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 Gα 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)

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF035151 and AF035152.

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‡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-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.
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’-ATATGCTCTCCAAAGGCAACTGAGACGG-3’ and antisense primer 5’-TACGGGGCCAAGGTGGAGGGATC-3’) and 3’-UTR of hRGS12 (sense primer 5’-ATCCCTCCACCTTG-GCCCGTAAGAC-3’ and antisense primer 5’-CTGTCGGAGGCTCCTCAGTTTC-3’) were designed based on cosmid sequences (9) and used to amplify the hRGS12 cDNA from 0.5 ng of Marathon-Ready™ human brain cDNA (CLONTECH) using the Expand™ 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-RGS12-(1–94), GST-RGS12-(664–885), and GST-RGS12-(1–110) fusion proteins was induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside for 8 h at 28 °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-nitrioltriacyl 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 Gα, myr-Gα, and myr-Gα were expressed and purified as described elsewhere (16). A point mutation of Gα, Arg-183 to Cys, which reduces without abolishing the intrinsic GTPase activity, was purified from SF9 cells co-infected with Gα(R183C), Gβ1, and Gγ2 baculoviruses (17). Recombinant Gα4 was purified from E. coli as described previously (18). Purification of recombinant RGS2 from SF9 cells will be described elsewhere.2

Purified Gα4(R183C) was loaded with [γ-32P]GTP (specific activity 500 cpm/pmol) for 3 h at 20 °C in the following buffer (150 mM final volume): 10 μM GTP, 5.5 mM CHAPS, 50 mM sodium HEPES, pH 7.5, 1 mM DTT, 1 mM EDTA, 0.9 mM MgSO4, 0.1 mg/ml bovine serum albumin, 30 mM (NH4)2SO4, and 4% glycerol. Other Gα subunits were loaded in the same manner in the absence of MgSO4; Gα and myr-Gα subunits were incubated for 20 min at 20 °C, and myr-Gα 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 sodium HEPES, pH 7.5, 1 mM EDTA, 0.9 mM MgSO4, 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 EDTA, 0.9 mM MgSO4, 0.01 mg/ml bovine serum albumin, and 1 mM GTP). GAP activity was initiated by adding Gz-GTP to RGS protein sample and OG buffer (supplemented with 9 mM MgSO4 when Gα, myr-Gα, or myr-Gα were used). Timed, 100–μl aliquots were withdrawn and quenched with 900 μl of a 5% (w/v) slurry of Norit A. After centrifugation, 600 μl of supernatant was counted for 32P content.

Northern Blot Analyses—Human MTN blots (CLONTECH), containing 2 μg 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

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Fig. 2. Northern blot analyses of four hRGS12 isoforms. Blots of poly(A)+ RNA (2 μg) 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-hβAR 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...
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 monoclonal antibody. The right 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.

**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 Go GTP hydrolysis was measured in single turnover assays using [γ-32P]GTP-loaded, recombinant Gq, Go, Gs, and Gα proteins. The slow rate of GDP dissociation and GTP binding observed for wild-type Gα protein relative to its intrinsic GTPase activity makes it difficult to prepare GTP-Gq protein for use in single turnover studies (21). Thus, a GTPase-deficient mutant of Go (R183C) was employed; this protein serves as an adequate substrate for the GST-rRGS12 (664–885) protein. GAP Activity and PDZ Domain Binding Specificities of RGS12

3 G. M. Brothers and D. P. Siderovski, unpublished data.
albeit modestly in comparison to recombinant, full-length RGS4 (Fig. 1, A and B). In contrast, Gα and Gβγ R183C  

GTPase activities were not enhanced by RGS12 (Fig. 1, C and D). Partially purified preparations of thioredoxin-rRGS12-1–1387 and thioredoxin-rRGS12-(140–1108) fusion proteins also demonstrated Gα and Gβγ 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 Gα class 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 rpholin (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 Rgs12 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 N31859 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
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 whole brain, kidney, thymus, and hippocampus. Hybridizing the same Northern blots with a PDZ domain-specific probe revealed two major mRNA transcripts of 6.3 and 5.7 kb. These transcripts of 6.3 and 3.7 kb. The 6.3-kb transcript was compared with the GenBank accession number, C terminus

| Protein                                      | Accession no. | C terminus |
|----------------------------------------------|---------------|------------|
| G-protein coupled receptors (GPCRs)          |               |            |
| Anaphylatoxin C3a receptor (C3aR)            | Z73157        | STTV       |
| C-C chemokine receptor type 5 (CCR5)         | P61862        | 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)      | Q66495        | 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)                     | P35554        | STEL       |
| Huntington-associated protein 1-binding protein (HAP1-BP) | U94190 | STYV       |
| Inducible nitric oxide synthase (iNOS)        | P29477        | ATRL       |
| Phosphatidylinositol 3-kinase (Cpk-m)        | U52193        | ATYV       |
| Phospholipase C β4 (PLCβ4)                   | 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 (p90-Rsk1)  | P18653        | STTL       |
| Ribosomal protein S6 kinase II α (p90-Rsk2)  | P51812        | STAL       |
| Ribosomal protein S6 kinase II α (p90-Rsk3)  | A57459        | STTL       |

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 β2-adrenergic receptor (22). Thus, we examined whether RGS12 might also bind the tail of β2AR 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 β2- or β3-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 β2AR 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 tail. Thioredoxin-rRGS12(1–440) protein bound to GST-hCXCR2 tail fusion protein in blot overlay experiments but failed to interact with either GST alone or GST-β2AR tail (Fig. 3C); mutation of Leu to Ala at the terminal position within the GST-β2AR 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 Gal4p transcription factor DBD and wild-type or mutated rCXCR2 tails fused to the Gal4p AD were expressed together. Growth on selective media lacking both histidine and adenine was compared with a positive control strain co-expressing the Gal4p transcription factor DBD and wild-type or mutated rCXCR2 tails fused to the Gal4p AD were expressed together.
type rCXCR2 receptor tail allowed prototrophic growth (Table I); however, truncation of the last five C-terminal amino acids of the rCXCR2 tail (∆555–559), 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 Cβ 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 and MGR5 interact with the PDZ domain of the neuron-specific protein Homer (25). The lack of binding of GST-RGS12(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 β1 and β3 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 amidoligated 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)-(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 indicated only CXCR2 as terminating with a polypeptide sequence conforming to the (T/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 interaction 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 G0-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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