3-3 ζ (Figure 2D).

Identification of the 14-3-3-binding site of RGS3

In order to identify the 14-3-3-binding site, the deletion mutants of RGS3 were generated and examined for their ability to bind 14-3-3. The Myc-tagged RGS3-deletion mutants, transiently expressed in CHO cells, were immunoprecipitated with Myc antibodies, followed by immunoblotting with 14-3-3 pan antibodies (recognizing all 14-3-3 isoforms). An example of these experiments is shown in Figures 3(A)–3(C), and the overall data are summarized schematically in Figure 3(F). The initial experiments employed deletion mutants generated by convenient restriction enzymes, and they identified the RGS3 (157–291) fragment as a potential 14-3-3-binding region (Figures 3A–3C). Because this region still contained at least two possible 14-3-3-binding sites, more RGS3 fragments were generated using PCR, narrowing the 14-3-3-binding region to residues 240–275. To confirm these data, a plasmid was generated, which encoded a fusion protein of RGS3 (240–275) with enhanced green fluorescent protein (EGFP). The EGFP-RGS3 (240–275) fusion protein, but not control EGFP, was pulled down by the MBP-14-3-3 ζ beads, as assessed by immunoblotting with GFP antibodies (Figures 3D and 3E). In addition, RGS3 (379–519), which comprises the RGS domain of RGS3, as well as RGS3 (314–519), termed RGS3T and expressed endogenously in a variety of tissues [29], failed to interact with 14-3-3 (results not shown). This suggests that the RGS domain of RGS3 does not mediate the interaction between RGS3 and 14-3-3.

Peptide sequence analysis of the RGS3 (240–275) fragment revealed the presence of a single putative 14-3-3-binding site, RTHSEGS²⁶⁷, which is consistent with the type-2 binding motif

Figure 3 Identification of RGS3 (240–275) as a 14-3-3-binding region

The CHO cells were transfected with the cDNAs for the Myc-tagged (A–C) or EGFP-tagged (D, E) RGS3 fragments. The cells were lysed as in Figure 1, and the cell extracts were subjected to immunoprecipitation with Myc antibodies (A, B), or to the pull down with the MBP-14-3-3 ζ beads (D). The binding complexes (A, B, D) or total cell lysates (C, E) were analysed by Western blotting with Myc antibodies (A), 14-3-3 pan antibodies (B, C) or GFP antibodies (D, E). (F) Schematic diagram of RGS3 fragments analysed on 14-3-3 binding. Box represents the RGS domain.

for 14-3-3 [18]. Because either of the serine residues in this motif could be important for binding 14-3-3, the following mutants were generated and examined for their ability to interact with 14-3-3: S²⁶⁴A, S²⁶⁷A and Δ (S²⁶⁴-S²⁶⁷). As shown in Figure 4(A), the S²⁶⁴A and Δ (S²⁶⁴-S²⁶⁷) mutants of RGS3 failed to bind endogenous 14-3-3. The S²⁶⁷A mutant still bound 14-3-3, but by a lesser extent than WT-RGS3 (Figure 4B). Similar results were obtained by pull-down experiments using MBP-14-3-3 ζ beads (results not shown). These data indicate that Ser²⁶⁴ is required for the interaction of RGS3 with 14-3-3 and that its mutation abrogates this interaction.

Role of 14-3-3 in the regulation of G-protein signalling by RGS3

To assess the role of 14-3-3 in the regulation of G-protein signalling by RGS3, we examined the effect of WT-RGS3 and S²⁶⁴A expression on ET1-induced, G_q-mediated activation of transcription factor Elk1, a sensitive and quantitative assay for the signalling of G-protein-coupled receptors [23]. After the transfection of CHO cells with the cDNA for ET_A, ET1 stimulated an approx. 10-fold increase in the transcriptional activity of Elk1, as measured by luciferase *trans*-reporter assay (Figure 5A). Comparison of the dose responses with the increasing concentrations of WT and S²⁶⁴A cDNAs revealed that S²⁶⁴A was at least 10-fold more potent in its inhibition of ET1-induced Elk1 activity. A maximal 50% decrease of Elk1 activity was

Figure 4 Identification of Ser²⁶⁴ as a 14-3-3-binding site of RGS3

CHO cells were transfected with empty vector, or with the cDNAs for Myc-tagged WT-RGS3 or various RGS3 mutants as indicated. The cells were lysed as in Figure 1, and the cell extracts were subjected to immunoprecipitation with Myc antibodies (A, B). The immune complexes (A, B) or total cell lysates (C) were analysed by Western blotting with Myc antibodies (A) or 14-3-3 pan antibodies (B, C).

achieved by the transfection of S²⁶⁴A cDNA at 100 ng/10⁶ cells, whereas a similar effect was achieved by transfection of WT-RGS3 cDNA at 1 μ g/10⁶ cells. The equal expression of both proteins at given concentrations of transfected DNA was confirmed by Western blotting with RGS antibodies (Figure 5B). Interestingly, at the high levels of expression, the inhibitory effects of WT and S²⁶⁴A appeared similar, reaching 50–60% inhibition depending on the experiment (Figure 5A). These data suggest that, when RGS3 is expressed at low levels, its interaction with 14-3-3 plays a negative role in inhibition of G-protein signalling, whereas at high levels of expression the effect of this interaction becomes negligible.

RGS3 exists in two distinct pools: 14-3-3-bound and G-protein-bound

The increased potency of S²⁶⁴A in the inhibition of G-protein signalling may be explained by the interference of 14-3-3 with WT-RGS3 binding to G-proteins, limiting the availability of RGS3 for G-proteins. To test this hypothesis, we first examined whether the 14-3-3-bound RGS3 was able to interact with G-proteins as effectively as the 14-3-3-unbound RGS3 in the presence of AlF₄⁻, which maintains the active state of G α subunits, providing for the binding of RGS proteins [4] (Figure 6). The experimental conditions were adjusted in such a way that the amounts of the total pool of RGS3 immunoprecipitated with Myc antibodies (Figure 6A, lane 1) would be similar to 14-3-3-bound RGS3, pulled down by MBP-14-3-3 ζ beads (Figure 6A, lane 4). This enabled an assessment of the relative amounts of endogenous G α_q , co-precipitated with these two pools of RGS3. As expected, the appreciable amounts of endogenous G α_q (Figure 6C, lane 1) and endogenous 14-3-3 (Figure 6B, lane 1) were present in the total pool of RGS3 immunoprecipitated with Myc antibodies. However, only a minor amount of G α_q was pulled down with MBP-14-3-3 ζ beads and it was not dependent on the presence of exogenous RGS3 (Figure 6C, lanes 4 and 5). This suggests that the 14-3-3-bound RGS3 is unable to interact with G α_q , or at least that its ability to bind G α_q is dramatically reduced.

Taking this into consideration, we next hypothesized that, at low levels of expression, the binding of RGS3 to 14-3-3 may

Figure 5 Effects of WT and S²⁶⁴A mutant of RGS3 on ET1-induced activation of transcription factor Elk1

(A) CHO cells grown in 24-well plates at a density of 0.2×10^6 cells/well were transfected with (per well): 200 ng of pFR-Luciferase (reporter plasmid), 12.5 ng of pFA2-ELK1 (fusion *trans*-activator plasmid), 50 ng of pCMV-LacZ (transfection-efficiency control plasmid) 50 ng of pME18SF-ETA (ET_A cDNA), together with increasing concentrations of the cDNAs for the WT or S²⁶⁴A proteins balanced with the empty vector to a total of 200 ng/well ($1 \mu\text{g}/10^6$ cells). The day before stimulation, the cells were serum-starved in 0.2% fetal bovine serum overnight, followed by incubation with 100 nM ET1 for 4 h. Luciferase activity was then measured in the cell extracts, normalized to β -galactosidase activity and finally expressed as a percentage of the maximal response to ET1. Data represent means $\pm$ S.D. from one of three independent experiments performed in quadruplicate. (B) The expression of WT and S²⁶⁴A proteins in cell lysates from the non-stimulated cells was determined by Western blotting with RGS3 antibodies.

result in a reduced availability of RGS3 to G-proteins. If this hypothesis is true, then the S²⁶⁴A mutant, which does not bind 14-3-3, should be more available for the interaction with G α_q , explaining the differential potency in the regulation of G-protein signalling. To test this hypothesis, we compared WT and S²⁶⁴A binding to G α_q when expressed at low (10 ng/10⁶ cells), medium (100 ng/10⁶ cells) and high (1 $\mu\text{g}/10^6$ cells) levels, proportional to that used in the signalling studies (Figure 5). As shown in Figure 7, overexpression and immunoprecipitation of increasing amounts of WT-RGS3 (Figure 7A) revealed increasing binding of WT-RGS3 to G α_q , reaching a maximum at medium levels and not increasing at higher levels of RGS3 expression (Figure 7B). By contrast, the binding of WT-RGS3 to 14-3-3 was not saturated at medium levels and was further increased at high levels of expression (Figure 7C). This suggests that in our cellular model the pool of 14-3-3 available for RGS3 is abundant and is larger than that of G-proteins. Furthermore, even though both WT and S²⁶⁴A bound G α_q at low levels of expression, the amounts of S²⁶⁴A-bound G α_q were significantly higher than that of the WT-bound G α_q (Figure 7B, compare lanes 2 and 3, reading left to right). This difference in G α_q binding was diminished when WT and S²⁶⁴A were expressed at higher levels, which is consistent with the signalling studies, wherein at high levels of expression the inhibitory effects of WT and S²⁶⁴A were similar (Figure 6). Some discrepancy between Figures 5 and 7 can be noticed at the medium levels of RGS3 expression (100 ng/10⁶ cells), wherein S²⁶⁴A elicited a stronger inhibition of ET1-induced Elk1 activity (Figure 5A), despite a similar binding of WT and S²⁶⁴A to G α_q (Figure 7B, compare lanes 4 and 5). However, one should not compare these data in a precise manner, but should consider the overall trend, given the differential design of the luciferase and co-immunoprecipitation experiments.

Taken together, our signalling data (Figure 5) and binding experiments (Figures 6 and 7) suggest that 14-3-3 interferes with

Figure 6 RGS3 exists in two distinct pools: 14-3-3-bound and G-protein-bound

CHO cells were transfected with the empty vector or with WT-RGS3 cDNA as indicated, and lysed in buffer containing 150 mM NaCl, 25 mM Hepes (pH 7.4), 0.5% Nonidet P-40, 2 mM MgCl₂, 10 mM NaF, 30 μM AlCl₃ and protease inhibitors. These lysis conditions provide the stabilized transition state of G α subunits, which is optimal for binding RGS proteins. Equal amounts of cell lysates were incubated with 8 μl of agarose beads conjugated to the Myc antibodies (lanes 1 and 2), or with 20 μl of Sepharose beads conjugated to MBP (lane 3) or MBP-14-3-3 (lanes 4 and 5) for 2 h. The beads were then washed three times with lysis buffer, and the bound proteins were analysed by Western blotting with the antibodies against Myc (A), 14-3-3 (B) and G α_q (C). (D) Schematic diagram of Myc-tagged RGS3 showing the binding sites for Myc antibodies, 14-3-3 and G α_q .

Figure 7 Binding of the WT and S²⁶⁴A mutant of RGS3 to G α_q /11 and 14-3-3 at various levels of expression

CHO cells (2×10^6 /transfection) were transfected with increasing concentrations of the cDNAs for the WT or S²⁶⁴A balanced with the empty vector to a total of 2 μg /transfection ($1 \mu\text{g}/10^6$ cells). The cells were lysed in buffer as in Figure 6, and the cell lysates were subjected to immunoprecipitation with 8 μl of agarose beads conjugated with Myc antibodies. The immune complexes (A–C) or total cell lysates (D, E) were analysed by Western blotting with antibodies against RGS3 (A), G α_q /11 (B, D), or 14-3-3 (C, E). V, vector.

the ability of RGS3 to bind G-proteins and to inhibit G-protein signalling, despite the fact that the 14-3-3-binding site of RGS3 is distant from its RGS domain (Figures 2 and 3).

DISCUSSION

The rationale for the present study was based on our observation that the non-RGS N-terminal region of RGS3 co-immunoprecipitated with proteins other than G α subunits, as mentioned in our previous report [28] and as demonstrated here (Figure 1). Furthermore, the yeast two-hybrid screening of the cDNA library identified 14-3-3 ϵ and 14-3-3 τ as RGS3-interacting proteins. The interaction between RGS3 and 14-3-3 was confirmed by co-immunoprecipitation of RGS3 with endogenous 14-3-3 (Figures 2A–2C), as well as by *in vitro* pull-down experiments employing MBP-14-3-3 ζ beads (Figure 2D).

While these studies were in progress, a report was published by Benzing et al. [30] showing the interaction between 14-3-3 and two RGS proteins, RGS7 and RGS3. The 14-3-3-binding site of RGS7 localized within its RGS domain at Ser⁴³⁴. The aspartate mutation S⁴³⁴D of its RGS domain provided binding to 14-3-3 *in vitro*. Based on the crystal structure of RGS4 complexed with activated G α_i [31], the conserved Ser⁴³⁴ residue of RGS7 aligns with the contact point of the RGS domain with activated G α_i . Therefore, it was surprising that *in vitro* phosphorylation of RGS7 (presumably at residue Ser⁴³⁴) had a modest effect on its GAP activity [30]. Nevertheless, the GAP activity of phosphorylated RGS7 was significantly reduced in the presence of 14-3-3, presumably due to the competition between 14-3-3 and G α_{11} for RGS7 binding, although the latter is still to be ascertained [30].

In the latter report, RGS3 also interacted with 14-3-3 [30]. However, the 14-3-3-binding site of RGS3 was not identified. It was speculated that Ser⁴⁹⁶ of RGS3, analogous to Ser⁴³⁴ of RGS7, may mediate the interaction between RGS3 and 14-3-3. However, the amino acid sequence of RGS3 flanking Ser⁴⁹⁶, MEKDS⁴⁹⁶YP, does not fit to the known 14-3-3-binding motifs [18]. Moreover, our data have shown that it was the N-terminus of RGS3, not its RGS domain, that interacted with 14-3-3 (Figures 2 and 3). In the present study we identified Ser²⁶⁴ as a 14-3-3-binding site of RGS3 and examined the functional significance of this interaction employing the S²⁶⁴A mutant, which was deficient in binding to 14-3-3. We show that 14-3-3-bound RGS3 loses its ability to interact with G-proteins, despite the fact that its 14-3-3-binding site is distant from the RGS domain. Accordingly, the S²⁶⁴A mutant, which is deficient in binding 14-3-3, elicits a higher potency in G-protein binding and in the regulation of G-protein signalling. To our knowledge, this is the first report showing that a point mutation of RGS protein can enhance its regulatory function towards G-proteins, which is of fundamental and practical importance.

Despite the differences between RGS3 and RGS7 in the 14-3-3-binding regions, our data and the results of Benzing et al. [30] have lead to similar conclusions, revealing a new level in the regulation of G-protein signalling. The activation cycle of heterotrimeric G-proteins, involving GDP/GTP exchange followed by GTP hydrolysis, is a well-established paradigm. In this cycle, the G-protein-coupled receptors promote the activation of G-proteins, whereas RGS proteins regulate G-protein signalling by binding the activated G α subunits and stimulating their GTPase activity [32]. On the other hand, RGS3 itself can be regulated by 14-3-3. When bound to 14-3-3, RGS3 is unable to bind G-proteins (Figure 6); therefore, the available pool of RGS3 for G-proteins is limited (Figure 7), resulting in the reduced potency of RGS3 in the inhibition of G-protein signalling (Figure 5).

The mechanism by which 14-3-3 interferes with the RGS3 binding to G-proteins has yet to be investigated. Considering that the 14-3-3-binding site of RGS3 is distant from the RGS

domain (more than 100 amino acids away) and that 14-3-3 exists in cells in the form of a dimer [33], one might suggest that the second molecule of the 14-3-3 dimer could be responsible for interfering with RGS3 binding to G-proteins. This possibility will be investigated in the future using dimerization-deficient mutants of 14-3-3. On the other hand, the second molecule of the 14-3-3 dimer can bind other ligands, providing a possibility for the indirect interaction between RGS3 and other proteins. The examples of 14-3-3 functioning as a 'bridge' between two interacting proteins include the 14-3-3-mediated complex of Raf and Bcr [34], as well as of Raf and Cdc25 [35], even though the functional significance of such interactions is unclear. In accordance with this notion is our observation that RGS3 and RGS3N co-immunoprecipitate with p100 and p130 proteins (Figure 1), although the role of 14-3-3 in these interactions is still to be examined.

The phosphorylation and dephosphorylation of RGS3 are important issues, which may provide the mechanism for RGS3 regulation by 14-3-3, and which are still to be investigated in detail. Our initial data indicate that in transfected CHO cells, RGS3 is phosphorylated under the basal conditions, and the stimulation of cells with forskolin, phorbol ester or serum does not significantly change the phosphorylation status of RGS3 nor its binding to 14-3-3 (results not shown). This may suggest that phosphorylation of RGS3 is not mediated by PKA, protein kinase C or other protein kinases stimulated by these agonists. Alternatively, it could be the basal activity of some protein kinase and/or a lack of dephosphorylation that provide conditions sufficient for RGS3 phosphorylation. Peptide sequence analysis of the 14-3-3-binding site suggested the possible role of PKA in phosphorylation of RGS3. In support of PKA involvement, the recombinant GST-RGS3N was phosphorylated by PKA and bound 14-3-3 ϵ *in vitro* (results not shown). Moreover, 14-3-3 ϵ protected the *in vitro*-phosphorylated GST-RGS3N from dephosphorylation by alkaline phosphatase (results not shown). If this is also true in intact cells one might suggest that 14-3-3 is a preferential binding target of RGS3; and once bound to 14-3-3, RGS3 is unlikely to be dephosphorylated and released from it.

RGS3 phosphorylation is not unique within the RGS family, as the phosphorylation of other RGS proteins has been reported. Interestingly, phosphorylation appears to elicit differential effects on the function of RGS proteins. The yeast Sst2p can be phosphorylated by mitogen-activated protein kinase, which does not affect its GAP activity, but decreases the rate of its degradation [36]. The phosphorylation of RGS2 by protein kinase C [37], of RGS9-1 by PKA [38] and of RGS16 by an as-yet unknown protein kinase [39], attenuates their GAP activity. By contrast, phosphorylation of GAIP by mitogen-activated protein kinase [40] and of RGS16 by the epidermal growth factor receptor [41] increases their GAP activity. However, in most cases, the mechanism by which phosphorylation affects the function of RGS proteins is not clear. In the present study we provide the mechanism by which phosphorylation of RGS3 regulates its function, wherein the phosphorylation-dependent interaction between RGS3 and 14-3-3 impairs the ability of RGS3 to bind G-proteins, and therefore reduces the pool of RGS3 available for G-proteins.

Phosphorylation of RGS proteins can also affect their intracellular localization. Phosphorylation of GAIP by casein kinase-2 is associated with its localization to the membrane fraction, particularly to clathrin-coated vesicles [42]. Phosphorylation of RGS10 by PKA does not impair its GAP activity, but results in its translocation from the cytoplasm to the nucleus [43]. Regarding nuclear localization, RGS3 possesses a putative

nuclear localization signal at residues 341–346, and its truncated isoform, RGS3T or RGS3 (314–519), which lacks a 14-3-3-binding site but still contains the nuclear localization signal, is localized to the nucleus [23]. Considering that an established function of 14-3-3 is to provide cytosolic retention of nuclear proteins such as Cdc25, FKHL1 (forkhead in rhabdomyosarcoma-like 1), NFAT (nuclear factor of activated T-cells) and others [21], one might suggest that 14-3-3 also retains RGS3 from the nucleus. In addition, 14-3-3 may provide the cytosolic retention of RGS3 away from the membrane, where G-proteins are localized, which may also contribute to the negative regulation of RGS3. This notion is in accord with our previous data, showing that RGS3N behaves similarly to FL-RGS3 in terms of cytosolic localization and agonist-induced translocation to the membrane in stably transfected human mesangial cells [28]. However, in the present study, due to a high degree of variability between expression levels in single cells after transient transfection, it appeared difficult to draw a definite conclusion about the differential localization of WT and S²⁶⁴A by employing immunofluorescence (results not shown). This issue will be addressed in the future, employing inducible stable cell lines with controlled and uniform expression of these proteins.

Finally, another important question is whether the interaction between RGS3 and 14-3-3 is a general phenomenon common for other RGS proteins. RGS3 and RGS7 have already been reported to bind 14-3-3 [30]. Moreover, the peptide sequence analysis revealed that other RGS proteins, such as RGS9, RGS12, RGS14 and GAIP, possess putative 14-3-3-binding sites, suggesting that they can potentially interact with 14-3-3. This, however, has to be confirmed, and the functional significance has to be investigated in each individual case.

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REFERENCES

- Koelle, M. R. and Horvitz, H. R. (1996) EGL-10 regulates G protein signaling in the *C. elegans* nervous system and shares a conserved domain with many mammalian proteins. *Cell*, **84**, 115–125.
- Druey, K. M., Blumer, K. J., Kang, V. H. and Kehrl, J. H. (1996) Inhibition of G-protein-mediated MAP kinase activation by a new mammalian gene family. *Nature (London)*, **379**, 742–746.
- Hunt, T. W., Fields, T. A., Casey, P. J. and Peralta, E. G. (1996) RGS10 is a selective activator of G α_i GTPase activity. *Nature (London)*, **383**, 175–177.
- Watson, N., Linder, M. E., Druey, K. M., Kehrl, J. H. and Blumer, K. J. (1996) RGS family members: GTPase-activating proteins for heterotrimeric G-protein α -subunits. *Nature (London)*, **383**, 172–175.
- De Vries, L., Zheng, B., Fischer, T., Elenko, E. and Farquhar, M. G. (2000) The regulator of G protein signaling family. *Annu. Rev. Pharmacol. Toxicol.*, **40**, 235–271.
- De Vries, L. and Gist Farquhar, M. (1999) RGS proteins: more than just GAPs for heterotrimeric G proteins. *Trends Cell Biol.*, **9**, 138–144.
- Huang, L. J., Durick, K., Weiner, J. A., Chun, J. and Taylor, S. S. (1997) D-AKAP2, a novel protein kinase A anchoring protein with a putative RGS domain. *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 11184–11189.
- Cabrera, J. L., de Freitas, F., Satpae, D. K. and Slepak, V. Z. (1998) Identification of the G β 5-RGS7 protein complex in the retina. *Biochem. Biophys. Res. Commun.*, **249**, 898–902.
- Snow, B. E., Krumins, A. M., Brothers, G. M., Lee, S. F., Wall, M. A., Chung, S., Mangion, J., Arya, S., Gilman, A. G. and Siderovski, D. P. (1998) A G protein γ subunit-like domain shared between RGS11 and other RGS proteins specifies binding to G β 5 subunits. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 13307–13312.
- Kozasa, T., Jiang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., Bollag, G. and Sternweis, P. C. (1998) p115 RhoGEF, a GTPase activating protein for G α_{12} and G α_{13} . *Science*, **280**, 2109–2111.
- Fukuhara, S., Chikumi, H. and Gutkind, J. S. (2001) RGS-containing RhoGEFs: the missing link between transforming G proteins and Rho? *Oncogene*, **20**, 1661–1668.
- De Vries, L., Lou, X., Zhao, G., Zheng, B. and Farquhar, M. G. (1998) GIPC, a PDZ domain containing protein, interacts specifically with the C terminus of RGS-GAIP. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 12340–12345.
- Snow, B. E., Hall, R. A., Krumins, A. M., Brothers, G. M., Bouchard, D., Brothers, C. A., Chung, S., Mangion, J., Gilman, A. G., Lefkowitz, R. J. and Siderovski, D. P. (1998) GTPase activating specificity of RGS12 and binding specificity of an alternatively spliced PDZ (PSD-95/Dlg/ZO-1) domain. *J. Biol. Chem.*, **273**, 17749–17755.
- Fukuhara, S., Chikumi, H. and Gutkind, J. S. (2000) Leukemia-associated Rho guanine nucleotide exchange factor (LARG) links heterotrimeric G proteins of the G(12) family to Rho. *FEBS Lett.*, **485**, 183–188.
- Schiff, M. L., Siderovski, D. P., Jordan, J. D., Brothers, G., Snow, B., De Vries, L., Ortiz, D. F. and Diverse-Pierluissi, M. (2000) Tyrosine-kinase-dependent recruitment of RGS12 to the N-type calcium channel. *Nature (London)*, **408**, 723–727.
- Lu, Q., Sun, E. E., Klein, R. S. and Flanagan, J. G. (2001) Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein and selectively inhibits G protein-coupled chemoattraction. *Cell*, **105**, 69–79.
- Zhong, H. and Neubig, R. R. (2001) Regulator of G protein signaling proteins: novel multifunctional drug targets. *J. Pharmacol. Exp. Ther.*, **297**, 837–845.
- Fu, H., Subramanian, R. R. and Masters, S. C. (2000) 14-3-3 proteins: structure, function, and regulation. *Annu. Rev. Pharmacol. Toxicol.*, **40**, 617–647.
- Reuther, G. W. and Pendergast, A. M. (1996) The roles of 14-3-3 proteins in signal transduction. *Vitam. Horm.*, **52**, 149–175.
- Baldin, V. (2000) 14-3-3 proteins and growth control. *Progr. Cell Cycle Res.*, **4**, 49–60.
- Muslin, A. J. and Xing, H. (2000) 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell Signal.*, **12**, 703–709.
- Gyuris, J., Golemis, E., Chertkov, H. and Brent, R. (1993) Cdt1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell*, **75**, 791–803.
- Dulin, N. O., Pratt, P., Tiruppathi, C., Niu, J., Voyno-Yasenetskaya, T. and Dunn, M. J. (2000) Regulator of G protein signaling RGS3T is localized to the nucleus and induces apoptosis. *J. Biol. Chem.*, **275**, 21317–21323.
- Huang, C., Hepler, J. R., Gilman, A. G. and Mumby, S. M. (1997) Attenuation of Gi- and Gq-mediated signaling by expression of RGS4 or GAIP in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 6159–6163.
- Wyllie, F., Heimann, K., Le, T. L., Brown, D., Rabnott, G. and Stow, J. L. (1999) GAIP, a G α_{i3} -binding protein, is associated with Golgi-derived vesicles and protein trafficking. *Am. J. Physiol.*, **276**, C497–C506.
- Adachi, M., Yang, Y. Y., Furuchi, Y. and Miyamoto, C. (1991) Cloning and characterization of cDNA encoding human A-type endothelin receptor. *Biochem. Biophys. Res. Commun.*, **180**, 1265–1272.
- Gu, M. and Du, X. (1998) A novel ligand-binding site in the ζ -form 14-3-3 protein recognizing the platelet glycoprotein Ib α and distinct from the c-Raf-binding site. *J. Biol. Chem.*, **273**, 33465–33471.
- Dulin, N. O., Sorokin, A., Reed, E., Elliott, S., Kehrl, J. H. and Dunn, M. J. (1999) RGS3 inhibits G protein-mediated signaling via translocation to the membrane and binding to G α_{11} . *Mol. Cell. Biol.*, **19**, 714–723.
- Chatterjee, T. K., Eapen, A. K. and Fisher, R. A. (1997) A truncated form of RGS3 negatively regulates G protein-coupled receptor stimulation of adenylate cyclase and phosphoinositide phospholipase C. *J. Biol. Chem.*, **272**, 15481–15487.
- Benzing, T., Yaffe, M. B., Arnould, T., Sellin, L., Schermer, B., Schilling, B., Schreiber, R., Kunzelmann, K., Lepar, G. G., Kim, E. and Walz, G. (2000) 14-3-3 interacts with regulator of G protein signaling proteins and modulates their activity. *J. Biol. Chem.*, **275**, 28167–28172.
- Tesmer, J. J., Berman, D. M., Gilman, A. G. and Sprang, S. R. (1997) Structure of RGS4 bound to AIF4-activated G(i α 1): stabilization of the transition state for GTP hydrolysis. *Cell*, **89**, 251–261.
- Ross, E. M. and Wilkie, T. M. (2000) GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu. Rev. Biochem.*, **69**, 795–827.
- Liu, D., Bienkowska, J., Petosa, C., Collier, R. J., Fu, H. and Liddington, R. (1995) Crystal structure of the ζ isoform of the 14-3-3 protein. *Nature (London)*, **376**, 191–194.
- Brasemann, S. and McCormick, F. (1995) Bcr and Raf form a complex *in vivo* via 14-3-3 proteins. *EMBO J.*, **14**, 4839–4848.
- Conklin, D. S., Galaktionov, K. and Beach, D. (1995) 14-3-3 proteins associate with cdc25 phosphatases. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 7892–7896.

- 36 Garrison, T. R., Zhang, Y., Pausch, M., Apanovitch, D., Aebersold, R. and Dohlman, H. G. (1999) Feedback phosphorylation of an RGS protein by MAP kinase in yeast. *J. Biol. Chem.* **274**, 36387–36391
- 37 Cunningham, M. L., Waldo, G. L., Hollinger, S., Hepler, J. R. and Harden, T. K. (2001) Protein kinase C phosphorylates RGS2 and modulates its capacity for negative regulation of G α 11 signaling. *J. Biol. Chem.* **276**, 5438–5444
- 38 Balasubramanian, N., Levay, K., Keren-Raifman, T., Faurobert, E. and Slepak, V. Z. (2001) Phosphorylation of the regulator of G protein signaling RGS9–1 by protein kinase a is a potential mechanism of light- and Ca²⁺-mediated regulation of g protein function in photoreceptors. *Biochemistry* **40**, 12619–12627
- 39 Chen, C., Wang, H., Fong, C. W. and Lin, S. C. (2001) Multiple phosphorylation sites in RGS16 differentially modulate its GAP activity. *FEBS Lett.* **504**, 16–22
- 40 Ogier-Denis, E., Pattingre, S., El Benna, J. and Codogno, P. (2000) Erk1/2-dependent phosphorylation of G α -interacting protein stimulates its GTPase accelerating activity and autophagy in human colon cancer cells. *J. Biol. Chem.* **275**, 39090–39095
- 41 Derrien, A. and Druey, K. M. (2001) RGS16 function is regulated by EGFR-mediated tyrosine phosphorylation. *J. Biol. Chem.* **276**, 48532–48538
- 42 Fischer, T., Elenko, E., Wan, L., Thomas, G. and Farquhar, M. G. (2000) Membrane-associated GAIP is a phosphoprotein and can be phosphorylated by clathrin-coated vesicles. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 4040–4045
- 43 Burgon, P. G., Lee, W. L., Nixon, A. B., Peralta, E. G. and Casey, P. J. (2001) Phosphorylation and nuclear translocation of a regulator of G protein signaling (RGS10). *J. Biol. Chem.* **276**, 32828–32834

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