14-3-3 interacts with Regulator of G Protein Signaling proteins
and modulates their activity

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Running title: 14-3-3 regulates RGS proteins

Abbreviations: RGS, regulator of G protein signaling; GST, glutathione S-transferase; GAP,
GTPase-activating protein.
Regulator of G Protein Signaling (RGS) proteins function as GTPase-activating proteins (GAPs) that stimulate the inactivation of heterotrimeric G proteins. We have recently shown that RGS proteins may be regulated on a posttranslational level (Benzing, T., Brandes, R., Sellin, L., Schermer, B., Lecker, S., Walz, G. and Kim, E. (1999) *Nat Med* **5**, 913-918). However, mechanisms controlling the GAP activity of RGS proteins are poorly understood. Here we show that 14-3-3 proteins associate with RGS7 and RGS3. Binding of 14-3-3 is mediated by a conserved phosphoserine located in the Gα-interacting portion of the RGS domain; interaction with 14-3-3 inhibits the GAP activity of RGS7, depends upon phosphorylation of a conserved residue within the RGS domain, and results in inhibition of GAP function. Collectively, these data indicate that phosphorylation-dependent binding of 14-3-3 may act as molecular switch that controls the GAP activity keeping a substantial fraction of RGS proteins in a dormant state.

Regulator of G protein signaling (RGS) proteins share a conserved RGS domain that binds α subunits of heterotrimeric G proteins and stimulates their intrinsic GTPase activity. By accelerating the inactivation of GTP-bound Gα subunits, RGS proteins serve as negative regulators of G protein-mediated signaling pathways and inhibit and redirect G protein-stimulated cellular responses (1-4). Heterotrimeric G proteins transduce a wide variety of receptor-mediated signals across the plasma membrane (5). The ability of RGS proteins to diminish the magnitude and duration of G protein-dependent signaling mandates tight regulation of their GAP activity. RGS proteins are subject to both transcriptional and posttranslational regulation (6-9). However, mechanisms directly controlling GAP activity of RGS proteins are poorly understood.
Ubiquitously expressed in all eukaryotic cells (10, 11) 14-3-3 proteins include nine distinct isotypes (α, β, γ, δ, ε, η, σ, τ, ζ) that modulate signaling events by binding to serine or threonine-phosphorylated target proteins. 14-3-3 proteins have been implicated in the activation of protein kinases, cell cycle control, and regulation of apoptosis (12-17).

Here we show that RGS3 and RGS7 contain a functional 14-3-3 binding site within their RGS domains and that a significant fraction of both proteins normally exists bound to endogenous 14-3-3. The binding of RGS3 and RGS7 to 14-3-3 is phosphorylation-dependent; the primary 14-3-3 binding site in RGS7 involves serine 434, a region implicated in interactions with Gα subunits and displaying sequence conservation with other RGS family members. Furthermore, phosphorylation and subsequent interaction with 14-3-3 results in a progressive decline in the GAP activity of RGS proteins. Our data suggest that regulated phosphorylation/14-3-3 binding and dephosphorylation within the RGS domain could function as a molecular switch to turn off and on the GAP function of RGS proteins in vivo.

Materials and Methods

Plasmids. Flag-tagged versions of human RGS7 (18, 7) and human RGS3 (a kind gift of Drs. K.M. Druey and J.H. Kehrl) were generated by PCR and standard cloning techniques. Site-directed mutagenesis was used to insert mutations in RGS7. Point mutations were verified by sequence analysis. A GST.14-3-3τ, kindly provided by Dr. Y.-C. Liu, was utilized to generate myc-, Flag-, and MBP-tagged versions. In some experiments, an extended myc-tagged 14-3-3τ version was used to differentiate transfected from endogenous 14-3-3 protein; this construct contained eight additional amino acids (ERDSRGSL) at the C-terminus. m2 muscarinic receptor (OB-m2) was a kind gift of Dr. Silvio Gutkind.
**Co-Immunoprecipitation.** Co-Immunoprecipitation experiments were performed as described (18). Briefly, HEK 293T cells were transiently transfected by the calcium phosphate method. After incubation for 24 hrs, cells were washed twice and lysed in a 1% Triton X-100 lysis buffer. After centrifugation (15,000xg, 15 min, 4°C) cell lysates containing equal amounts of total protein were incubated for 1 hr at 4°C with the appropriate antibody followed by incubation with 40 µl of protein G-sepharose beads (mouse monoclonal antibodies) or protein A-sepharose (rabbit polyclonal antisera) (Amersham Pharmacia Biotech) for approximately 3 hrs. The beads were washed extensively with lysis buffer and bound proteins were resolved by 10% SDS-PAGE.

**Metabolic Labeling and Immunoprecipitation.** HEK 293T cells were transiently transfected with plasmid DNA as indicated. After 24 hrs methionine/cysteine-free DMEM containing 1% dialyzed fetal bovine serum was applied for 30 min, then 1 mCi Tran35S-Label (ICN) containing [35S]methionine/cysteine was added for 6 hrs. Cells were harvested, lysates immunoprecipitated and precipitates subjected to SDS-PAGE. Densitometric analysis of non-saturating autoradiographs utilizing NIH image software was corrected for the number of incorporated methionines and cysteines of the respective proteins.

**RGS3 Pull-Down Assay.** HEK 293T cells were transiently transfected with F.RGS3. After incubation with staurosporine cells were lysed in 1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM NaF, 15 mM Na2P2O7, 2 mM Na3VO4, 1 mM EDTA, and protease inhibitors for 15 min. on ice. Following centrifugation the supernatant was incubated for 1 hr at 4°C with 6 µg of recombinant purified GST.14-3-3τ prebound to glutathione sepharose beads (Amersham
Pharmacia Biotech). Bound proteins were separated by 10% SDS-PAGE and RGS3 was visualized with anti-Flag antibody. Equal loading of GST.14-3-3\(\tau\) was confirmed by coomassie blue staining of the gels.

**In Vivo Co-Immunoprecipitation from Brain.** For preparation of brain protein extracts whole brains of female BALB/c mice (20 g in body weight, Charles River) were removed and homogenized in 4 ml of brain lysis buffer (20 mM Tris, pH 7.5, 0.1% Triton X-100, 40 mM NaCl, 50 mM NaF, 15 mM Na\(_2\)P\(_2\)O\(_7\), 2 mM Na\(_3\)VO\(_4\), 1 mM EDTA, containing protease inhibitor mix and 44 \(\mu\)g/ml PMSF) for 15 min. on ice. Following centrifugation and ultracentrifugation (100,000 x g, 4°C, 30 min.), the supernatant was divided into 2 fractions and immunoprecipitated with specific anti-RGS7 antiserum and control antibody followed by incubation with protein G-sepharose. Resulting precipitates were subjected to immunoblot analysis with anti-14-3-3 mAb (Santa Cruz) followed by incubation with HRP-coupled secondary antiserum and enhanced chemiluminescence.

**Pull-Down of Native RGS7 from Brain.** GST and GST.14-3-3\(\tau\) fusion protein was immobilized on affinity chromatography mini columns (BioRad) using glutathione sepharose beads; columns were washed extensively and pre-equilibrated by three washes with lysis buffer (1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM NaF, 15 mM Na\(_2\)P\(_2\)O\(_7\), 2 mM Na\(_3\)VO\(_4\), 1 mM EDTA, and protease inhibitors) at 4°C. Whole brains of healthy adult WKY rats were frozen in liquid nitrogen, homogenized in 4 ml of lysis buffer, incubated on ice for 20 minutes, and centrifuged at 20,000 x g for 20 min. Supernatants were loaded on a GST or a GST.14-3-3\(\tau\) column. Flow-through (identical volumes) was subjected SDS-PAGE and immunoblot analysis with anti-RGS7 antiserum, PKC\(\alpha\) and \(\beta\)-catenin antibodies.
In Vitro Phosphorylation and Interaction. In vitro phosphorylation of GST.RGS7\textsuperscript{315-469} and MBP.RGS7\textsuperscript{315-469} was performed for 30 min at 37°C in a 100-µl reaction in a buffer containing 20 mM HEPES, pH 7.4, 10 mM MgCl\textsubscript{2}, 0.1 mM CaCl\textsubscript{2}, 100 µM ATP, 20 µg/ml DAG, 100 µg/ml phosphatidylserine, 0.03% Triton X-100, and the indicated amount of recombinant RGS7 protein. The phosphorylation was initiated by the addition of 0.5 U of recombinant protein kinase C α (1850 U/mg, Panvera) in enzyme dilution buffer or enzyme dilution buffer alone (control). To monitor the incorporation of phosphate, the unlabeled ATP was supplemented with 10 µCi $^{32}$P]ATP, and radiolabeled MBP.RGS7\textsuperscript{315-469} or GST.RGS7\textsuperscript{315-469} was visualized by SDS-PAGE and autoradiography or spotted on nitrocellulose filter and counted in a scintillation counter.

For in vitro interaction studies, purified recombinant protein (1 µg of phosphorylated or unphosphorylated GST.RGS7\textsuperscript{315-469} or GST alone) was immobilized to glutathione sepharose beads and incubated with bacterial lysates containing 2.5 µg/ml of recombinant MBP.14-3-3τ for 90 min. in 450 µl of binding buffer containing 50 mM potassium phosphate, pH 7.5, 150 mM KCl, 1 mM MgCl\textsubscript{2}, 10% (v/v) glycerol, 1% Triton X-100, and protease inhibitors. The washed precipitate was separated on a 10% SDS acrylamide gel. Bound MBP.14-3-3τ was detected by immunoblotting using an anti-MBP rabbit antiserum (New England Biolabs).

Fluorescence Polarization Studies. For fluorescence polarization assays GST.14-3-3τ fusion proteins were expressed in bacteria and purified on glutathione sepharose beads as described previously (19, 20). Fluorescent peptides were synthesized using N-α-FMOC-protected amino acids and standard BOP/HOBt coupling chemistry on an ABI 431A Peptide BioSynthesizer, with fluorescein isothiocyanate connected to the peptide amino-terminus via a β-alanine linker. Fluorescence polarization anisotropy was measured using a Panvera Beacon 2000 Variable
Temperature Fluorescence Polarization System. Low fluorescence buffers and reagents (Panvera Corp) were used throughout. Binding curves were measured independently in 3 separate experiments. 14-3-3 proteins were serially diluted (0-223 µM) to a final volume of 150 µl in PBS in 6 x 50 mm borosilicate glass tubes. Fluorescein-tagged peptide was added (59 nM final concentration), mixed, and fluorescence polarization measured at 22°C after a 120 s delay with a 16 s integration. Background fluorescence was measured for each sample prior to peptide addition. Binding data was analyzed by assuming that fluorescence polarization was a linear function of ligand binding (21), and that each 14-3-3 monomer contained a single peptide binding site (19). Curves were fit to the equation: \( \frac{L_b}{L_{tot}} = \frac{L_r}{(k_d+L_r)} \) where \( L_b \) is bound ligand, \( L_{tot} \) is total ligand, \( L_r \) is free ligand and \( k_d \) is the dissociation constant, in closed form using non-linear regression analysis (Kaleidograph).

**GTP Hydrolysis Assays.** Single turnover GTPase activity measurements were carried out as described (22, 23, 24). Recombinant myristoylated G\( \alpha_i \) subunits (Calbiochem, 250 nM) were loaded with \( [\gamma^{32}P]GTP \) (1.0 µM) for 20 min. at 30°C in 500 µl of buffer containing 50 mM HEPES, pH 8.0, 5 mM EDTA, 2 mM DTT, and 0.1% lubrol. The stoichiometry of GTP binding of G\( \alpha_i \) subunits was 25-40%. For zero time point a 12.5 µl aliquot was removed and added to 375 µl of 5% (w/v) Norit in 50 mM NaH\(_2\)PO\(_4\). GTP hydrolysis was initiated at 4°C by adding 150 µl of the loaded G\( \alpha_i \) subunits on ice to MgCl\(_2\) (15 mM final concentration) and unlabelled GTP (150 µM final concentration), with or without purified, phosphorylated or unphosphorylated MPB.RGS7\( ^{315-469} \) (1.0 µM final concentration), that was preincubated with GST or GST.14-3-3\( \tau \) (final concentration 5 µM, 30 min. on ice) as indicated. Aliquots of 25 µl were removed from the hydrolysis reaction, mixed with 375 µl of 5% (w/v) Norit in 50 mM NaH\(_2\)PO\(_4\) on ice, centrifuged at 10,000 rpm for 5 min. and counted by liquid scintillation spectrometry. Zero time values were
subtracted from all experimental points. Statistical analysis was performed using the statistical and curve fitting functions of SigmaPlot 4.01 (Jandel Scientific). Hydrolysis rate constants were calculated according to Wang et al. (27). To demonstrate statistical significance of differences in GTP hydrolysis, hydrolysis rate constants were normalized, expressed as fold increase of basal hydrolysis rate constant, and averages of these constants were depicted in a table.

**ERK1/2 phosphorylation.** For the determination of ERK1/2 phosphorylation HEK293T cells were transfected with the plasmid DNA as indicated. After transfection cells were serum-starved overnight and incubated in the absence/presence of carbachol. After 15 min. the stimulation was stopped by placing the cells on ice and exchange of the media with ice-cold phosphate-buffered saline. Cells were harvested, lysed in a 1% Triton X-100 lysis buffer containing 20 mM Tris, pH 7.5, 50 mM NaCl, 50 mM NaF, 15 mM Na₃P₂O₇, 2 mM Na₃VO₄, 1 mM EDTA, protease inhibitors. The lysate was cleared by centrifugation and equal amounts of protein were separated by 12% SDS-PAGE. Dually phosphorylated ERK1/2 was visualized with phosphospecific antisera (New England Biolabs) that detects ERK1/2 only when phosphorylated at threonine 202 and tyrosine 204 (pT E pY motif). Equal loading was confirmed by reprobing the membrane with β-actin and amidoblack staining. The degree of dual phosphorylation of ERK1/2 was quantitated by densitometric analysis of non-saturated radiographs with the NIH Image software.

**Results and Discussion**

To test whether 14-3-3 binds and regulates RGS proteins we used RGS3, RGS7, 14-3-3τ and 14-3-3β as model proteins. Both RGS3 and RGS7 specifically interacted with 14-3-3 proteins in transfected HEK 293T cells (Fig. 1). Epitope-tagged 14-3-3 co-precipitated with RGS3 and RGS7 but not with control proteins and vice versa. Truncations of RGS3 and RGS7 were
generated to localize the site of interaction with 14-3-3 to the RGS domains (Fig. 1a and e). To more quantitatively assess the amount of RGS3 complexed with 14-3-3 we labeled transiently transfected HEK 293T cells with [35S]methionine/cysteine. Immunoprecipitation from labeled cells revealed that approximately 70% of the immunoprecipitated RGS3 was complexed with 14-3-3 (Fig. 2a). Since immunoprecipitation of myc-tagged 14-3-3 immobilized only 15% of the RGS3, it appears that most of the RGS3 is bound to 14-3-3, whereas the majority of 14-3-3 is complexed with other cellular proteins. 14-3-3 proteins generally recognize their ligands only following serine/threonine phosphorylation (19, 20, 25). We therefore examined whether treatment of HEK 293T cells with staurosporine, a broad spectrum inhibitor of protein kinases, would prevent the phosphorylation of RGS proteins and their subsequent interaction with 14-3-3. Staurosporine nearly abrogated the interaction between RGS3 and 14-3-3 in vivo and in vitro. Only trace amounts of 14-3-3τ were co-immunoprecipitated in staurosporine-treated HEK 293T cells (Fig. 2b). Similarly, treatment of RGS3-expressing HEK 293T cells with staurosporine dramatically reduced binding of RGS3 to immobilized 14-3-3τ in vitro (Fig. 2c). Note that the staurosporine treatment did not cause a nonspecific reduction in the cellular amounts of either RGS3 or 14-3-3τ (Fig. 2b and c, lower panels). Similar experiments with RGS7 were precluded by the destabilization of RGS7 by staurosporine treatment. Both RGS7 and 14-3-3 are highly abundant in mouse brain. When we examined their endogenous interaction by co-immunoprecipitation of mouse brain lysates (Fig. 3a), 14-3-3 specifically co-precipitated with RGS7 indicating an endogenous in vivo interaction (Fig. 3b). To quantitatively assess the capacity of endogenous RGS7 to interact with 14-3-3, we determined the fraction of endogenous RGS7 retained by a recombinant glutathione-S-transferase-14-3-3 fusion protein immobilized to a glutathione sepharose column. Unbound RGS7 was measured in the flow-through by immunoblotting; PKCα and β-catenin levels were used to correct for unspecific binding and equal
loading (Fig. 3c). More than 50% of the RGS7 contained in brain lysates was retained on a GST.14-3-3τ column (Fig. 3d). Collectively, these data indicate that RGS3 and RGS7 interact with 14-3-3 in a phosphorylation-dependent manner. This interaction does not only occur in transfected cells but can also be demonstrated with endogenous proteins. Furthermore, the data suggest that a substantial fraction of RGS proteins is bound to 14-3-3 in vivo.

Mutational analysis revealed that serine 434 of RGS7 was critical for binding to 14-3-3. Replacement of the serine residue at position 434 of RGS7 by alanine completely abolished binding of 14-3-3τ to both full-length RGS7 and to the isolated RGS domain (RGS7315-469) (Fig. 1d and e). To demonstrate that phosphorylation of serine 434 is required for the interaction of RGS7 and 14-3-3, the interaction of 14-3-3 with unphosphorylated and phosphorylated RGS7 was tested in vitro. As expected, no interaction was detectable between bacterially expressed, unphosphorylated RGS7315-469 fused to GST (GST.RGS7315-469) and 14-3-3τ fused to MBP (MBP.14-3-3τ) (Fig. 4a), whereas phosphorylation by PKCα enabled GST.RGS7315-469 to bind MBP.14-3-3τ. Replacement of serine 434 in RGS7 by aspartate to mimic serine phosphorylation facilitated a constitutive association between RGS7 and 14-3-3 that remained unaffected by subsequent phosphorylation with PKCα (data not shown), suggesting that serine 434 is the relevant PKCα phosphorylation site.

Fluorescence polarization measurements confirmed that 14-3-3τ rapidly binds to a 14-mer phosphopeptide containing the serine 434 14-3-3 binding site of RGS7 with a k_D of 15.9 µM (Fig. 4b and c). Most published affinities for 14-3-3 interacting peptide sequences range from 0.1-2 µM, using surface plasmon resonance (Biacore); however, the interpretation of these affinities is complicated by an avidity effect since dimeric 14-3-3 may simultaneously bind to two phosphopeptides immobilized on the Biacore chip (19), while the fluorescence polarization experiments were performed with solubilized molecules. Given the strong interaction of 14-3-3
with RGS7 and RGS3 in the co-immunoprecipitation and pull-down experiments, this moderate $k_d$ suggests that additional factors such as dimerization of RGS proteins or tandem binding of 14-3-3 may contribute to the interaction of RGS proteins with 14-3-3 in vivo. Indeed, both RGS3 and RGS7 contain at least two additional potential 14-3-3 binding sites in close proximity within the RGS domain. Although our data clearly implicate serine 434 as a critical 14-3-3 binding residue in RGS7, it is conceivable that simultaneous binding to additional sites increases the affinity and stability of this interaction. Several 14-3-3 binding proteins such as c-Raf-1, Cbl, and BAD contain two 14-3-3 binding sequences separated by polypeptide segments of various length, and tandem binding to adjacent 14-3-3 sites has been shown to facilitate the formation of a high-affinity, bidentate complex (19).

Based on the resolution crystal structure of RGS4 complexed with activated $G_{\alpha_i}$ subunits (26) the 14-3-3 binding site at serine 434 in RGS7 aligns with one of the three putative domains required for $G_{\alpha_i}$ interaction and stimulation of GTPase activity of $G_{\alpha_i}$ (Fig. 5c). To test whether binding of 14-3-3 interferes with the GTPase accelerating activity of RGS7, we measured the GAP activity of RGS7 in single-turnover GTP hydrolysis assays (22). GTP hydrolysis follows a single exponential time course equivalent to a first order reaction which can be expressed by means of the rate constant of a first order reaction (27). Addition of 14-3-3 reduced the hydrolysis rate constant of phosphorylated RGS7 from 10-fold to 3.5-fold (Fig. 5a), while addition of 14-3-3 to unphosphorylated RGS7 or $G_{\alpha_i}$ had no effect on the hydrolysis rate constant (data not shown). In order to more quantitatively assess the effect of phosphorylation and/or 14-3-3 interaction on GAP activity of RGS7 hydrolysis rate constants of several experiments were averaged and expressed as fold increase of basal hydrolysis rate (Table 1). Addition of 14-3-3 to phosphorylated RGS7 almost completely abrogated the GTPase-stimulatory effect, suggesting that phosphorylation of serine 434 and the subsequent interaction
with 14-3-3 dramatically interferes with binding of activated $\alpha_i$. These findings suggest that a phosphorylation-dependent interaction between 14-3-3 and the RGS domain may regulate the GAP activity of RGS proteins; however, the kinase responsible for this phosphorylation \textit{in vivo} has yet to be determined. RGS3 impairs MAP kinase activation by mammalian G protein-linked receptors in human embryonic kidney (HEK) cells (28). To illustrate the functional consequences of the interaction between 14-3-3 and RGS proteins, we co-transfected the $\alpha_i$-linked m2 cholinergic receptor with and without RGS3 and 14-3-3 into HEK cells expressing Rap1 and Rap1GAPII. Stimulation with carbachol (30 $\mu$M, 15 min) resulted in a strong dual phosphorylation of ERK1 and ERK2 on threonine 202 and tyrosine 204 (Fig. 5b). Dual phosphorylation of ERK1/2 within the T E Y motif leads to the activation of the kinase and represents a sensitive measure of ERK1/2 activity. RGS3 inhibited the carbachol-mediated MAP kinase phosphorylation. Co-expression of 14-3-3 rescued the $\alpha_i$-induced MAP kinase phosphorylation from this inhibitory effect of RGS3 (Fig. 5b), but did not affect MAP kinase activation in the absence of RGS3 (data not shown). Equal protein loading was ensured by reprobing the blot against actin and by amidoblack staining. These data provide further evidence that binding of 14-3-3 counteracts the inhibitory effect of RGS proteins on G protein-initiated signaling.

Alignment of the sequence bordering serine 434 in RGS7 with other RGS members reveals a putative 14-3-3 binding motif: K/E K/R D pS Y P (Fig. 5c) (19, 20). Since the 14-3-3 binding site in RGS7 is conserved in other RGS members, we speculate that 14-3-3 binding may similarly regulate the GAP activity of other RGS proteins. It is unclear whether the reduction of RGS GAP activity depends upon a conformation change induced by 14-3-3 binding or on the physical impedance of the association of RGS and $\alpha_i$. Our data suggest that phosphorylation-
dependent interaction of RGS proteins with regulatory proteins such as 14-3-3 may rapidly and dynamically control RGS GAP activity without altering their expression.

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Figure Legends

Fig. 1. The RGS domains of RGS3 and RGS7 specifically interact with 14-3-3. Lysates were prepared from HEK 293T cells, transfected with epitope-tagged constructs as indicated, and immunoprecipitated with Flag- or myc-specific antibodies, and resolved by SDS-PAGE. (a) 14-3-3-τ co-precipitates with RGS3 as well as with truncations RGS3314-520 and RGS3421-520, but not with RGS31-389 or the control protein GFP (upper left panel); HC denotes IgG heavy chain and LC light chain. Expression levels of myc-tagged 14-3-3-τ in cell lysates are shown in the lower panel, the expression levels of Flag-tagged RGS3 truncations are shown in the right panel. (b) RGS3 co-precipitates with 14-3-3-τ, but not with TRAF2 (upper panel). Expression levels of Flag-tagged RGS3 in cell lysate are shown in the lower panel; expression levels of myc.14-3-3-τ and myc.TRAF2 are shown in the right panel. (c) 14-3-3-β co-precipitates with RGS3, but not with Tau protein (upper panel). Expression levels of Flag-tagged RGS3, Tau and a HA-tagged 14-3-3-β in the lysate are shown in the lower panel. (d-e) 14-3-3-τ co-precipitates with RGS7 and the RGS7315-469 truncation that contains the RGS domain of RGS7 (amino acid 333 to 448). Serine-to-alanine substitution at position 434 of RGS7 (F.RGS7S434A, RGS7315-469 S434A) abrogates the interaction with 14-3-3-τ (upper panels). Expression levels of myc.14-3-3-τ in cell lysates are shown in the lower panel (d) and middle panel (e), respectively. Expression levels of RGS7315-469 and RGS7315-469 S434A are shown in the lower panel (e).

Fig. 2. The interaction of RGS proteins with 14-3-3 is phosphorylation-dependent. (a) Autoradiograph after in vivo labeling with [35S]methionine/cysteine, and immunoprecipitation of transiently transfected HEK 293T cells with anti-Flag or anti-myc antibodies. An extended myc.14-3-3-τ construct was used that is easily distinguished from endogenous 14-3-3, labeled “14-3-3”. (b) Co-precipitation of 14-3-3-τ with RGS3 is decreased after staurosporine
pretreatment (0.5 μM, 2 hrs) (upper panel). Expression levels of transiently transfected Flag-tagged RGS3, Tau and myc-tagged 14-3-3τ in HEK 293T cellular lysates prior to IP are shown in the lower panel. (c) Pull-down of RGS3 with GST.14-3-3τ prebound to glutathione sepharose beads is diminished after staurosporine pretreatment. Precipitated RGS3 was detected with anti-Flag antibody (upper panel). Expression of Flag-tagged RGS3 and Tau in lysates of transiently transfected HEK 293T cells are shown in the middle panel. Coomassie staining of GST.14-3-3τ, used to pull down RGS protein, is shown in the lower panel.

**Fig. 3.** Endogenous interaction of RGS7 with 14-3-3. (a) Western blot analysis reveals that RGS7 and 14-3-3 are highly abundant in mouse brain. (b) Co-immunoprecipitation of endogenous RGS7 and 14-3-3 from mouse brain. Mouse brain lysates were immunoprecipitated with control or anti-RGS7 antiserum, and resolved by 15% SDS/PAGE. Co-precipitated 14-3-3 protein was visualized with an anti-14-3-3 antibody. The first lane is a positive control (myc.14-3-3τ); the second lane represents brain lysates before IP; the third lane represents immunoprecipitates from mouse brain with control antiserum; and the fourth lane demonstrates the co-immunoprecipitation of 14-3-3 with RGS7 from mouse brain using a specific anti-RGS7 polyclonal antiserum. LC denotes the position of the light chain. (c) Precleared brain lysates containing native RGS7 were incubated with either GST (control) or GST.14-3-3τ, immobilized on glutathione sepharose beads. Equal amounts of the flow-through were separated by SDS-PAGE and sequentially probed with anti-RGS7, anti-PKCα, and anti-β-catenin. (d) Densitometric analysis, performed on non-saturating autoradiographs using the NIH image software and corrected for levels of the control proteins PKCα and β-catenin.
Fig. 4. Requisite phosphorylation of serine 434 of RGS7 mediates interaction with 14-3-3. (a) In vitro interaction of MBP.14-3-3\(\tau\) (lane 1) with GST (before and after treatment with PKC\(\alpha\), lanes 2 and 3), GST.RGS7\(^{315-469}\) WT (wild-type) (before and after treatment with PKC\(\alpha\), lanes 4 and 5), and a pseudophosphorylated RGS7 mutant containing a serine 434 to aspartate mutation (GST.RGS7\(^{315-469}\) S434D, lane 6). Interaction of recombinant GST proteins with bacterially expressed MBP.14-3-3\(\tau\) was detected by immunoblotting with anti-MBP antiserum. (b-c) Fluorescence polarization was used to determine the affinity between RGS7 and 14-3-3\(\tau\). A fluorescein-tagged RGS7 phosphopeptide CLMKSDpSYPRFIRS, derived from the putative 14-3-3 binding site of RGS7, was incubated with GST.14-3-3\(\tau\). (b) depicts the time-dependent binding between the RGS7 peptide and 14-3-3\(\tau\). (c) Fluorescein-tagged RGS7 peptide (59 nM final concentration) was added to serially diluted 14-3-3 proteins (0-223 \(\mu\)M) to determine the dissociation constant.

Fig. 5. 14-3-3 inhibits the GAP function of RGS7. (a) Single turnover GTP hydrolysis assay was initiated on ice by the addition of Mg\(^{2+}\) and GTP to activated G\(\alpha_{i1}\) subunits preloaded with \([\gamma-\text{32P}]\)GTP (22). Representative paired experiment with the black curve showing GTP hydrolysis by G\(\alpha_{i1}\) subunits alone (with control GST and MBP). Phosphorylated MBP.RGS7\(^{315-469}\) (blue curve) enhances the rate of GTP hydrolysis by purified G\(\alpha_{i1}\). Preincubation of phosphorylated MBP.RGS7 with GST.14-3-3\(\tau\) dramatically inhibits stimulation of the GTPase activity of purified G\(\alpha_{i1}\) (green curve). (b) HEK 293T cells were co-transfected with the m2 muscarinic acetylcholine receptor, F.RGS3, and myc.14-3-3\(\tau\) as indicated. Cells were stimulated with carbachol (15 min., 30 \(\mu\)M), harvested and equal amounts of protein were separated by SDS-PAGE, immunoblotted and probed with antibody against phosphorylated ERK1/2. Co-expression of 14-3-3 rescued the G\(\alpha_{i}\)-induced MAP kinase activation from the inhibitory effects of RGS3.
The middle panels reveals the expression of RGS3 and 14-3-3; densitometric analysis of the ERK2 phosphorylation is depicted in the lower panel. (c) Alignment of the sequence bordering serine 434 in RGS7 with other RGS members reveals a putative 14-3-3 binding motif: K/E K/R D pS Y P in several RGS proteins. Putative contact points of the RGS domains with activated Gαi subunits are shaded in dark gray (26).

**Table 1.** Phosphorylation and interaction of RGS7 with 14-3-3 inhibits GAP activity. Hydrolysis rate constants were determined as described (27), and expressed as fold increase of basal GTP hydrolysis rate constant to compare three independent experiments. The average of the relative hydrolysis rate constants of these three experiments is shown in Table 1.
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**Figure a**

- myc.14-3-3
- F.Tau + myc.14-3-3
- myc.TRAF2 + F.RGS3
- myc.14-3-3 + F.RGS3

**Figure b**

- Stauroporine
  - myc.14-3-3
  - F.RGS3
  - F.Tau
  - Lysates
- IP anti-Flag

**Figure c**

- Stauroporine (µM)
  - 0.5 1.0 0.5 1.0

- F.RGS3
- F.Tau
- GST.14-3-3

- Coomassie
PKCα

Brain lysates

GST

14-3-3τ

β-catenin

Whole Brain

Brain Stem

Cerebrum

RGS7 (32.5 kD)

14-3-3 (62 kD)

GST

myc.14-3-3τ

Brain lysate

IP control ab

IP anti-RGS7

- 14-3-3

- LC

c

Brain lysates

RGS7

PKCα

β-catenin

d

RGS7 protein (% of control)

0

GST

14-3-3τ

50

100

0

GST

14-3-3τ

50
**a**

Fluorescence polarization (mPa)

| PKCα | pos. control | GST | GST RGS7 | GST RGS7 WT | GST RGS7 S434D |
|------|--------------|-----|----------|-------------|----------------|
| -    | -            | +   | -        | -           | -              |

**b**

Fluorescence Polarization (mPa)

Time (min)

- 0
- 5
- 10
- 15
- 60

**c**

Fraction Bound

[kD = 15.9 µM]

[14-3-3τ]
|                  | fold increase in GTP hydrolysis rate |
|------------------|---------------------------------------|
| control          | 1                                     |
| RGS7 unphosphorylated | 11.5                                 |
| RGS7 phosphorylated         | 9.4                                  |
| RGS7 phosphorylated + 14-3-3 | 2.2                                  |
14-3-3 interacts with regulator of G protein signaling proteins and modulates their activity

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