

GTPase Activating Specificity of RGS12 and Binding Specificity of an Alternatively Spliced PDZ (PSD-95/Dlg/ZO-1) Domain*

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Regulator of G-protein signaling (RGS) proteins increase the intrinsic guanosine triphosphatase (GTPase) activity of G-protein α subunits *in vitro*, but how specific G-protein-coupled receptor systems are targeted for down-regulation by RGS proteins remains uncharacterized. Here, we describe the GTPase specificity of RGS12 and identify four alternatively spliced forms of human RGS12 mRNA. Two RGS12 isoforms of 6.3 and 5.7 kilobases (kb), encoding both an N-terminal PDZ (PSD-95/Dlg/ZO-1) domain and the RGS domain, are expressed in most tissues, with highest levels observed in testis, ovary, spleen, cerebellum, and caudate nucleus. The 5.7-kb isoform has an alternative 3' end encoding a putative C-terminal PDZ domain docking site. Two smaller isoforms, of 3.1 and 3.7 kb, which lack the PDZ domain and encode the RGS domain with and without the alternative 3' end, respectively, are most abundantly expressed in brain, kidney, thymus, and prostate. *In vitro* biochemical assays indicate that RGS12 is a GTPase-activating protein for G_i class α subunits. Biochemical and interaction trap experiments suggest that the RGS12 N terminus acts as a classical PDZ domain, binding selectively to C-terminal (A/S)-T-X-(L/V) motifs as found within both the interleukin-8 receptor B (CXCR2) and the alternative 3' exon form of RGS12. The presence of an alternatively spliced PDZ domain within RGS12 suggests a mechanism by which RGS proteins may target specific G-protein-coupled receptor systems for desensitization.

The mammalian “regulators of G-protein signaling” (RGS)¹

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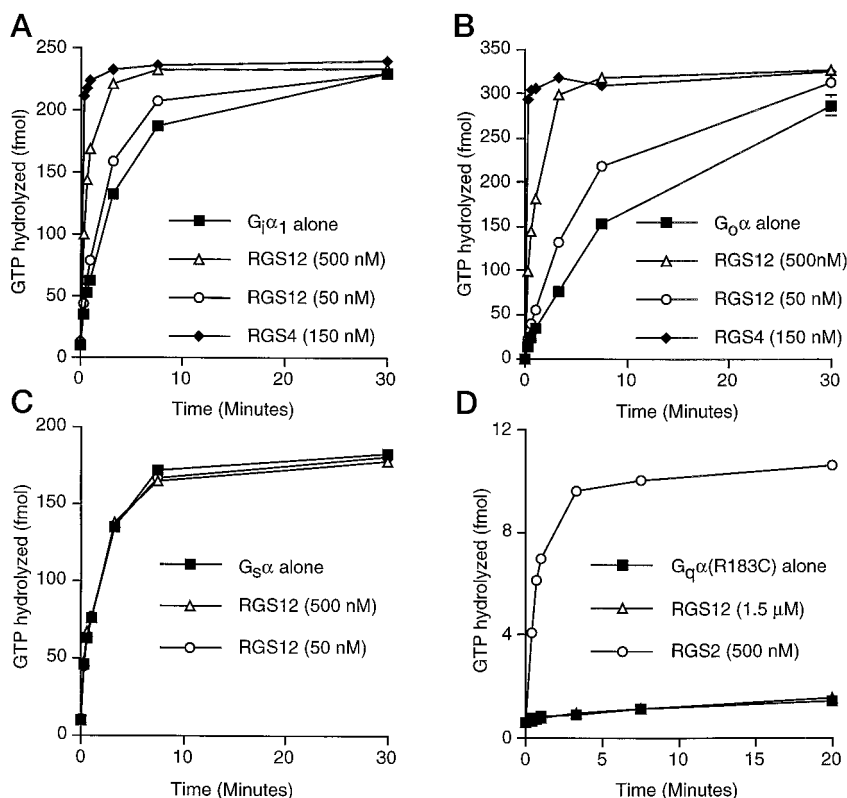
¹ The abbreviations used are: RGS, regulator of G-protein signaling; AD, activation domain; AR, adrenergic receptor; bp, base pair(s); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DBD, DNA binding domain; DTT, dithiothreitol; GAP, GTPase-activating protein; GPCR, G-protein-coupled receptor; GST, glutathione S-transferase; GTPase, guanosine triphosphatase; h, human; kb, kilo-

gene family was first identified by sequence and functional similarity to fungal and nematode genes captured in genetic screens for negative regulators of specific G-protein-coupled receptor (GPCR) signals (1–3). *In vitro* biochemical analyses soon established that this gene family encodes potent accelerators (“GAPs”) of the intrinsic GTP hydrolysis activity of G-protein α subunits, revealing a molecular mechanism by which RGS proteins drive G-proteins into their inactive GDP-bound form and hence down-regulate GPCR signal transduction *in vivo* (reviewed in Refs. 4 and 5). However, the mechanisms by which individual RGS proteins desensitize pathways activated by particular GPCRs remain to be elucidated. Tightly regulated transcription has been described for RGS1 (3), RGS2 (6), and RGS3-RGS11 (7), and palmitoylation of the cysteine-rich N terminus of $G\alpha$ -interacting protein (GAIP) has also been observed (8); however, transcriptional regulation and post-translational modifications of particular RGS family members can each only be expected to afford a gross level of intracellular control over the temporal and spatial expression of $G\alpha$ -directed GAP activity.

We and others have hypothesized that regions outside the RGS fold contribute to regulation of $G\alpha$ GAP activity and/or targeting of individual RGS proteins to particular receptor signaling pathways (4, 5, 9, 10). Here, we report the GAP activity of RGS12 and identify a PDZ-like N-terminal sequence within two splice forms. PDZ domains are protein-protein interaction modules that bind to three or four amino acid motifs at the extreme carboxyl termini of target proteins (reviewed in Ref. 11). PDZ domains of many proteins localize enzymatic activities and other protein-protein interaction domains to specific submembranous regions. Members of the PSD-93/95 protein family cluster NMDA receptors, nitric oxide synthase activity, and Shaker K^+ channels at neuronal synapses (12). The protein tyrosine phosphatase FAP-1 interacts via a PDZ domain with the C terminus of CD95, receptor of the apoptosis-inducing Fas ligand (13). Most recently, the five-PDZ domain-containing protein InaD was shown to organize the G-protein-coupled phototransduction machinery in *Drosophila*, with individual PDZ domains binding to phospholipase C, transient receptor potential ion channel, and protein kinase C subunits (14). Using yeast two-hybrid, surface plasmon resonance, and protein overlay analyses, we demonstrate specific binding of the RGS12 PDZ domain to C-terminal (A/S)-T-X-(L/V) motifs. Our findings suggest that, by virtue of an alternatively spliced N terminus, specific isoforms of RGS12 may localize *in vivo* to

base(s); myr, myristoylated; NHERF, Na^+/H^+ exchanger regulatory factor; PCR, polymerase chain reaction; PDZ, PSD-95, Disc-large, and ZO-1; r, rat; RU, response unit(s); SPR, surface plasmon resonance; Trx, thioredoxin; UTR, untranslated region.

FIG. 1. Increase in the single turnover GTPase activity of $G_i\alpha$ subunits by RGS12. A solution containing Mg^{2+} and indicated concentrations of recombinant GST-rRGS12-(664–885) fusion protein, or control RGS proteins, was added to the following final concentrations of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ -bound $G\alpha$ proteins: A, 2.4 nM myr- $G_i\alpha_1$; B, 3.25 nM myr- $G_o\alpha$; C, 1.85 nM $G_s\alpha$; D, 0.105 nM $G_q\alpha(R183C)$ proteins. Reactions were performed at 4 °C for A–C and 20 °C for D. Aliquots were taken at the indicated times for measurement of free ^{32}P .



site(s) of specific G-protein-coupled signaling complexes. In addition, affinity of the RGS12 PDZ domain for an alternatively spliced C-terminal motif within RGS12 itself presents the possibility of RGS12 autoregulation by intra- and/or intermolecular association.

EXPERIMENTAL PROCEDURES

Cloning of Human RGS12—Oligonucleotides flanking both the open reading frame (sense primer 5'-ATATGGCTCCAAGGGAACAATGAG-ACG-3' and antisense primer 5'-TACGGGGCCAAGGTGGAGGGATC-AG-3') and 3'-UTR of *hRGS12* (sense primer 5'-ATCCCTCCACCTTG-GCCCCGTAAGC-3' and antisense primer 5'-CTGCTGGGAGCCTCG-CCTCAGTTTC-3') were designed based on cosmid sequences (9) and used to amplify the *hRGS12* cDNA from 0.5 ng of Marathon-ReadyTM human brain cDNA (CLONTECH) using the ExpandTM long template PCR system (Boehringer Mannheim) as described previously for *hRGS16* (15). The resulting PCR products were then cloned and sequenced as described previously (15).

Protein Expression and Purification—cDNA fragments from rat *RGS12* encoding amino acids 1–94 and 664–885, and from human *RGS12* encoding amino acids 1–110, were each amplified by PCR, cloned into the GST fusion vector pGEX4T3 (Amersham Pharmacia Biotech), sequenced, and transformed into *Escherichia coli* strain BL21 (Stratagene). Expression of GST-rRGS12-(1–94), GST-rRGS12-(664–885), and GST-hRGS12-(1–110) fusion proteins was induced with 0.8 mM isopropyl- β -D-thiogalactopyranoside for 4 h at 37 °C in bacterial cultures at an OD_{600 nm} of 0.7. Cells were lysed by lysozyme treatment and sonication in lysis buffer (50 mM sodium phosphate, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors) and cleared by centrifugation at 27,000 \times g for 30 min. Supernatant was passed through a glutathione-Sepharose column (Amersham Pharmacia Biotech) pre-equilibrated with lysis buffer and the column was washed with lysis buffer and eluted with 10 mM glutathione in 50 mM Tris-HCl, pH 8.0. Eluted proteins were dialyzed against storage buffer (25 mM HEPES, pH 7.9, 2 mM EDTA, 2 mM DTT, 150 mM NaCl), concentrated by ultrafiltration, and stored at –80 °C.

cDNA fragments from rat *RGS12* encoding amino acids 1–440, 440–1108, and 1–1387 were each amplified by PCR, cloned into the thioredoxin-hexahistidine tag (Trx) fusion vector pET32b (Invitrogen), sequenced, and transformed into *E. coli* strain BL21(DE3) (Stratagene). Expression of Trx-rRGS12-(1–440), Trx-rRGS12-(440–1108), and Trx-rRGS12-(1–1387) was induced with 0.8 mM isopropyl- β -D-thiogalacto-

pyranoside for 8 h at 26 °C. Cells were lysed as above in Trx-lysis buffer (25 mM HEPES, pH 7.9, 100 mM NaCl, 20 mM imidazole, 10% glycerol, 5 mM β -mercaptoethanol, 0.5% Nonidet P-40, and protease inhibitors). Cell lysate was cleared by centrifugation and the supernatant was passed through nickel-nitrilotriacetic acid resin (Qiagen). The resin was washed with Trx-lysis buffer, and the hexahistidine-tagged fusion proteins were eluted using an imidazole concentration gradient and dialyzed against storage buffer.

GTPase Assays—G-protein α subunits $G_s\alpha$, myr- $G_i\alpha_1$, and myr- $G_o\alpha$ were expressed and purified as described elsewhere (16). A point mutant of $G_q\alpha$, Arg-183 to Cys, which reduces without abolishing the intrinsic GTPase activity, was purified from Sf9 cells co-infected with $G_q\alpha(R183C)$, G β 1, and G γ 2 baculoviruses (17). Recombinant RGS4 was purified from *E. coli* as described previously (18). Purification of recombinant RGS2 from Sf9 cells will be described elsewhere.²

Purified $G_q\alpha(R183C)$ was loaded with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (specific activity 500 cpm/pmol) for 3 h at 20 °C in the following buffer (150 μ l final volume): 10 μ M GTP, 5.5 mM CHAPS, 50 mM sodium HEPES, pH 7.5, 1 mM DTT, 1 mM EDTA, 0.9 mM $MgSO_4$, 0.1 mg/ml bovine serum albumin, 30 mM $(NH_4)_2SO_4$, and 4% glycerol. Other $G\alpha$ subunits were loaded in the same manner in the absence of $MgSO_4$; $G_s\alpha$ and myr- $G_o\alpha$ subunits were incubated for 20 min at 20 °C, and myr- $G_i\alpha_1$ was incubated for 30 min at 30 °C. Following loading, reaction mixtures were exchanged by Sephadex G-25 chromatography into 1 mM CHAPS, 50 mM HEPES, pH 7.5, 1 mM DTT, 1 mM EDTA, 0.9 mM $MgSO_4$, 0.018 mg/ml bovine serum albumin. Protein eluants were then diluted 4-fold in ice-cold OG buffer (0.1% octyl glucopyranoside, 20 mM sodium HEPES pH 7.5, 80 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.9 mM $MgSO_4$ (for $G_q\alpha$ only), 0.01 mg/ml bovine serum albumin, and 1 mM GTP). GAP activity was initiated by adding $G\alpha$ -GTP to RGS protein sample and OG buffer (supplemented with 9 mM $MgSO_4$ when $G_s\alpha$, myr- $G_o\alpha$, or myr- $G_i\alpha_1$ were used). Timed, 100- μ l aliquots were withdrawn and quenched with 900 μ l of a 5% (w/v) slurry of Norit A. After centrifugation, 600 μ l of supernatant was counted for ^{32}P content.

Northern Blot Analyses—Human MTN blots (CLONTECH), containing 2 μ g of poly(A)⁺ RNA from multiple adult human tissues and brain regions, were hybridized consecutively with a 686-bp *hRGS12* 3'-UTR cDNA probe (nucleotides 4185–4870 of GenBank AF035152), a 330-bp

² T. Ingi, A. M. Krumins, P. Chidiac, G. M. Brothers, S. Chung, B. E. Snow, C. A. Barnes, A. A. Lanahan, D. P. Siderovski, E. M. Ross, A. G. Gilman, and P. F. Worley, submitted for publication.

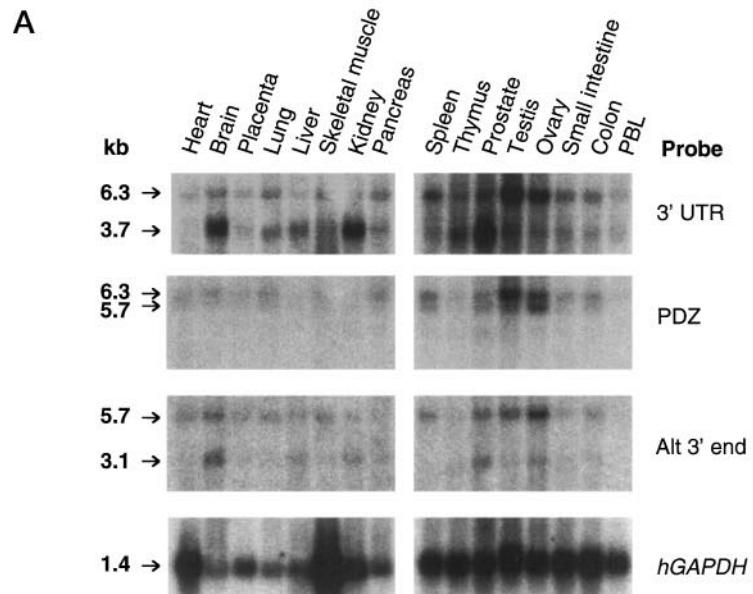
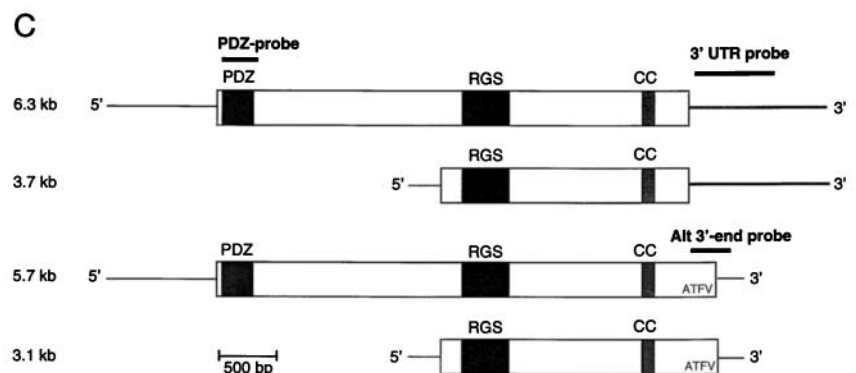
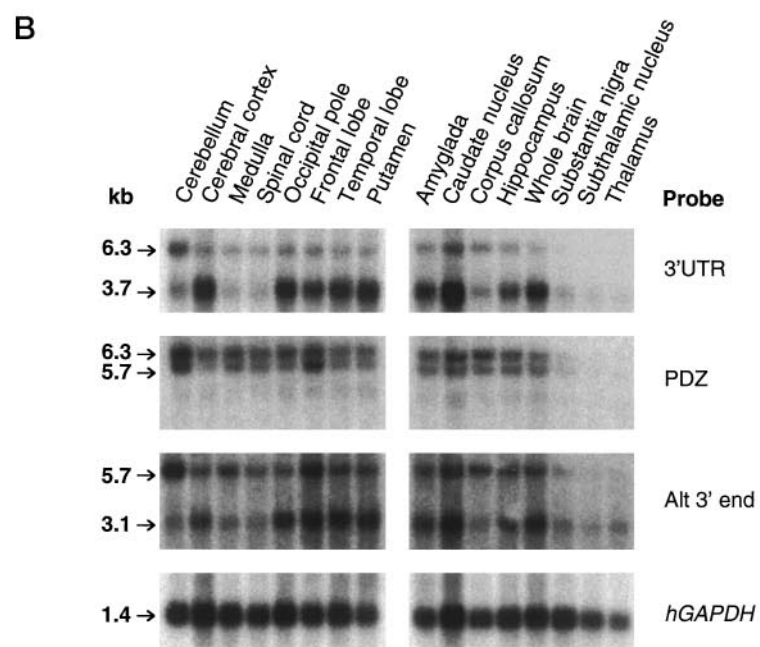


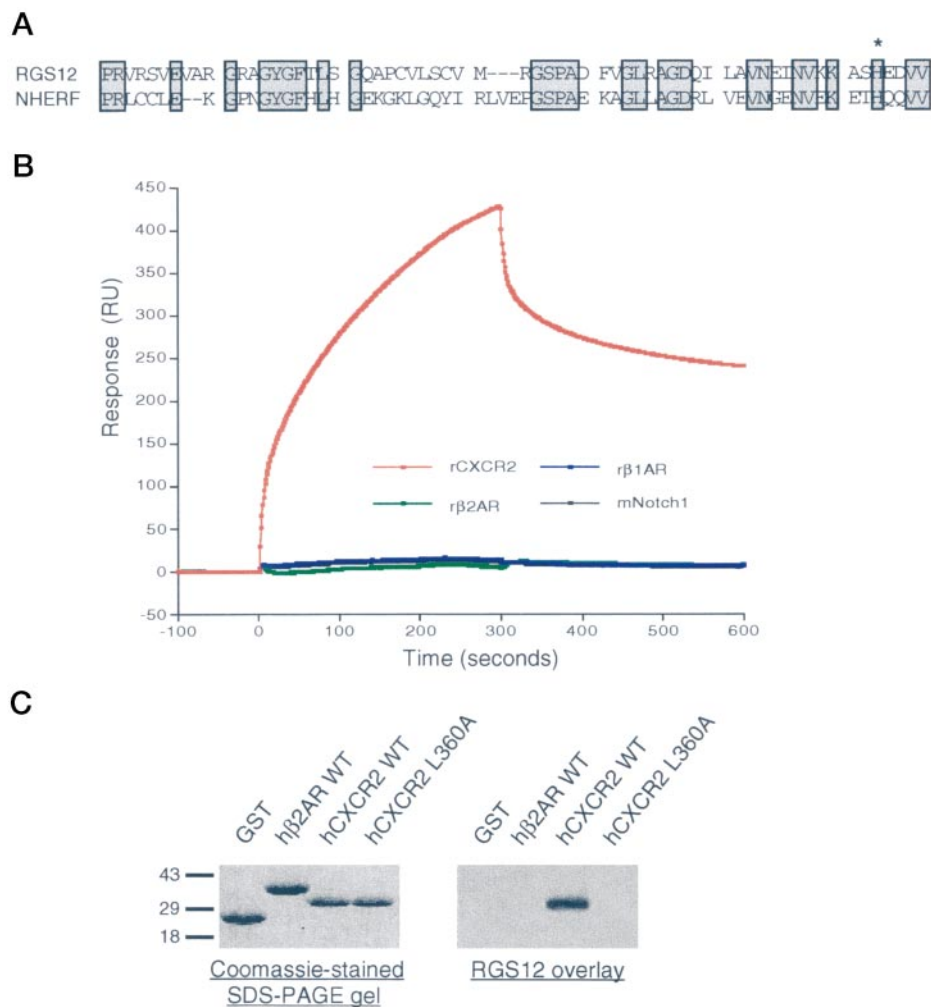
FIG. 2. Northern blot analyses of four *hRGS12* isoforms. Blots of poly(A)⁺ RNA (2 μ g) from various human tissues (A) and brain anatomical features (B) were probed with randomly primed ³²P-labeled human *RGS12* cDNA fragments corresponding to the 3'-UTR described previously (9), the N-terminal PDZ domain (PDZ), and the alternative 3' exon (*Alt 3'-end*) described under "Results and Discussion." Blots were then probed with ³²P-labeled human *GAPDH* cDNA (*hGAPDH*) to assess loading. C, schematic representation of the four *hRGS12* transcripts detected by Northern blot analyses. CC, location of predicted coiled-coil heptad repeats previously described (9). ATFV, C-terminal four amino acids encoded by the alternative 3' end.



cDNA probe spanning the entire PDZ domain (nucleotides 55–384 of GenBank AF035152), and a 350-bp alternative 3' end probe (nucleotides 2441–2790 of GenBank AF030109). Blots were then hybridized with a 1.3-kb fragment of human *GAPDH* cDNA to correct for RNA loading. Probe labelings and blot hybridizations were performed as described previously (15).

Overlay Assay—GST-h β_2 AR tail (80 amino acids) and GST-hCXCR2 tail (40 amino acids) fusion proteins were produced via PCR amplification of the tails and insertion of the PCR products into a pGEX-2T vector (Amersham Pharmacia Biotech). Mutation to the hCXCR2 tail was induced by the use of a mutant sequence oligonucleotide during PCR and confirmed by sequencing. GST fusion proteins were expressed

FIG. 3. Specific binding of the rat RGS12 PDZ domain to the C terminus of interleukin-8 receptor B (CXCR2) as measured by SPR and protein blot overlay assays. *A*, sequence similarity between the N-terminal PDZ domain of human RGS12 and PDZ domain 1 of human NHERF. Asterisk denotes conserved histidine residue starting the second α -helix (αB) of the PDZ fold, previously shown within the PSD-95 PDZ-3/peptide co-crystal structure to interact with threonine at position -2 of the QTSV-COOH peptide binding site (26). *B*, simultaneous SPR measurement of binding to C-terminal peptides from rat interleukin-8 receptor B (rCXCR2), β_1 - and β_2 -adrenergic receptors (r β_1 AR, r β_2 AR), and mouse Notch1 protein (mNotch1) after injection (time 0 s, flow rate 10 μ l/min) of 50 μ l of 2.5 μ M GST-rRGS12-(1–94) fusion protein at 25 $^{\circ}$ C (see Table II for peptide sequences). *C*, equal amounts (25 μ g) of GST and GST fused to the C termini of human β_2 -adrenergic receptor (h β_2 AR), interleukin-8 receptor B wild-type (hCXCR2 WT), or a mutant of the interleukin-8 receptor B where the final residue was changed from leucine to alanine (hCXCR2 L360A) were resolved on an SDS-polyacrylamide gel, blotted, and overlaid with thioredoxin-rRGS12-(1–440) fusion protein (50 nM). Bound RGS12 was detected by Western analysis using an anti-thioredoxin monoclonal antibody. The *left panel* shows a Coomassie stain of the fusion proteins; the *right panel* shows the binding of RGS12 following a blot overlay experiment. The positions of molecular weight markers are shown on the *left*.



and purified as described above. GST and GST fusion proteins were resolved by SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose, and incubated with recombinant thioredoxin-rRGS12-(1–440) fusion protein. Bound protein was detected using an anti-thioredoxin mouse monoclonal antibody.³ Horseradish peroxidase-conjugated goat anti-mouse secondary antibody, and enhanced chemiluminescence (Amersham Pharmacia Biotech).

Biosensor Measurements—Surface plasmon resonance measurements were performed on the BIAcore 2000 (Biacore Inc., Piscataway, NJ). N-terminally biotinylated, synthetic polypeptides were bound to a streptavidin-coated sensor surface (Sensor Chip SA) as per manufacturer's instructions to a density of 300 response units (RU). GST and thioredoxin fusion proteins were 2-fold serially diluted in running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20) and injected at a 10 μ l/min flow rate; surface regeneration was performed by 10- μ l injections of 1 M NaCl in 50 mM NaOH at a 20 μ l/min flow rate. Relative binding to test peptides (test) was calculated based on the increase in response units relative to simultaneous measurement of binding to wild-type CXCR2 peptide (wt) and negative control peptide (ctrl) surfaces at 286 s after the start of a 300 s total protein injection: relative binding = $(RU_{286s}^{test} - RU_{286s}^{ctrl}) / (RU_{286s}^{wt} - RU_{286s}^{ctrl})$.

Yeast Two-hybrid Analysis—Yeast strain PJ69–4A and the plasmids pAS1 and pACTII have been described (19, 20). Rat RGS12 cDNA (amino acids 1–94) was amplified from rat brain cDNA using PCR, cloned into pCR2.1 (Invitrogen), sequenced, and subcloned in-frame downstream of the Gal4p DNA binding domain (DBD) (amino acids 1–147) in pAS1 using the *EcoRI* and *SalI* sites. cDNA encoding amino acids 322–359 of the rat high affinity interleukin-8 receptor B (rCXCR2; Swiss-Prot no. P35407) was amplified from rat brain cDNA using PCR, cloned and sequenced in pCR2.1, and subcloned in-frame downstream of the Gal4p activation domain (AD) (amino acids 768–881) in pACTII

using the *EcoRI* and *XhoI* sites. Nonsense and missense mutations to the rCXCR2 C-terminal tail were engineered within the pCR2.1 plasmid using QuikChangeTM site-directed mutagenesis (Stratagene, La Jolla, CA), sequenced, and subcloned into pACTII as above.

Yeast strain PJ69–4A was co-transformed with pAS1- and pACTII-based plasmids using the Gietz lab transformation kit (Bio/Can Scientific, Mississauga, Ontario, Canada) and plated on synthetic complete drop-out medium (SC) lacking tryptophan and leucine (BIO 101, Vista, CA). Six independent yeast colonies from at least two independent transformations were then plated onto SC lacking tryptophan, leucine, histidine, and adenine, supplemented with 2 mM 3-aminotriazole.

RESULTS AND DISCUSSION

We recently described the cloning of *Rgs12* from rat brain using a degenerate PCR strategy directed toward conserved regions of the RGS domain (9). To ascertain whether RGS12 is capable of stimulating the GTPase activity of G-protein α subunits, the conserved RGS domain of rRGS12 (amino acids 664–885) was expressed as a GST fusion protein in *E. coli* and purified by glutathione-Sepharose chromatography. The ability of GST-rRGS12-(664–885) protein to enhance G α GTP hydrolysis was measured in single turnover assays using [γ -³²P]GTP-loaded, recombinant G α_1 , G α_o , G α_q , and G α_s proteins. The slow rate of GDP dissociation and GTP binding observed for wild-type G α_q protein relative to its intrinsic GTPase activity makes it difficult to prepare GTP-G α_q for use in single turnover studies (21). Thus, a GTPase-deficient mutant of G α_q (R183C) was employed; this protein serves as an adequate substrate for detection of the GAP activities of RGS2 and RGS4.² The GTPase activities of myr-G α_1 and myr-G α_o proteins were increased by the addition of GST-rRGS12-(664–885) protein,

³ G. M. Brothers and D. P. Siderovski, unpublished data.

TABLE I
Yeast two-hybrid analysis of rat RGS12 PDZ domain binding specificity

Gal4p-DBD fusion	Gal4p-AD fusion	C terminus of Gal4p-AD	Growth ^a on -Leu-Trp -His-Ade + 2 mM 3-aminotriazole
None	None		—
Snf1p	Snf4p		++
rRGS12PDZ-(1-94)	Snf4p		—
Snf1p	rCXCR2	VGSSSANTSTTL-COOH	—
rRGS12PDZ-(1-94)	rCXCR2-Δ355-359	VGSSSAN-COOH	—
rRGS12PDZ-(1-94)	rCXCR2	VGSSSANTSTTL-COOH	++
rRGS12PDZ-(1-94)	rCXCR2-L359AA-COOH	—
rRGS12PDZ-(1-94)	rCXCR2-T358AA.-COOH	++
rRGS12PDZ-(1-94)	rCXCR2-T358CC.-COOH	++
rRGS12PDZ-(1-94)	rCXCR2-T358DD.-COOH	+
rRGS12PDZ-(1-94)	rCXCR2-T358FF.-COOH	++
rRGS12PDZ-(1-94)	rCXCR2-T358SS.-COOH	++
rRGS12PDZ-(1-94)	rCXCR2-T357SS...-COOH	—

^a —, 0/6 independent colonies tested; +, 4/6 tested; ++, 6/6 tested.

TABLE II
Surface plasmon resonance analysis of RGS12 PDZ domain binding specificity

Receptor or effector	Peptide	Peptide sequence ^a	Relative binding ^b
A. Rat RGS12 PDZ domain ^c			
β-Adrenergic receptor 1	rβ1AR	PGRQGFSSSESKV-COOH	0
β-Adrenergic receptor 2	rβ2AR	QGRNCNTNDSPL-COOH	0
β-Adrenergic receptor 3	rβ3AR	FDGYEGERPFPPT-COOH	0
Melanocortin receptor 4	rMC4-R	PLGGICELPGRY-COOH	0
Interleukin-8 receptor A	rCXCR1	SASFRTSLTTIY-COOH	0.04 ± 0.01
Interleukin-8 receptor B	rCXCR2	VGSSSANTSTTL-COOH	1.0
Metabotropic glutamate 1α	rMGR1α	ILRDYKQSSSTL-COOH	0.03 ± 0.01
Metabotropic glutamate 2	rMGR2	GREVVDSTTSSL-COOH	0.02 ± 0.01
Metabotropic glutamate 5	rMGR5	IIRDYQSSSSL-COOH	0.07 ± 0.01
Phospholipase C β1	rPLCβ1	GENAGREFDTPL-COOH	0.17 ± 0.01
B. Human RGS12 PDZ domain ^d			
Interleukin-8 receptor A	hCXCR1	YTSSSVNVSSNL-COOH	0.09 ± 0.02
Interleukin-8 receptor B	hCXCR2	VGSSSGHTSTTL-COOH	1.0
	hCXCR2-cap-CONH ₂	0
	hCXCR2-L360AA-COOH	0
	hCXCR2-T359AA.-COOH	1.05 ± 0.04
	hCXCR2-T359AcapA.-CONH ₂	0
	hCXCR2-T358SS...-COOH	0.16 ± 0.05
	hCXCR2-T358AA...-COOH	0.02 ± 0.01
	hCXCR2-S357AA...-COOH	0.22 ± 0.03
	hCXCR2-T356SS...-COOH	0.46 ± 0.02
	hCXCR2-T356AA...-COOH	0.86 ± 0.02
	hCXCR2-H355AA...-COOH	0.78 ± 0.03
Melanocortin receptor 4	hMC4-R	PLGGICDLSSRY-COOH	0
Neuropeptide Y receptor 2	hNPYR2	GPNDSFTEATNV-COOH	0.02 ± 0.01
Phospholipase C β3	hPLCβ3	ADSESQEENTQL-COOH	0.26 ± 0.02
RGS12	hRGS12Tail1	VPGPSRPGTSRF-COOH	0
RGS12 alternative 3' end	hRGS12Tail2	KPKTSAHHATFV-COOH	0.35 ± 0.01

^a -COOH, acid C terminus; -CONH₂, amide C terminus.

^b Binding to wild-type CXCR2 peptide is defined as 1.0 (see "Experimental Procedures").

^c Relative to control surface of mouse Notch1 C terminus (PSQITHIPEAFK-COOH).

^d Relative to control surface of rat β-adrenergic receptor 1 C terminus (rβ₁AR).

albeit modestly in comparison to recombinant, full-length RGS4 (Fig. 1, A and B). In contrast, G_sα and G_qα(R183C) GTPase activities were not enhanced by RGS12 (Fig. 1, C and D). Partially purified preparations of thioredoxin-rRGS12-(1-1387) and thioredoxin-rRGS12-(440-1108) fusion proteins also demonstrated G_iα₁ and G_oα GAP activity (data not shown). Based on these *in vitro* results, we conclude that RGS12 is a *bona fide* member of the RGS family, in that it acts as a GAP for at least G_i class α subunits.

In our initial report of the cloning of rat *Rgs12*, we also identified the human ortholog (9) within cosmid sequences mapping to human chromosome 4p16.3. PCR was used to amplify both the predicted open-reading frame and 3'-UTR of *hRGS12* from human brain cDNA. These two cDNA clones overlapped to form a contiguous sequence encompassing 4870 nucleotides and encoding 1376 amino acids (GenBank

AF035152). Comparison of rat and human RGS12 protein sequences previously revealed nearly identical RGS domains and a highly conserved N terminus with similarity to a C-terminal region of mouse rhophilin (9); subsequent sequence and biochemical analyses (see below) indicate that the latter region encodes a PDZ domain. During the cloning of *rRgs12* cDNA, we observed an alternatively spliced variant lacking the PDZ domain (GenBank AF035151); an alternative exon 1 with a unique 5'-UTR was found to replace exons 1 and 2 of the longer *rRgs12* cDNA (GenBank U92280). Sequence of several human expressed sequence tags suggests that alternative exon usage also occurs in the same position in human *RGS12* (e.g. GenBank N31659 and AA455449).

To explore the tissue distribution of human *RGS12* mRNA isoforms containing or lacking the PDZ domain region, Northern blot analyses were performed with probes specific to either

TABLE III
Potential RGS12 PDZ domain-binding proteins

Protein	Accession no.	C terminus
G-protein coupled receptors (GPCRs)		
Anaphylatoxin C3a receptor (C3aR)	Z73157	STTV
C-C chemokine receptor type 5 (CCR5)	P51682	STGL
Interleukin-8 receptor B (CXCR2)	P25025	STTL
Neuropeptide Y receptor type 2 (NPY-R2)	P49146	ATNV
Transmembrane proteins		
Delta-like protein 1 (DLL1)	Q61483	ATEV
Glutamate receptor 1 (AMPA1)	P42261	ATGL
N-methyl-D-aspartate receptor chain 1 (NMDA-R1)	A47551	STVV
Sodium/phosphate cotransporter 2 (NPT2)	Q06495	ATRL
Others		
Active breakpoint cluster region-related protein (ABR)	A49307	STDV
Breakpoint cluster region protein (BCR)	P11274	STEV
Cyclooxygenase-1 (COX-1)	P23219	STEL
Cyclooxygenase-2 (COX-2)	P35354	STEL
Huntingtin-associated protein 1-binding protein (HAP1-BP)	U94190	STYV
Inducible nitric oxide synthase (iNOS)	P29477	ATRL
Phosphatidylinositol 3-kinase (Cpk-m)	U52193	ATYL
Phospholipase C β 4 (PLC β 4)	A48047	ATVV
Protein kinase A anchoring protein (D-AKAP2)	AF037439	STKL
Regulator of G-protein signaling-12 (RGS12)	AF030111	ATFV
Ribosomal protein S6 kinase II α 1 (p90-Rsk1)	P18653	STTL
Ribosomal protein S6 kinase II α 2 (p90-Rsk2)	P51812	STAL
Ribosomal protein S6 kinase II α 3 (p90-Rsk3)	A57459	STTL

the 3'-UTR or the PDZ domain. Hybridization of the 3'-UTR cDNA probe to poly(A)⁺ RNA prepared from various human tissues (Fig. 2A) and brain regions (Fig. 2B) revealed two major *hRGS12* transcripts of 6.3 and 3.7 kb. The 6.3-kb transcript was expressed abundantly in spleen, testis, ovary, cerebellum, and caudate nucleus, whereas the 3.7-kb transcript was most abundant in whole brain, kidney, thymus, and prostate, and detected at lower levels in all other tissues examined. Within specific brain regions, the 3.7-kb mRNA was most abundant in the cerebral cortex, occipital pole, frontal lobe, temporal lobe, putamen, amygdala, caudate nucleus, and hippocampus. Hybridizing the same Northern blots with a PDZ domain-specific probe revealed two *hRGS12* transcripts of 6.3 and 5.7 kb (Fig. 2, A and B); the absence of hybridization to lower molecular weight mRNA indicates that the 3.7-kb *hRGS12* transcript corresponds to an isoform lacking the PDZ domain. The 6.3-kb mRNA detected by the PDZ domain-specific probe corresponds to the size and tissue distribution of the 6.3-kb transcript detected by the 3'-UTR probe. The additional, 5.7-kb transcript was observed in both the same tissue distribution and abundance as seen for the 6.3-kb transcript.

To determine how the 5.7- and 6.3-kb isoforms differed, the sequence of human *RGS12* was compared with the GenBank data base. Alignments to the predicted amino acid sequences of human expressed sequence tags (e.g. GenBank AA523013) and several other *hRGS12* cDNA clones (e.g. GenBank AF030109 and AF030111) revealed several isoforms of *RGS12* possessing an alternative 3' end. Alternative splicing removes the last four amino acids of RGS12 (GenBank AF035152) and inserts an additional 75 amino acids of open-reading frame and an alternative 3'-UTR. Hybridization of Northern blots with a probe specific for the alternative 3' end detected *hRGS12* mRNA of 5.7 and 3.1 kb (Fig. 2A and B), with the size and tissue distribution of the 5.7-kb transcript identical to that detected with the PDZ domain-specific probe. The 3.1-kb isoform was observed to be expressed with the same tissue distribution and abundance as the 3.7-kb isoform. These data indicate the presence of at least four alternatively spliced isoforms of *hRGS12*, containing or lacking an N-terminal PDZ domain, and encoding one of two alternative C termini (Fig. 2C).

Alternative splicing of the N terminus of human and rat

RGS12 suggests that this region may be functionally important. Sequence analysis revealed highest homology of this region to the PDZ domains of the Na⁺/H⁺ exchanger regulatory factor (NHERF; Fig. 3A), a protein that has recently been shown to bind the C-terminal tail of the β_2 -adrenergic receptor (22). Thus, we examined whether RGS12 might also bind the tail of β_2 AR or other G-protein-coupled receptors (GPCRs). Full-length and truncated forms of human and rat RGS12 were expressed as GST or thioredoxin fusion proteins and their binding to various peptides was investigated by surface plasmon resonance (SPR) analysis. Streptavidin-coated biosensor surfaces were pre-adsorbed with N-terminally biotinylated, synthetic peptides encompassing the last 12 amino acids of various GPCRs. Neither thioredoxin-rRGS12-(1-1387), thioredoxin-rRGS12-(1-440), nor GST-rRGS12-(1-94) fusion proteins bound appreciably to a biosensor surface coated with rat β_1 - or β_2 -adrenergic receptor tail peptides. However, the RGS12 fusion proteins did bind the C terminus of the rat interleukin-8 receptor B (rCXCR2; Fig. 3B and data not shown). GST and thioredoxin proteins alone did not bind any peptide surface tested, whereas full-length NHERF and NHERF domain 1 proteins bound both the rat β_2 AR and CXCR2 tail peptides (data not shown).

We tested the C-terminal-specific nature of this interaction in a protein blot overlay using a point mutant of the CXCR2 C-terminal motif. Thioredoxin-rRGS12-(1-440) protein bound a GST-hCXCR2 tail fusion protein in blot overlay experiments but failed to interact with either GST alone or GST- β_2 AR tail (Fig. 3C); mutation of Leu to Ala at the terminal position within the hCXCR2 tail (L360A) abolished all binding. Specificity for the extreme C terminus of CXCR2 was also shown by yeast two-hybrid analysis. Rat RGS12 PDZ domain fused to the Gal4p transcription factor DBD and wild-type or mutated rCXCR2 tails fused to the Gal4p AD were expressed together in a yeast strain containing *HIS3* and *ADE2* reporter genes under the control of the *GAL1* and *GAL2* promoters, respectively (19). Growth on selective media lacking both histidine and adenine was compared with a positive control strain co-expressing the strongly interacting yeast proteins Snf1p and Snf4p (23) and a negative control strain co-expressing solely the Gal4p DBD and AD proteins. Co-expression of rRGS12 PDZ domain with wild-

type rCXCR2 receptor tail allowed prototrophic growth (Table I); however, truncation of the last five C-terminal amino acids of the rCXCR2 tail ($\Delta 355$ –359), or mutation of the -2 or terminal residues, abolished the interaction. Several missense mutations at the -1 position within the rCXCR2 tail did not disrupt the interaction, consistent with previous reports of PDZ domain binding specificity (24).

We were intrigued by the selectivity shown by RGS12 for threonine at the -2 position of the CXCR2 tail, as removal of the C β methyl group by mutation of threonine to serine was observed to abolish completely the interaction in yeast (Table I). To confirm this specificity, we tested three different rat metabotropic glutamate GPCR tails with Ser/Thr-rich carboxyl termini ending in S-(S/T)-L, of which MGR1 α and MGR5 interact with the PDZ domain of the neuron-specific protein Homer (25). The lack of binding of GST-rRGS12-(1–94) to these three MGR tail peptides, as measured by SPR (Table II, A), confirms the preference of RGS12 for the motif T-X-L *versus* S-X-L. While necessary, the C-terminal T-X-L motif alone is not the sole determinant of RGS12 PDZ domain binding, as interaction with phospholipase C $\beta 1$ and $\beta 3$ carboxyl termini, both possessing the T-X-L motif, was at least 4-fold weaker than with the CXCR2 tail (Table II, A and B). Alanine/serine scanning mutagenesis of the last six residues of human CXCR2 tail peptide revealed the most important residues for hRGS12 PDZ binding specificity as the -3 , -2 , and 0 positions (S-T-X-L), with lesser contributions from the -4 and -5 positions (Table II, B). In addition, the failure of GST-hRGS12PDZ-(1–110) to bind amide-blocked C-terminal CXCR2 peptides confirms a critical role for the free carboxylic acid moiety in the binding interaction, as previously observed for other PDZ domains (26).

The predicted C terminus of the alternative 3' end splice isoform of hRGS12 is similar to that of human CXCR2, with both tails conforming to a consensus of (T/S)-S-(A/G)-H-X-(A/S)-T-X-(L/V). We therefore tested binding of the human RGS12 PDZ domain to synthetic peptides encompassing the last 12 amino acids of the two, alternative carboxyl termini of RGS12. Although no binding was detected to the T-S-R-F C terminus conserved between human and rat RGS12 (hRGS12Tail1; Table II, B), GST-hRGS12PDZ-(1–110) protein was shown to bind the A-T-F-V C-terminal RGS12 peptide (hRGS12Tail2; Table II, B). While a pattern search of the Swiss-Prot data base identified only CXCR2 as terminating with a polypeptide sequence conforming to the (T/S)-S-(A/G)-H-X-(A/S)-T-X-(L/V) motif, several other GPCRs, membrane-spanning proteins, and intracellular proteins with GAP or other enzymatic activities were identified as terminating in the shorter (A/S)-T-X-(L/V) motif. These potential RGS12 PDZ domain-binding proteins and their C-terminal sequences are summarized in Table III. It must be noted, however, that a terminal (A/S)-T-X-(L/V) motif alone is clearly not sufficient to specify RGS12 PDZ domain binding, as no appreciable binding has been observed to the A-T-N-V C-terminal tail of the human neuropeptide Y receptor type 2 (Table II, B). We are currently evaluating other proteins from Table III for their ability to bind RGS12.

Binding of the RGS12 PDZ domain to the RGS12 A-T-F-V tail was somewhat weaker than binding to the CXCR2 tail. However, among those form(s) of RGS12 containing both the N-terminal PDZ domain and the A-T-F-V C terminus, the in-

teraction could be kinetically favored given its intramolecular nature. Such a scenario is reminiscent of the mechanism of reversible autoinhibition of Src family kinases, which adopt a "closed" conformation upon intramolecular association of their N-terminal SH2 and SH3 domains with C-terminal motifs (27). Intramolecular association of the N and C termini within RGS12 isoform(s) may serve as a source of regulation, either of its G α -directed GAP activity or of PDZ-mediated binding to other proteins. The ability of the alternative C terminus to serve as a docking site for PDZ domain-containing proteins also presents the possibility of *in vivo* concatemerization and/or organization of RGS12 within multi-component signaling complexes. Compartmentalization by virtue of the N- and/or C-terminal domains of RGS12 could thereby associate desensitizing GAP activity within specific receptor complexes, analogous to inclusion of the negative regulatory function of protein kinase C within the *Drosophila* phototransduction complex by InaD (14).

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