Regulators of G protein signaling (RGS) proteins that contain DEP (disheveled, EGL-10, pleckstrin) and GGL (G protein \( \gamma \) subunit-like) domains form a subfamily that includes the mammalian RGS proteins RGS6, RGS7, RGS9, and RGS11. We describe the cloning of RGS6 cDNA, the specificity of interaction of RGS6 and RGS7 with G protein \( \beta \) subunits, and certain biochemical properties of RGS6/\( \beta \) and RGS7/\( \beta \) complexes. After expression in Sf9 cells, complexes of both RGS6 and RGS7 with the G\( \beta \) subunit (but not G\( \beta \) 1–4) are found in the cytosol. When purified, these complexes are similar to RGS11/\( \beta \)5 in that they act as GTPase-activating proteins specifically toward G\( \alpha \). Unlike conventional G\( \rho \)5 complexes, RGS6/\( \beta \)5 and RGS7/\( \beta \)5 do not form heterotrimeric complexes with either G\( \alpha \)5-GDP or G\( \alpha \)5-GTP. Neither RGS6/\( \beta \)5 nor RGS7/\( \beta \)5 altered the activity of adenylcyclases types I, II, or V, nor were they able to activate either phospholipase C-\( \beta \)1 or -\( \beta \)2. However, the RGS/\( \beta \)5 complexes inhibited \( \beta \)12-\( \gamma \)2-mediated activation of phospholipase C-\( \beta \)2. RGS/\( \beta \)5 complexes may contribute to the selectivity of signal transduction initiated by receptors coupled to G\( \alpha \) and G\( \alpha \)5 by binding to phospholipase C and stimulating the GTPase activity of G\( \alpha \)5.

Initial studies of proteins that belong to the regulators of G protein signaling (RGS) family demonstrated that they are GTPase-activating proteins (GAPs) that accelerate hydrolysis of GTP bound to the \( \alpha \) subunits of certain heterotrimeric G proteins (1–3). As such, RGS proteins can function as negative regulators of G protein-mediated signal transduction by speeding deactivation of the active form of G\( \alpha \), subunits, thereby promoting formation of inactive G protein heterotrimers (G\( \alpha \)GDP/\( \gamma \)). It is now clear that some proteins that contain an RGS domain have more complex functions. For example, p115 RhoGEF, which contains an RGS domain near its amino terminus, is an effector for G protein action, catalyzing guanine nucleotide exchange on the monomeric G protein Rho more efficiently when stimulated by G\( \alpha \)13-GTP. The capacity of G\( \alpha \)13 to activate p115 RhoGEF is dependent on the RGS domain of p115, which also accelerates hydrolysis of GTP by G\( \alpha \)13 (4, 5). p115 RhoGEF thus resembles phospholipase C\( \beta \), a well-characterized effector for G protein action that also deactivates its regulator (G\( \alpha \)) by acting as a GAP (6–8).

A subfamily of RGS proteins has been identified in which each member possesses so-called DEP (disheveled, EGL-10, pleckstrin) and GGL (G protein \( \gamma \) subunit-like) domains in addition to an RGS domain (9, 10). Members of this group include mammalian RGS proteins (RGS6, RGS7, RGS9, and RGS11), a Drosophila RGS protein (dRGS7), and EGL-10, an RGS protein found in Caenorhabditis elegans (10–12). Functionally, the GGL domain was shown to specify binding of RGS11 or a fragment of RGS7 to the G protein \( \beta \) subunit (10). It has also been shown that both RGS7 and RGS9 can be isolated from brain and retina as a complex with G\( \beta \) (13–15) and that the distribution of mRNA for the GGL-containing RGS proteins and G\( \beta \) overlap in these tissues (10, 11, 16–20). In addition, Snow and co-workers (10) demonstrated that the RGS11/\( \beta \) complex accelerates hydrolysis of GTP bound to the \( \alpha \) subunit of the G protein G\( \alpha \). The functional significance of the DEP domains of these proteins remains unknown. The mammalian members of this subfamily of RGS proteins are found predominantly in the central nervous system (10, 11, 16, 17), and to date, their role in G protein-mediated signal transduction is unknown.

The superficial resemblance of the complex formed by G\( \beta \) and a GGL-containing RGS protein to a G protein \( \beta \)\( \gamma \) subunit complex suggests potential targets for modulation of G protein signaling. G protein \( \beta \)\( \gamma \) subunits regulate the activity of a diverse array of effectors (adenylcyclases, ion channels, and phospholipase C-\( \beta \) isoforms, among others), play a significant role in interactions of G proteins and G protein-coupled receptor kinases with receptors, and facilitate signal transfer from G protein-coupled receptors to mitogen-activated protein kinase cascades (21). However, relatively little is known about the signaling properties of G\( \beta \). Of the five known G\( \beta \) subunits, G\( \beta \)5 is the clear outlier. Although G\( \beta \) 1–4 are 80–90% identical to each other, G\( \beta \)5 is only about 50% homologous to its relatives (18). Functionally, the recombinant \( \beta \)5\|2 dimer appears capable of stimulating the phospholipase activity of PLC-\( \beta \)2 (18, 22, 23) and coupling G\( \alpha \) with ET\( \alpha \) or m1 muscarinic receptors in crude reconstituted systems (23). However, this complex was unable to stimulate either type II adenyl cyclase in vitro (23) or the mitogen-activated protein kinase pathway in COS cells (22).

We describe herein the cloning of RGS6 and explore the biochemical properties of RGS6 and RGS7, the most closely related member of this group of RGS proteins. We demonstrate that both RGS6 and RGS7 associate strongly and specifically

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* A. Krumins and A. G. Gilman, unpublished observations.
with Gβ5. These RGS/β5 complexes have been purified following expression directed by recombinant baculoviruses and tested for interactions with certain recombinant G protein α subunits and known effectors for G protein action.

**Experimental Procedures**

**Materials—γ-32P GTP/γ-32P GTP** were obtained from NEW Life Science Products. Spodoptera frugiperda (Sf9) cells were maintained and recombinant baculoviruses were amplified as described previously (24). Baculoviruses encoding amino-terminally hexahistidine-tagged Gβ5 and 5 have been described previously (10, 49). Viruses encoding amino-terminally hexahistidine-tagged Gβ3 and 4 were prepared similarly. Gαz, Gαs, and p115 Rhoger were generously provided by Dr. Tohru Kozasa (University of Texas Southwestern Medical Center); PLC-β1 and PLC-β2 by Dr. Paul Sternweis (University of Texas Southwestern Medical Center); antisera against Gαs (SGS-1) by Dr. William Simonds (National Institutes of Health); an antisem that reacts with Gαi1–4 subunits (B600) by Dr. Susanne Mumby (University of Texas Southwestern Medical Center); and a recombinant baculovirus encoding GαS by Dr. Andrejs Krumins (University of Texas Southwestern Medical Center).

**Cloning of Human RGS6 cDNA**—A partial rat RGS6 cDNA sequence (GenBank: RNU32436), identified by homology to the mouse Systems (St. Louis, MO), and sequences derived from its 5′ National Laboratory collection of expressed sequence tags through Ge- tag data base identified g87443 as identical to HUMORFE except with a frameshift change. Further BLAST alignments with a human expressed sequence tag data base derived from human RGS6 cDNA clone were Gαz, Gαs, and p115 Rhoger were generously provided by Dr. Tohru Kozasa (University of Texas Southwestern Medical Center); PLC-β1 and PLC-β2 by Dr. Paul Sternweis (University of Texas Southwestern Medical Center); antisera against Gαs (SGS-1) by Dr. William Simonds (National Institutes of Health); an antisem that reacts with Gαi1–4 subunits (B600) by Dr. Susanne Mumby (University of Texas Southwestern Medical Center); and a recombinant baculovirus encoding GαS by Dr. Andrejs Krumins (University of Texas Southwestern Medical Center).

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the required elements for interaction with G-domain, defined by Snow signaling proteins (9). The second region of interest is the GGL domain, which is a highly conserved hydrophobic domain found in RGS proteins. The DEP domain comprises amino acid residues 42–510 with a deduced molecular weight of 54,422.

FIG. 1. Cloning of human RGS6 cDNA and comparison with murine RGS7. A, clone g874443 (open rectangle) containing human 5’ RGS6 cDNA sequences was fused to a 1143-base pair polymerase chain reaction product obtained from sequential amplification of human brain RGS6 cDNA with primers B413 and AP1 followed by B413 and B415 (boxed). Areas of sequence identity are boxed. Open bar, DEP domain; gray bar, GGL domain; black bar, RGS domain. Amino acids conserved among other RGS proteins that contact G protein subunits are indicated with an asterisk.

adenylyl cyclase activity were conducted according to published protocols (31, 32).

RESULTS

Cloning of Human RGS6 cDNA—A full-length coding sequence for RGS6 was obtained by joining a human expressed sequence tag clone (g874443) encoding all of the 5’ region to a primer-extended and polymerase chain reaction-amplified cDNA encoding the complete 3’ region of RGS6 (Fig. 1). The full-length clone contains 2946 nucleotides and has an open reading frame encoding a protein of 472 amino acid residues with a deduced molecular weight of 54,422.

There are three regions of particular interest in the RGS6 protein. The DEP domain comprises amino acid residues 42–121. It is a highly conserved hydrophobic domain found in RGS6, 7, 9, and 11, in addition to a wide variety of other signaling proteins (9). The second region of interest is the GGL domain, defined by Snow et al. (10) as a domain that contains the required elements for interaction with Gβ5. Within the full-length RGS6 protein, the GGL domain is found between amino acid residues 261 and 309 (Fig. 1). The third recognizable motif, the RGS domain, is found between amino acid residues 325 and 451 of RGS6. A series of critical amino acid residues in the RGS domain that contact Gαi was identified from the crystal structure of the complex formed between RGS4 and Gαi, (33). Although there is a substantial tolerance for amino acid substitutions within the consensus RGS domain of the family, there is an expected conservation of those residues that are critical for the direct interaction with the switch domains of the Gα subunit (at least for those RGS proteins that interact with members of the Gα subfamily). Within RGS6, those critical residues are: Glu357-Phe-Ser359, Glu361-Asn362, Asn401, Asp403, Leu432, Asp436-Ser437 and Arg440. Based on the conservation of these critical residues, we predicted that RGS6 would function as a GTPase-activating protein.

Association of RGS6 or RGS7 with G Protein β Subunits—In a previous study with RGS11 (10), Snow and co-workers demonstrated that full-length RGS11 or a fragment of RGS7 formed specific and high affinity complexes with Gβ5. This association required the GGL domain present in RGS11 and RGS7; this domain can also be recognized in RGS6 and RGS9. Although several studies have now shown that RGS7 and RGS9 form heterodimers with Gβ5 (10, 13–15), the specificity of this interaction with Gβ5 has yet to be generalized for the entire subfamily of RGS proteins.

We have addressed this question for full-length RGS6 and RGS7 with a recombinant baculovirus expression system. In these experiments, S9 cells were infected with recombinant baculoviruses encoding RGS6 or RGS7 and hexahistidine-tagged Gβ subunits 1–5. Cells were then processed as described under “Experimental Procedures,” and immunoblots were analyzed for the presence of RGS6 or RGS7 and Gβs 1–5. Upper panel, RGS7; this membrane fraction (M, 3 μg), the cytosolic fraction (C, 20 μg), and the eluted material from the Ni-NTA resin (B, 250 ng) are shown for each experiment. The exposure time for the main immunoblot was 30 s. Longer exposures were necessary to detect Gβs 1–4 in the cytosolic fraction and eluted material from the Ni-NTA resin. The lower series of panels represent a region of the blot shown above, where RGS7 and Gβs 1–4 would be present if detected. The time (in minutes) for each exposure is shown to the left of each panel. Bottom panel, RGS6. The membrane fraction (M, 2 μg), the cytosolic fraction (C, 15 μg), and the eluted material from the Ni-NTA resin (B, 1.5%) are shown for each experiment. The exposure time of the immunoblot shown was 30 s; a longer exposure (5 min) was necessary to detect Gβ4 in the cytosolic fraction and eluted material from the Ni-NTA resin.
Although attempts to purify an RGS/Gβ complex from the cytosol were unsuccessful in experiments with Gβs 1–4, both RGS6 and RGS7 co-purified with Gβ5 reproducibly (see below). Gel filtration of crude cytosolic fractions also revealed that only Gβ5 formed a heterodimer with these RGS proteins (data not shown). In summary, these data demonstrate strong and specific association of the mammalian GGL-containing RGS family with Gβ5.

**Purification of the RGS6/β5 and RGS7/β5 Complexes**—The distribution of the RGS6/β5 and RGS7/β5 complexes between the soluble and particulate fractions is similar to that observed for Gβ5 in brain homogenates (18). Both complexes can be purified to homogeneity from the soluble fraction (Fig. 3) and remain intact when passed through anion and cation exchange resins. Gel filtration analyses indicate a 1:1 complex of RGS7 with Gβ5 (data not shown). Two protein bands are visualized with the RGS7 antibody and migrate electrophoretically near the expected position for the protein (Fig. 3, left panel). The upper band in these preparations disappears after treatment with phosphoprotein phosphatases (data not shown). RGS6 migrates as a single protein species (Fig. 3, right panel).

Efforts to purify these RGS/β5 complexes from the particulate fraction have not been successful. This may be due to several factors, including an instability of the complex in the presence of detergent and/or the presence of a large amount of poorly folded or aggregated recombinant protein in the particulate fraction.

**Stimulation of Gα GTPase Activity by RGS6/β5 and RGS7/β5**—Although GAP activity that is largely specific for Gα5-GTP was observed previously with the GRS1I/β5 complex (10), Levay et al. (14) claim that the nucleotide-dependent interaction of RGS7 with Gαq is abolished in the presence of Gβ5. Despite this claim, GAP activity of purified RGS6/β5 or RGS7/β5 complexes was detected readily. Of interest, both of these complexes showed the same remarkable specificity for Gαq observed previously with GRS1/β5 (Fig. 4). The GTPase activity of Gαq was clearly enhanced by both RGS6/β5 and RGS7/β5, but the activities of Gα11, Gα12 (data not shown), Gα13 (data not shown), Gαq, Gαq, Gαq, and Gαq were not.

Substrate specificity was not altered when crude membranes containing RGS6/β5 or RGS7/β5 were tested for GAP activity (data not shown). Interestingly, RGS6 and RGS7 in crude membrane fractions exhibited modest GAP activity toward Gαq-GTP in the absence of recombinant Gβ5 (data not shown). Although we cannot rule out the presence of a *S. frugiperda* ortholog of Gβ5 in these experiments, it is certainly possible (or likely) that association with Gβ5 is not a prerequisite for the GAP activity of these proteins. The GAP activity of membrane-associated RGS7 was consistently greater in the presence of recombinant Gβ5 but was not augmented by coexpression of RGS7 with other Gβ subunits. However, there may be several explanations for this difference, including activation of RGS7 by Gβ5 and/or greater stability or proper folding of RGS7 protein in the presence of Gβ5.

**Interactions of RGS6/β5 or RGS7/β5 with Gα5—We** have also assessed the capacity of RGS6 or 7/β5 complexes to form heterotrimers with Gαq or myristoylated Gαq proteins (Fig. 5). Purified (His6)RGS6/β5, RGS7/(His6)β5, or a truncated RGS6 protein lacking the RGS domain (His6)(RGS6ΔR/β5) were incubated on ice for 30 min with GDP and purified Gαq proteins. We then attempted to detect heterotrimeric complexes by adsorption to and elution from Ni-NTA resin. Both Gαq and myristoylated Gαq readily formed stable heterotrimers with β1/(His6)β2. By contrast, heterotrimeric complexes were not detected when RGS6, RGS7, or RGS6ΔR/β5 complexes were tested. In at least this sense, these proteins do not appear to function as G protein βγ subunit-like complexes.

**Interactions with Adenyl Cyclase and Phospholipase C-β**—Although previous studies have demonstrated several roles for βγ complexes in G protein-mediated signaling (21), the Gβ5 subunit appears to be functionally restricted when compared with Gβs 1–4 (18, 22, 23). Although the βγ/2 dimer can stimulate PLC-β2, it does not activate type II adenylyl cyclase. β5/γ2 also associates preferentially with members of the Gαq
subfamily of Gα proteins in vitro and may be selectively released by Gα-linked receptors. Nevertheless, Gβ5 forms heterodimers with γ3, γ4, γ5, and γ7 subunits, as well as the GGL-containing RGS proteins (10, 18). Thus, the role of Gβ5 in signaling may be quite extensive.

We have considered the potential of the RGS6/β5 and RGS7/β5 complexes to interact with three isoforms of adenylyl cyclase and two isoforms of PLC-β. Several βγ dimers inhibit the Gα5-stimulated activity of type I adenylyl cyclase and activate (conditionally with Gαs) type II adenylyl cyclase (34); βγ subunits do not appear to interact with type V adenylyl cyclase. Interestingly, neither RGS6/β5 nor RGS7/β5 could be modulate the activity of any of these adenylyl cyclases in the presence or absence of activated Gαs (Fig. 6). In addition, neither complex was able to interfere with the capacity of β1γ2 to activate type II adenylyl cyclase (data not shown). We considered the possibility that the RGS domain might influence interactions of the complex with effectors. However, neither deletion of the RGS domain of RGS6 nor inclusion of activated Gαs in these assays failed to alter the earlier results.

G protein βγ subunits are also capable of stimulating the activity of selected isoforms of PLC-β. PLC-β2 can be stimulated up to 20-fold by βγ subunits, while the activity of PLC-β1 is relatively insensitive to the βγ dimer (32, 35). However, both RGS/β5 complexes failed to influence the activity of PLC-β1 or PLC-β2 in the presence or absence of activated Gαs (Fig. 7, A and B); removal of the RGS domain did not alter these results (Fig. 7B). We did observe a moderate, concentration-dependent inhibition by RGS/β5 complexes of the ability of β1γ2 to activate PLC-β2 (Fig. 7C). At the highest concentration of RGS/β5 tested, βγ-stimulated phospholipase activity was reduced roughly 25% and 40% by RGS6/β5 and RGS7/β5, respectively. The inclusion of activated Gαs did not influence these results (data not shown).

**Discussion**

We describe herein the cloning of RGS6, the specificity of interaction of RGS6 and RGS7 with the G protein β5 subunit, and biochemical properties of the RGS6/β5 and RGS7/β5 complexes. Sequence alignments of RGS6 with known RGS proteins reveal high sequence homology to a subfamily of RGS proteins that includes RGS7, dRGS7, RGS9, RGS11, and EGL-10. The members of this subfamily each contain a DEP domain near the carboxyl terminus, a GGL domain roughly in the middle of the protein, and an RGS domain near the amino terminus. The mRNA transcripts of the mammalian members of this subfamily are found primarily in the central nervous system, including the retina (10, 11, 16, 17, 19, 36). Among the subfamily members, RGS7 is most similar in sequence to RGS6. It thus seemed likely that RGS6 and RGS7 would share other important properties. Interestingly, their distribution in the central nervous system is somewhat distinct, which may suggest subtle differences in their roles in G protein-mediated signaling (17).

Both RGS6 and RGS7 can be expressed in Sf9 cells and purified to homogeneity from cytosolic extracts as complexes with Gβ5. Like RGS11 (10), the GAP activities of the RGS6/β5 and RGS7/β5 complexes in solution (in vitro) appear specific for Gαs. Previous studies conducted with the RGS homology domain of RGS7 fused to glutathione S-transferase suggested a
proteins such as RGS4 and GAIP (39), the affinity of the RGS7β/β complex for Goα-GDP-αF4 is modest, and interaction between these proteins would likely go undetected in assays designed to measure binding rather than catalytic activity. It has also been suggested that RGS7 may be important in Gq-mediated signaling (38). To date, we have failed to detect GAP activity for these RGS/β complexes with Goα in single turnover assays (Fig. 4) or in steady-state GTPase assays in which the m1 muscarinic cholinergic receptor and heterotrimeric Go were reconstituted in phospholipid vesicles (data not shown).

The specific and high affinity association of the GGL domains of RGS6, RGS7, and RGS11 with Goβ appears to be a general property of this subfamily of RGS proteins. RGS9 has been isolated from rod outer segment membranes as a complex with GoβL, a long splice variant of Goβ found primarily in retina (18). In addition, RGS9 can also form a complex with Goβ when the two genes are coexpressed in S9 cells using a recombinant baculovirus system; however, the specificity of the RGS9 GGL domain for Goβ has not yet been tested. There is an apparent ortholog of Goβ in C. elegans (Q206636), but its association with EGL-10 has not been described (14).

Many of the experiments that we have performed to date have been guided by the hypothesis that RGS/β complexes may play roles analogous to more conventional G protein βγ subunits. Many of these experiments have proven negative, particularly including those designed to detect interactions between RGS/β complexes and GDP-bound Goα subunits. Similarly, these RGS/β complexes by themselves appear unable to modulate the activities of at least certain effectors for Goβ proteins. However, both complexes are capable of inhibiting stimulation of PLC-β2 by βγγ2 (Fig. 7C). When the magnitude of the inhibition appears modest, the affinity of the interaction may be sufficient to target the RGS domain of these proteins to PLC-β2. Deactivation of Goα in the vicinity of PLC-β2 by the RGS domain would ensure rapid sequestration of βγ and termination of phosphoinositide-mediated signaling from the relevant subset of receptors. Importantly, these RGS/β complexes do not inhibit βγγ2 stimulation of type II adenylyl cyclase, and they do not display significant GAP activity (at least in vitro) for the three isoforms of Goα. This constellation of effects raises the possibility of a role of RGS/β6 and RGS7/β as enforcers of selectivity in signaling initiated from receptors that can activate Goα and Goβ.

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