A novel protein class, termed regulators of G protein signaling (RGS), negatively regulates G protein pathways through a direct interaction with Ga subunits and stimulation of GTP hydrolysis. An RGS subfamily including RGS6, -7, -9, and -11, which contain a characteristic Gy-like domain, also has the unique ability to interact with the G protein β subunit Gβ5. Here, we examined the behavior of Gβ5, RGS7, RGS9, and Ga in tissue extracts using immunoprecipitation and conventional chromatography. Native Gβ5 and RGS7 from brain, as well as photoreceptor-specific Gβ5L and RGS9, always co-purified as tightly associated dimers, and neither RGS-free Gβ5 nor Gβ5-free RGS could be detected. Co-expression in COS-7 cells of Gβ5 dramatically increased the protein level of RGS7 and vice versa, indicating that cells maintain Gβγ complex stoichiometry in a manner similar to Gβγ complexes. This mechanism is non-transcriptional and is based on increased protein stability upon dimerization. Thus, analysis of native Gβ5-RGS and their coupled expression argued that in vivo, Gβ5 and Gy-like domain-containing RGSs only exist as heterodimers. Native Gβ5-RGS7 did not co-precipitate or co-purify with Ga, or Ga,G; nor did Gβ5L-RGS9 with Ga. However, in transfected cells, RGS7 and Gβ5-RGS7 inhibited Ga,G-activated Ca2+ response to muscarinic M3 receptor activation. Thus, Gβ5-RGS dimers differ from other RGS proteins in that they do not bind to Ga with high affinity, but they can still inhibit G protein signaling.

Heterotrimeric G proteins transduce information from ligand-activated G protein-coupled receptors to their appropriate intracellular effectors. Upon receptor activation, the Ga subunit of the heterotrimer binds GTP, and the signal is terminated when the bound GTP is hydrolyzed. The intrinsic GTPase activity of Ga subunits is relatively slow compared with the signaling seen in many physiologic responses suggesting that additional factors are needed to accelerate GTPase activity in vivo. One class of GTPase-activating proteins (GAPs) for heterotrimeric G proteins are their effectors; cGMP phosphodiesterase (1), phospholipase C (2), and adenyl cyclase (3). Recently, a new class of GAPs, termed regulators of G protein signaling (RGS) proteins, has emerged (4–7). The currently established role of RGS proteins is to negatively regulate G protein-linked signaling pathways by reducing the lifetime of Ga proteins in the active GTP-bound state. RGS proteins achieve this by functioning as GAPs for Ga subunits (4, 8, 9) and/or through a GTPase-independent mechanism (10, 11).

RGS proteins are characterized by a homologous amino region, referred to as the RGS domain, that is responsible for binding Ga and stimulating its GTPase rate (12, 13). Outside of this RGS domain, however, the more than 30 members of the family are structurally diverse. Other structural elements found in RGS proteins include PDZ, pleckstrin homology, proline-rich, and Dbl-homology (DH) domains, which might mediate subcellular targeting, assemble signaling complexes, or be involved in the regulation of RGS GAP activity (6, 7).

A distinct subfamily of RGS proteins, including RGS6, -7, -9, and -11, contains two unique domains. One is the Disheveled/Egl-10/pleckstrin (DEP) domain, which is found at the N-terminal part of the proteins, and has an unknown function. The other domain is the Gy-like (GGL) domain, which is responsible for the specific interaction with the neurospecific G protein β subunit, Gβ5 (14–16). Reconstitution of Gβ5 and RGS7 in vitro has shown that Gβ5 preferentially forms a heterodimer with RGS7 over Gyγ (15). The dimers of Gβ5 and RGS6, -7, and -11 display GAP activity toward Ga,G in vitro, but not other Ga subunits (14, 17). The Gβ5L-RGS9 complex from photoreceptor outer segments can act as a GAP for Ga,G in vivo.
concent with its effecter, the γ subunit of cGMP phosphodies-
terase (PDEγ) (18).

Importantly, previous works have shown that Gβγ-RGS complexes
exist in vivo (18–20). In contrast, despite the fact that Gβγ has
been shown to interact with Gγ subunits in vitro (21–24), Gβγ complexes have never been detected in native tissues. Here, we ask if Gβγ is always complexed with RGS proteins, does it exist as a monomer, or is it associated with Gα in native tissues. We found that in the cytosol and detergent extracts of membranes from both the retina and brain, Gβγ exists exclusively as a dimer with RGS. We also demonstrate through co-expression of Gβ and RGS7 in cultured cells that a mechanism regulating the stoichiometry of these proteins ex-
ists. Furthermore, although Gβγ-RGS complexes do not bind to Gα subunits with high affinity, the complex can inhibit Gαqmediated signaling through the muscarinic M3 receptor.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Polyclonal antisera were raised against a synthetic pep-tide corresponding to amino acids 463–477 of bovine RGS9 by Alpha Diagnostic International. Antibodies against RGS7, Gβ, and Gγ were described previously (15, 19). RGS7 and G9 antisera were affinity-purified by passing the serum over a Sulfolink column (Pierce) with covalently attached immunizing peptide. Anti-Gαq antibody was kindly provided by Dr. Allen Spiegel (National Institutes of Health, Bethesda, MD), anti-Gγ by Dr. Melvin Simon (Caltech, Pasadena, CA), anti-Gαq by Dr. David Manning (University of Pennsylvania, Philadelphia, PA), anti-PDEγ by Dr. Eva Faurobert (IPMC-CNRS, Valbone, France), anti-arrestin by Dr. Vsevolod Gurevich (Sun Health Research Institute, Sun City, AZ), and anti-phosducin by Dr. Rehwa Lee (UCLA, Los Angeles, CA). Antibodies against the various Gγ subunits were pur-
chased from Santa Cruz Biotechnology, Inc. Secondary antibodies were obtained from Jackson Immunologicals.

**Gel Electrophoresis and Immunoblotting—SDS-PAGE** and Western blot analysis were performed as described previously (15, 19). Visual-
ization of protein bands was performed using ECL reagents obtained
from Pierce, Inc.

**Isolation of Native Gβγ Complexes**—The Gβγ-containing complex was purified from the soluble fraction of bovine retina as described previ-
ously (19). For purification of brain complexes, rat brains were removed
post-mortem and homogenized in 10 ml of buffer containing 20
mM Tris-HCl, 1 mM EDTA, 50 mM NaCl, 2 mM β-mercaptoethanol, pH 7.5 (TEBS). Total brain homogenate was centrifuged at 50,000 × g for
20 min. at 4°C. The supernatant was then dialyzed against 1 ml of buffer
and further resolved on SP-Sepharose. For analysis of the membrane proteins, the pellet was washed twice in ice-cold TEBS buffer. The pellet was then resuspended in TEBS buffer also containing 1% sodium cholate or 1% Genapol C-100. The suspensions were supplemented with 4°C for 1 h before centrifugation at 50,000 × g for
30 min. The supernatant was diluted 1:2 using TEBS buffer and loaded onto
a 5-ml Q-Sepharose column. Bound proteins were eluted with a linear gradient of NaCl from 50 mM to 500 mM in a total volume of 25
ml. Fractions containing Gβγ and RGS7, as detected by Western blot,
were pooled and diluted 4-fold in TEBS buffer without NaCl and ap-
plied to a 2-ml SP-Sepharose column and eluted with a 10-ml gradient
from 50 to 500 mM NaCl in TEBS. Fractions (500 μl) were collected and
analyzed by Western blot.

**Expression of Gβγ and RGS7 in COS-7 Cells**—COS-7 monkey kidney cells were cultured in DMEM with 10% fetal bovine serum under 5% CO2 at 37°C. 2 × 105 cells/well were plated into 12-well plates 1 day before transfection. 1 μg of total plasmid DNA, typically containing 0.5 μg of Gβγ, RGS7, RGS72 (15), or LacZ, was mixed with 5 μl of Lipofecta
MINE (Life Technologies, Inc.) in 200 μl of OPTI-MEM and added to
the washed cells. For RGS7, 0.9 μg of plasmid DNA needed to be used to reliably detect the protein using Western blot. Five hours later, the transfection mixture was removed and replaced with 1 ml of 10% fetal bovine serum in DMEM. Twenty-four and 48 h after transfection, cells were harvested and assayed by Western blot. All cDNAs were carried by the plasmid pBluescript KS+ vector (Stratagene). After linearization, antisense (−32P)UTP-labeled probe was synthesized with Maxiscrypt T7 kit (Ambion). Full-length transcript was purified by electrophoresis on a 5% polyacrylamide, 8 × 0.8-cm Tris borate/EDTA gel.

Total RNA was isolated with TRIzol reagent (Life Technologies, Inc.)
from 104 of COS-7 cells 48 h after transfection with pcDNA3 plasmids
encoding LacZ, RGS7, and Gβγ. The RNase protection assay was performed with 2–10 μg of total RNA and 80,000 cpm of RGS7 probe using
an RPA III kit (Ambion). After hybridization overnight or 45 and 45°C
for 5 and 30 min, RNase digestion, separation, and detection of protected RNA probe was performed by trichloroacetic acid as described by Pham et al. (25). The protected RNA probe was precipitated with 1 ml of 0.75% sodium
pyrophosphate in 5% trichloroacetic acid and 0.025% bovine serum
albumin. The precipitated RNA probes were collected onto glass micro-
fiber filters (GF grade, Whatman) by vacuum filtration. The filters were washed first with 5% trichloroacetic acid, dried, and radioactivity
measured in 4 ml of scintillation mixture in a scintillation counter. To verify
the integrity of the RNA probe after RNase digestion, an aliquot of the
samples was subjected to 5% polyacrylamide electrophoresis and sub-
sequently visualized using autoradiography (BioMax MR, Eastman
Kodak Co.).

**Northern Blot Analysis**—Total RNA (5–15 μg) prepared from trans-
fected COS-7 cells was denatured with glyoxal/dimethyl sulfoxide, res-
olved on a 1% agarose gel, blotted onto BrightStar-Plus membrane
(Ambion), and subsequently hybridized to RGS7-specific [32P]UTP-
labeled riboprobe prepared as described above. Pre-hybridization was
carried out for 30 min at 68°C in ULTRAhyb buffer (Ambion) in
hybridization oven. The blot was hybridized with 5 × 105 cpm probe/ml
membrane at 68°C, washed at 68°C, washed at 68°C, washed at room
temperature for 20 min (twice for 10 min), followed by another 20 min (twice for 15
min) wash at 68°C. The blot was exposed to autoradiography film
(BioMax MR, Kodak).

**Pulse-Chase Labeling and Immunoprecipitation—COS-7 cells
were transfected with RGS7, Gβγ, RGS7 + Gβγ, or LacZ cDNA constructs
as described earlier. Twenty-four hours after transfection, the cells (3–105)
were incubated at 37°C in 60 × 15-mm dishes in methionine- and
cysteine-free DMEM (Life Technologies, Inc.) for 1 h. A pulse of 200
μCi/dish of [35S]methionine and [35S]cysteine (NEN Life Science Products)
was given for 1 h in methionine- and cysteine-free DMEM contain-
ing 10% diazoylated fetal bovine serum. After washing the cells twice
using phosphate-buffered saline (PBS), the cells were chased with se-
um-free DMEM at 37°C. The cells were harvested at the indicated times
in 500 μl of PBS containing 10 mM EDTA and 10 mM phenyl-
ethylsulfonyl fluoride. Following freeze-thawing, the cells were cen-
trifuged for 30 min at 14,000 × g. The supernatants were then pre-
cleared with Protein A-Sepharose beads, and 200 μl of the supernatant was
immunoprecipitated using affinity-purified anti-RGS7 antibody (1
μg of IgG/20 μl of Protein A) or anti-Gβγ antibody for the Gβγ monomer.
Approximately 10 μg of beads were used for each sample. The beads
were washed twice with PBS and then eluted using 2 × SDS-PAGE
loading buffer. The [35S]methionine/cysteine-labeled proteins were resolved by 12% SDS-PAGE, transferred to nitrocellulose, visualized by autoradiography (BioMax MR, Kodak), and quantified using Scion Image.

**Localization of RGS and Gβγ in Retinal Fractions**—Bovine retinas were processed according to the procedure for the isolation of trans-
duced COS-7 cells described above. Bovine retinas were homogenized in
deciliated DMEM (26). Brioni retinas were homogenized and the
resuspended, in an isotonic buffer (10 mM Tris-HCl, 100 mM KCl, 2 mM
MgCl2, 1 mM DTT, pH 7.5) containing 45% sucrose. The suspension was
passed through cheesecloth and centrifuged. Unsolubilized material
was pelleted (P1) at 5,000 × g for 10 min. The supernatant (S1) was
diluted 1:1 in isotonic buffer. Crude rod outer segments (ROS) were
collected (P2) by centrifugation at 15,000 × g for 30 min. The crude
outer segment (OS) pellet was further subjected to ultracentrifugation on a stepwise sucrose density gradient and the purified OS were recov-
ered at the interface of the 1.115 and 1.135 density gradient steps.

**Immunocytochemistry of Mouse Retinas**—The enucleated eye from
an euthanized mouse was placed in 4% paraformaldehyde in PBS for 5
min. The lens and cornea were removed, and the remaining eyeball was
fixed for 1 h. After fixation, the eye was rinsed three times, 15 min
each, in cold PBS, and infiltrated with 30% sucrose overnight. The next
day, the eye cup was embedded in OCT and quickly frozen in liquid
nitrogen, and 10-μm cryosections were obtained. The retinal sections
were blocked for 30 min in PBS containing 1% bovine serum albumin,
1% goat serum, and 0.3% Triton X-100. Affinity-purified RGS7 polyno-
mer antibody was diluted 1:100 in the block solution, applied to the
sections, and incubated at room temperature for 1 h, after which the
sections were washed three times, 5 min each, in PBS containing 1%
bovine serum albumin and 1% goat serum. The fluorescein isothio-
 cyanate-conjugated secondary antibody (Vector laboratories) was diluted
1:50 and incubated for 30 min at room temperature. After rinsing three
times for 5 min each time in PBS, the sections were mounted in Vectashield and viewed under a fluorescence microscope.
**Immunoprecipitation**—Protein A-Sepharose beads (15 μl) were washed with TEBS buffer and then incubated on ice with 1 μg of affinity-purified RGS7 or RGS9 antibody for 30 min. After collecting the unbound material, the antibody-containing beads were mixed with 75 μl of either brain or outer segment extract for 1 h on ice. After washing the beads twice with a large volume of TEBS buffer, a third wash using 75 μl was collected for analysis on Western blot. The proteins were eluted using 75 μl of 2× SDS-PAGE loading buffer and subjected to Western blot analysis. For immunoprecipitation of photoreceptor outer segments, the membranes were first solubilized with 60 mM n-octyl-β-D-glucopyranoside (Sigma) as described in He et al. (27) in either the presence or absence of AMP (25 μM AlCl₃, 10 mM MgCl₂, 10 mM NaF). In initial experiments, a control in which pre-immune serum was bound to the Protein A-Sepharose was tested and revealed no binding of RGS and Gβγ proteins. In subsequent immunoprecipitations, the negative control was extract-mixed with Protein A-Sepharose beads without antibody.

**Effect of Gβ5-RGS7 Complex on Signaling through the Muscarinic M3 Receptor**—CHO cells in six-well plates were either not transfected or transfected with the indicated combinations of cDNAs encoding the human muscarinic M3 receptor (3 μg/well), RGS7 (5 μg/well), and Gβγ (5 μg/well). Twenty-four hours following transfection, cells were harvested in trypsin/EDTA and re-plated in 96 well plates. Approximately 40 h later, cells were loaded for 1 h at room temperature in a balanced salt solution with 5 μM fluo-3-AM in the presence of 0.2% Pluronic F-127. Cells were washed and subsequently excited at 505 nm with emission recorded at 530 nm as an index of 

**RESULTS**

**Purification of Gβ5-RGS Complexes from Retina and Brain**—Although complexes of Gβγ with RGS proteins have been reconstituted in vitro and found in native tissues, Gβγ-Gγ complexes have only been studied in vitro (21–24, 29, 30). In an attempt to detect native Gβγ-Gγ, the Gβγ-containing fraction from the soluble fraction of bovine retina was purified to homogeneity as described previously in Cabrera et al. (19). This purified sample was analyzed using silver stain and Western blot. As shown previously in Cabrera et al. (19), Gβγ and RGS co-migrated throughout the purification. Fig. 1 shows that this Gβγ-RGS complex does not contain a Gγ subunit. As a control for the presence of a Gγ subunit, the SDS-PAGE lane with an equivalent amount of purified transducin heterotrimer shows a band characteristic of Gγ at approximately 10 kDa. These results show that in the retinal cytosol Gβγ is bound to RGS and is not associated with Gγ.

We reasoned that Gβγ could exist in a complex with Gγ in certain non-retinal cell types and thus attempted to detect Gβγ-Gγ complexes in brain extracts. Because Gβ5, in cytosolic and detergent-extracted membrane fractions of rat brain. Fig. 2A shows that in brain cytosol, similarly to the retina, Gβ5 co-migrates with RGS7 upon consecutive anion- and cation-exchange chromatographies. We previously demonstrated that cation-exchange chromatography on SP-Sepharose can be used as an assay for the association of Gβγ with an RGS (15). Since the majority of proteins are negatively charged at physiologic pH, at least 90% of proteins in crude extracts do not bind to this matrix. RGS7 and other GGL-containing RGS proteins contain positively charged amino acid clusters, which perhaps interact with the cation-exchanger and allow the Gβγ-RGS complexes to be absorbed. Thus, co-elution of Gβ5 with RGS7 as a single peak through ion-exchange chromatographic steps indicates that they are associated. In some experiments (one of which is represented in Fig. 2B), a small portion of Gβ5 did not bind SP-Sepharose. However, even if Gβ5 could be detected in the unbound fraction, it represented less than 1% of the total Gβ5. Furthermore, with prolonged development of the Western blots, RGS7 could also be seen in the same sample. In one experiment, we re-loaded the unbound fraction on fresh SP-Sepharose and both Gβ5 and RGS7 then absorbed to the column. This indicates that the small portion of Gβ5 in the unbound fraction is likely due to incomplete absorption on SP-Sepharose due to some variations of experimental conditions rather than Gβ5 existing without an RGS.

To confirm the interaction between Gβ5 and RGS7 using an alternative approach, we co-immunoprecipitated Gβ5 with RGS7 using an affinity-purified antibody to RGS7 (Fig. 2C). Despite the fact that both the immunoprecipitating and detect-
the only RGS species present, 100% of Gβ5 is always present in a dimer with either Gγ2 or Gγ3 and Gα subunits from the membranes. The protein extracts were analyzed by chromatography and immunoprecipitation using the protocols developed for the cytosol. Following 10-fold enrichment on Q-Sepharose, the Gβγ-RGS-containing fractions were pooled and further resolved on SP-Sepharose (Fig. 3A). Chromatography on SP-Sepharose resulted in at least an additional 100-fold purification, and both Gβ5 and RGS7 co-eluted during the procedure. Similarly to the results in Fig. 2 (A and B), regardless of which detergent was used, only a small portion (≤1%) of Gβ5 could be detected in the unbound fraction in some experiments (Fig. 3A). The chromatographic behavior of the membrane-bound Gβγ-RGS7 complex suggests that it is essentially identical to the cytosolic form, as well as the reconstituted dimer (15). Importantly, since neither Gβ5 nor Gγ2 bound to SP-Sepharose, Gβγ is completely resolved from “classic” Gβγ subunits (Fig. 3B). Finally, Gβ5 was immunoprecipitated from the detergent extracts using the anti-RGS7 antibody (Fig. 3C). Recent work by Zhang and Simonds has also shown that Gβ5 and RGS7 can be co-immunoprecipitated (20). Based on these results, we have to conclude that in brain membranes, as well as in the cytosol, Gβ5 is always present in a dimer with either RGS7 or one of the other GGL-containing RGS proteins.

**Coupled Expression of Gβγ and RGS7 in Transfected Cells**—
Supported by previous data on the co-localization of these proteins (31–34), co-purification of Gβγ and RGS proteins indicated that Gβγ and RGS7 are always dimerized in native tissues. This led us to the idea that the stoichiometry between Gβγ and RGS7 must be regulated. To examine this, we tested to see if expression of Gβγ would affect RGS7 levels and vice versa.
Fig. 4 shows that when COS-7 cells were co-transfected with both Gβ5 and RGS7 expression cassettes, the levels of both proteins were increased compared with transfection with the individual constructs. Importantly, the two proteins formed a complex as detected by Gβ5 immunoprecipitation using an anti-RGS7 antibody (Fig. 4B). Co-transfection of an RGS7 mutant lacking the Gβ5-binding GGL domain (RGS7Δ) with Gβ5 did not result in such an increase. This demonstrates the necessity of the protein-protein interaction in stabilizing the Gβ5-RGS5 dimer. Quantification of three independent experiments showed that levels of Gβ5 and RGS7 increased 6- and 10-fold, respectively, when co-transfected (Fig. 4C). In many experiments, particularly when less than 0.9 μg of RGS7 plasmid was used for transfection, RGS7 could not be detected without Gβ5, thereby making quantification of the -fold stimulation impossible. However, in the presence of Gβ5, RGS7 could always be detected even when lesser amounts of RGS7 cDNA were used. This result indicates that cells have a mechanism for regulating Gβ5-RGS7 stoichiometry and further supports the idea that Gβ5 and RGS7 are always together in a complex.

To examine the mechanism by which the Gβ5-RGS7 dimer was regulated in transfected cells, we first performed an RNase protection assay using a labeled RGS7 riboprobe (Fig. 5, A and B). Despite the fact there is more RGS7 protein in COS-7 cells transfected with Gβ5, the level of RGS7 mRNA does not change. Similar experiments have demonstrated that endogenous Gβ5 mRNA also remains unchanged upon infection of rat pituitary cells with an RGS7 adenovirus, while the amount of Gβ5 protein is increased 10-fold. Additionally, Northern blot analysis shows identical levels of RGS7 RNA in cells transfected with RGS7 alone or RGS7 + Gβ5 (Fig. 5C). This suggests that protein degradation and/or synthesis is the major regulator of RGS7 levels in cells. Subsequently, protein degradation in COS-7 cells was studied using pulse-chase labeling and immunoprecipitation (Fig. 6). In cells transfected with RGS7 or Gβ5 alone, the labeled protein was completely degraded within 18 h, with a half-life of about 1.5 h. Conversely, in the presence of Gβ5, the RGS7 protein was stabilized considerably (and vice versa), with a half-life of over 24 h. Along with the RNA data, these results clearly demonstrate that the increase in RGS7 protein levels in the presence of Gβ5 is due to the slowed proteolytic degradation of dimerized RGS7 compared with the monomer.

Native Gβ5-RGS5 Complexes Do Not Bind to Go with High Affinity—Since at least one of the functions of Gβ5-RGS5 complexes is regulation of G protein GAP activity, we sought to determine if Gα subunits could be detected in complex with the Gβ5-RGS5 dimer in native tissue extracts. In photoreceptor OS, transducin is the only G protein and the role of the Gβ5L-RGS9 complex in its regulation has been established. T. Wensel’s laboratory (27, 35) showed that RGS9, which is located in OS, is likely to be the only RGS in the system. However, the polyclonal antibody used in these prior studies was derived against the entire RGS domain of RGS9 and has a minor cross-reactivity with RGS7 (19, 27). Therefore, using RGS7- and RGS9-specific anti-peptide antibodies, we first confirmed that RGS7 is not present in OS. Purified OS were obtained from bovine retinas, and the localization of the RGS proteins was studied by Western blot throughout the fractionation procedure (Fig. 7A). Since we have previously shown that retinal Gβ5 (“short”) is 100% soluble, while Gβ5L is strictly OS membrane-associated

Fig. 6. Stability of RGS7 and Gβ5 monomers compared with the Gβ5-RGS7 dimer in the transfected COS-7 cells. A, COS-7 cells were metabolically labeled with a pulse of [35S]methionine/cysteine and RGS7 was immunoprecipitated at the indicated chase times as described under “Experimental Procedures.” The identity of the indicated bands as RGS7 and Gβ5 were confirmed by Western blot. The data shown are representative of two independent experiments. B, autoradiographs from two independent pulse-chase experiments were quantified using Scion Image and graphed as mean ± S.E. Gβ5 alone, triangles; RGS7 alone, circles; Gβ5 + RGS7, squares.

Gβ5 protein is increased 10-fold. Additionally, Northern blot analysis shows identical levels of RGS7 RNA in cells transfected with RGS7 alone or RGS7 + Gβ5 (Fig. 5C). This suggests that protein degradation and/or synthesis is the major regulator of RGS7 levels in cells. Subsequently, protein degradation in COS-7 cells was studied using pulse-chase labeling and immunoprecipitation (Fig. 6). In cells transfected with RGS7 or Gβ5 alone, the labeled protein was completely degraded within 18 h, with a half-life of about 1.5 h. Conversely, in the presence of Gβ5, the RGS7 protein was stabilized considerably (and vice versa), with a half-life of over 24 h. Along with the RNA data, these results clearly demonstrate that the increase in RGS7 protein levels in the presence of Gβ5 is due to the slowed proteolytic degradation of dimerized RGS7 compared with the monomer.

Native Gβ5-RGS5 Complexes Do Not Bind to Go with High Affinity—Since at least one of the functions of Gβ5-RGS5 complexes is regulation of G protein GAP activity, we sought to determine if Gα subunits could be detected in complex with the Gβ5-RGS5 dimer in native tissue extracts. In photoreceptor OS, transducin is the only G protein and the role of the Gβ5L-RGS9 complex in its regulation has been established. T. Wensel’s laboratory (27, 35) showed that RGS9, which is located in OS, is likely to be the only RGS in the system. However, the polyclonal antibody used in these prior studies was derived against the entire RGS domain of RGS9 and has a minor cross-reactivity with RGS7 (19, 27). Therefore, using RGS7- and RGS9-specific anti-peptide antibodies, we first confirmed that RGS7 is not present in OS. Purified OS were obtained from bovine retinas, and the localization of the RGS proteins was studied by Western blot throughout the fractionation procedure (Fig. 7A). Since we have previously shown that retinal Gβ5 (“short”) is 100% soluble, while Gβ5L is strictly OS membrane-associated

J. D. Neill, L. C. Musgrove, J. C. Sellers, L. W. Duck, D. S. Witherow, K. Levay, and V. S. Slepak, submitted for publication.
Fig. 7. Localization of RGS7 and RGS9 in the retina. A, bovine retinas were fractionated by ultracentrifugation as described under "Experimental Procedures" and the fractions (see text for details) were probed with antibodies to RGS7, RGS9, and Gβ5, B, sections of mouse retinas were prepared as described under "Experimental Procedures" and stained with affinity-purified RGS7 antibody. Bipolar cells with somata in the upper aspect of the inner nuclear layer are immunoreactive for RGS7. The axonal arbors of these bipolar cells extend to the lower aspect of the inner nuclear layer are immunoreactive for RGS9, and stained with affinity-purified RGS7 antibody. Bipolar cells with somata in the upper aspect of the inner nuclear layer are immunoreactive for RGS7. The axonal arbors of these bipolar cells extend to the outer portions of the inner plexiform layer and ends with large varicosities (GO). Some amacrine cells also are immunopositive. RPE, retinal pigmented epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; OPL, outer plexiform layer; GCL, ganglion cell layer.

(19, 30), these molecules were used as internal markers for the fractions. Fig. 7A shows that RGS7 is found in the soluble fraction (S2) along with Gβ5. Immunostaining with anti-RGS7 antibody confirmed that it is not found in OS, but rather is localized mostly to rod bipolar cells (Fig. 7B). RGS9 is located along with Gβ5 in the membrane-associated form in the OS (P2), in accord with previous data utilizing immunostaining (35). Next, we solubilized the OS with n-octyl-β-D-glucopyranoside under conditions preserving the GAP activity of the native RGS9 complex (27) and immunoprecipitated the extract with anti-RGS9 antibody. Probing the obtained fractions with antibodies to Gβ5 and Gα revealed that 100% of Gβ5 was absorbed on the beads while Gα was not co-immunoprecipitated (Fig. 8A). Incubation of the samples with 100 μM AlCl3, 10 mM MgCl2, and 10 mM NaF (AMF), which is known to activate Gβ5-L GAP activity (17), was measured in the presence and absence of Gβ5 and RGS7 using a FLIPR. Fig. 9 (A and B) shows that both RGS7 alone and RGS7 co-expressed with Gβ5 can inhibit Goq-mediated Ca2+ release in response to the muscarinic agonist metacholine. We observed that, in the presence of Gβ5, this inhibition is stronger. However, as shown in Fig. 9C, RGS7 levels are increased dramatically in the presence of Gβ5. Therefore, the stimulation of RGS7 activity by Gβ5 could be due to either increased RGS7 levels or the increased potency of the Gβ5-RGS dimer. Despite the difficulty with the quantitative aspects of these results, it is clear that the Gβ5-RGS complexes, as well as monomeric RGS7, can inhibit Goq-mediated signaling through the M3 receptor.

DISCUSSION

In Native Tissues Gβ5 and RGS7 Are Only Present as a Complex—Previous studies have shown that Gβ5-RGS complexes are found in tissue extracts (18, 19), but it has not been

3 D. S. Witherow and V. S. Slepak, unpublished observations.

FIG. 8. Ga subunits do not co-immunoprecipitate with Gβ5-RGS. A, photoreceptor outer segments were solubilized in the presence and absence of AMF using 60 mM n-octyl-β-D-glucopyranoside. The extract was then immunoprecipitated using anti-RGS9 antibody, and the fractions were examined by immunoblots using Gβ5L and Goq antibodies. B and C, Western blots illustrating the immunoprecipitation of brain membrane extracts stained with either Goq (B) or Gα (C) antibodies. For the control, a 1:1 mixture of Genapol and cholate extracts was used. In A–C, the control represents the extracts mixed with beads not containing any antibody as described under "Experimental Procedures." U, unbound; W, wash; E, eluate.
branched pool of $G_b$ and RGS7, we solubilized membranes with mild detergents and examined the behavior of $G_b$ and RGS7 by chromatography and immunoprecipitation similarly to the cytosolic forms.

In our assays we made an emphasis to quantitatively evaluate the yield and distribution of the complex in all fractions, including in the unbound material. Cation-exchange chromatography completely separates $G_b$ from $G_b$ and $G_y$ subunits and, importantly, shows that essentially 100% of the $G_b$ pool is bound to RGS proteins (Fig. 3). This conclusion is corroborated by our experiments utilizing immunoprecipitation with RGS7 and RGS9 antibodies, as well as the very recent work of Zhang and Simonds (20), who in a reciprocal experiment immunoprecipitated RGS6 and RGS7, but not $G_y$, from brain extract using a $G_b$ antibody.

As in all experiments with membrane proteins, it is possible that detergent could alter the composition of native complexes, i.e. $G_y$ could dissociate from $G_b$, allowing RGS to bind. Indeed, Jones and Garrison (21) have recently demonstrated that the ionic detergents CHAPS and cholate can dissociate the recombinant $G_b$-$G_y$ dimer. In contrast, the non-ionic detergent Genapol C-100 did not disrupt the interaction (21). In light of this, we studied membrane extracts using different detergents including cholate, which has been used to purify $G$ proteins (49). Genapol C-100, shown to preserve $G_b$-$G_y$ (21), and $n$-octyl-$\beta$-$D$-glucopyranoside, which preserves the GAP activity of the native RGS9 complex (27). The results presented in Fig. 3 show that, regardless of the detergent used, $G_b$ and RGS7 from brain membranes exist as a complex that is similar in stability to that of the "classic" $G_b$-$G_y$ dimers. The question why some of the heterodimer is cytosolic and some is membrane-bound remains open and requires additional investigation. The studies of retinal and brain $G_b$-RGS presented here are in agreement with our previous data with in vitro translated proteins that $G_b$ preferentially binds to RGS7 even in the presence of excess $G_y$ (15). Although it is possible in principle that in a specific small population of cells, $G_b$ and RGS proteins may exist apart from each other, current analysis of native extracts indicates that in situ $G_b$ and RGS are present as a tightly associated complex regardless of their subcellular localization.

Control of $G_\beta$-RGS Balance in Cells—The apparent absence of monomeric forms of $G_b$ and RGS7 and $G_\beta$-$G_y$ complexes in situ, as well as the co-localization of $G_b$ and RGS7 in brain (31–34), suggested that their association is tightly controlled. Indeed, as we show here, in cells transiently transfected with RGS7 and $G_b$, the level of expression of one protein is significantly increased in the presence of the other (Fig. 4). The increased protein level is based on the enhanced stabilization of the complex and requires direct protein-protein interaction between $G_b$ and RGS7 (Figs. 4–6). In another recent study, we have shown that infection of neuroendocrine cells in primary culture with an adenovirus expressing RGS7 increases endogenous $G_b$ levels through a post-transcriptional mechanism requiring direct interaction between RGS7 and $G_b$.\(^2\) Our finding of post-transcriptional regulation of $G_b$ and RGS7 is supported by research by Benzing et al. (50), who demonstrated degradation of RGS7 through the ubiquitin-proteosome pathway. Co-regulation of $G_b$ and RGS protein levels has also independently been shown by others and thus appears to be a universal phenomenon. The knockout of the RGS9 gene in mouse leads to disappearance of $G_b$ from photoreceptors (51). Snow et al. (16) observed greater levels of RGS6 and $G_b$ in cell lysates when they were co-expressed. In contrast, Kovoor et al. (52) recently reported that the amount of RGS7 expressed in Xenopus oocytes does not change upon co-expression of $G_b$.
It is clear that Gβ₅ does not co-immunoprecipitate with RGS7 because the complex was denatured due to the presence of 4% SDS during the protein extraction. Furthermore, even though the SDS was diluted to 0.4% for immunoprecipitation, it is possible that some of the IgG was denatured, making quantitative analysis of RGS7 inaccurate. Even if RGS7 levels do not increase when co-expressed with Gβ₅ in the oocytes, it is clear that Gβ₅-RGS balance is maintained in mammalian cells. Our results show this mechanism to be analogous to regulation of Gβγ dimers where the Gβ₂γ stoichiometry is also controlled through proteolytic degradation of unassociated subunits (53–56).

**Function of the Gβ₅-RGS7 Complex**—The hallmark function of RGS proteins is to act as GAPs for Go subunits, and therefore studies have been performed to analyze the GAP activity of recombinant Gβ₅-RGS complexes. In contrast to the isolated RGS domains of large RGS proteins or relatively small monomeric RGSs such as RGS4, the functionally active, full-length GGL-containing RGS proteins cannot be easily obtained in the large quantities required for GAP assays. Despite this difficulty, initial experiments presented by Snow et al. (14) showed that the complex of Gβ₅ with RGS11 lacking the DEP domain can be produced in a baculovirus system, and clearly demonstrated that this truncated mutant had GAP activity toward Goₐ. In later studies of recombinant complexes of Gβ₅ with full-length RGS6 and RGS7, Posner et al. (17) confirmed the GAP activity of Gβ₅-RGS dimers. This GAP activity is remarkably specific to Goₐ but not Goₕ (14, 17). Interestingly, in cell-based assays, RGS7 can inhibit Goₐ-mediated (52, 56) as well as Goₕ-mediated signaling (41). Here, in accord with these previous studies, we show that Gβ₅-RGS7 can inhibit Goₕ-mediating Goₐ²⁺ mobilization caused by stimulation of the M3 muscarinic acetylcholine receptor in CHO cells (Fig. 9). The controversy between the GAP analysis and functional cellular assays can be reconciled by two considerations. First, it is possible that Gβ₅-RGS7 acts via a non-GAP mechanism, similarly to that described for RGS4 and GAIP (10, 11). Second, the interaction of Gβ₅-RGS7 with the G protein and the GAP activity may require the presence of other components of the pathway, such as effector or receptor. It is known that the photoreceptor RGS protein, RGS9, acts only in the presence of the G protein effector PDEγ (18, 27). Furthermore, partial purification of native Gβ₅L-RGS9 complex leads to the loss of GAP activity even in the presence of PDEγ, suggesting the necessity of an additional component (18). Importantly, it appears that RGS7 can only inhibit Goₐ-mediating signal transduction from certain receptors, as a different Goₐ-coupled receptor (gonadotropin-releasing hormone receptor) cannot be inhibited by RGS7. The receptor specificity of RGS action has been previously demonstrated for RGS1, -4, and -16 (58, 59).

In an attempt to identify molecules that interact with Gβ₅-RGS in situ, we carried out a series of experiments utilizing chromatography and immunoprecipitation. Because Gβ₅-RGS7 is a GAP for Goₐ, and RGS7 inhibits Goₐ-mediating signaling, these Go subunits are obvious candidates for such a direct protein-protein interaction. We found that neither Goₕ nor Goₐ co-immunoprecipitates with Gβ₅-RGS7 complex during purification (Fig. 8). No binding was detected in the presence or absence of aluminum fluoride (AMF), a promoter of G protein-RGS binding. It is unlikely that we tested the samples for the “wrong” Go subunits, as these assays also did not reveal the interaction between Gβ₅L-RGS9 and transducin (Fig. 8C) for which the interaction has been established (18, 27, 51). We have also conserved studies using recombinant Gβ₅-RGS complexes in accord with previous data using recombinant Gβ₅-RGS dimers (15, 17) showing that Gβ₅-RGS7 interacts with Goₕ with low affinity. This affinity, however, must be sufficient to confer the functional effects upon Goₐ and Goₕ in cells and on Goₕ in GAP assays.

Although it is clear that the Gβ₅-RGS complexes can act as GAPs for Goₐ subunits, the role of Gβ₅ in this interaction is unknown. In pull-down binding assays, full-length RGS7, as well as its RGS domain alone, binds to Goₐ (15, 57), while Gβ₅-RGS7 complexes do not (15, 17). This implicates the role of Gβ₅ as an inhibitor of Goₐ-RGS binding. Similarly, the RGS domain of RGS9 exerts GAP activity toward Goₕ alone, while the native Gβ₅L-RGS9 complex does not. The GAP activity of the native complex requires the presence of the effector enzyme PDEγ. Also consistent with the idea that Gβ₅ constrains RGS in a less active form is the fact that the GAP activity of the RGS7 domain (41), and also full-length RGS4, is much stronger (>10-fold) than that of the Gβ₅-RGS7 complexes (17). Another possible role of Gβ₅ is to increase the specificity of the complex toward Goₐ subunits. For example, the RGS7 domain alone binds to Goₕ and Goₐ (57), while the Gβ₅-RGS7 complex has only been shown to be a GAP for Goₐ (17). Thus, data obtained in vitro indirectly suggests that Gβ₅ may either attenuate RGS function or confine Goₐ specificity. In contrast, Kvoor et al. (52) have recently shown that Gβ₅ augments RGS7-mediated inhibition of Goₕ signaling in oocytes. Our current results also allude that Gβ₅ increases RGS7 activity. However, since Gβ₅ enhances the amount of RGS7, the correct interpretation of the data must require further experimentation where the expression levels of Gβ₅, RGS, and other molecules are controlled.

The data presented here show that Gβ₅ and RGS7 always exist as a complex in vivo, and as with Gβγ subunits, cells coordinate their stoichiometry. Gβ₅-RGS7 dimers do not bind to Goₐ subunits with a high affinity, but can inhibit Goₐ-mediating signaling through the M3 receptor in a cellular assay. Previous experiments, even very recent studies (34, 50, 61, 62), investigated the roles of recombinant Gβ₅ (i.e. Gβ₅γₐ complexes) and GGL-containing RGS proteins outside the context of each other. However, the current data dictate that studying Gβ₅ and these RGS proteins requires viewing them not as separate entities, but as heterodimeric proteins that potentially have several functions.

**REFERENCES**

1. Arshavsky, V. Y. & Bownds, M. D. (1992) *Nature* **357**, 416–417
2. Berstein, G., Blank, J. L., Jhon, D. Y., Exton, J. H., Rhee, S. G. & Ross, E. M. (1992) *Cell* **70**, 411–418
3. Scholich, K., Mullinix, J. B., Witt科普, C., Poppleton, H. M., Pierre, S. C., Lindorfer, M. A. & Garrison, J. C. & Patel, T. B. (1999) *Science* **1328–1331
4. Berman, D. M., Wilkie, T. M. & Gilman, A. G. (1996) *Cell* **96**, 425–452
5. Dobshott, G. H. & Thorne, J. (1997) *Biol. Chem.** **272**, 3871–3874
6. Hepler, J. B. (1999) *Trends Pharmacol. Sci.** **20**, 376–382
7. De Vries, L. & Farquhar, M. G. (1999) *Trends Cell Biol.** **9**, 138–144
8. Hunt, T. W., Fields, T. A., Casey, P. J. & Peralta, E. G. (1996) *Nature* **383**, 175–177
9. Watson, N., Linder, M. E., Druery, M. K., Kehr, H. J. & Blumer, K. J. (1996) *Nature* **383**, 172–175
10. Hepler, J. K., Herman, D. M., Gilman, A. G. & Kazusa, T. (1997) *Proc. Natl. Acad. Sci. U. S. A* **94**, 428–432
11. Yan, Y., Chi, P. P. & Bourne, H. R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11924–11927
12. Popov, S., Yu, K., Kazusa, T. & Wilkie, T. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 7216–7220
13. Faurobert, E. & Hurley, J. B. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2945–2950
14. Snow, B. E., Krumins, A. M., Brothers, G. M., Lee, S. F., Wall, M. A., Chung, S., Mangion, J., Arya, S., Gilman, A. G. & Siderovski, D. P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13307–13312

**D. K. Satpaev and V. S. Slepak, unpublished observations.**
Gβ5-RGS Complexes in Vivo and in Vitro

15. Levay, K., Cabrera, J. L., Satpaev, D. K. & Slepak, V. Z. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2503–2507

16. Snow, B. E., Betts, L., Mangion, J., Sondek, J. & Siderovski, D. P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6489–6494

17. Posner, B. A., Gilman, A. G. & Harris, B. A. (1999) J. Biol. Chem. 274, 31087–31093

18. Makino, E. R., Handy, J. W., Li, T. & Arshavsky, V. Y. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1947–1952

19. Cabrera, J. L., de Freitas, F., Satpaev, D. K. & Slepak, V. Z. (1998) Biochim. Biophys. Res. Commun. 249, 898–902

20. Zhang, J. H. & Simonds, W. F. (2000) J. Neurosci. 20, 1964–1972

21. Jones, M. B. & Garrison, J. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 33575–33579

22. Watson, A. J., Katz, A. & Simon, M. I. (1994) J. Biol. Chem. 269, 198–204

23. Watson, A. J., Aragay, A. M., Slepak, V. Z. & Simon, M. I. (1996) J. Biol. Chem. 271, 28154–28160

24. Zhang, S., Coso, O. A., Lee, C., Gutkind, J. S. & Simonds, W. F. (1996) J. Biol. Chem. 271, 32815–32820

25. Liang, J. J., Cockett, M. & Khawaja, X. Z. (1998) J. Biol. Chem. 273, 35757–35759

26. Bigay, J. & Chabre, M. (1994) Methods Enzymol. 237, 139–246

27. He, W., Cowan, C. W. & Wensel, T. G. (1998) Neuron 20, 95–162

28. Sullivan, E., Tucker, E. M. & Dale, I. L. (1999) Methods Mol. Biol. 114, 123–123

29. Fletcher, J. E., Lindorfer, M. A., DeFilippo, J. M., Yasuda, H., Guillemard, M. & Garrison, J. C. (1998) J. Biol. Chem. 273, 636–644

30. Bayewitch, M. L., Avidor-Reiss, T., Levy, R., Pfeuffer, T., Nevo, I., Simonds, W. F. & Vogel, Z. (1998) FEBS Lett. 421, 1019–1025

31. Betty, M., Jones, P. G., Khawaja, X. Z. & Cockett, M. I. (1998) Neuroscience 85, 475–486

32. Liang, J. J., Cockett, M. & Khawaja, X. Z. (1998) J. Neurochem. 71, 345–355

33. Gold, S. J., Ni, Y. G., Dohlman, H. G. & Nestler, E. J. (1997) J. Neurosci. 17, 8024–8037

34. Khawaja, X. Z., Liang, J. J., Saugstad, J. A., Jones, P. G., Harnish, S., Conn, P. J. & Cockett, M. I. (1999) J. Neurochem. 72, 174–184

35. Cowan, C. W., Fariss, R. N., Sokal, I., Palczewski, K. & Wensel, T. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5351–5356

36. Bigay, J., M. Kozusa, T. & Gilman, A. G. (1996) J. Biol. Chem. 271, 27209–27212

37. Tesmer, J. J., Berman, D. M., Gilman, A. G. & Sprang, S. R. (1997) Cell 89, 151–162

38. Wieland, T., Chen, C. K. & Simon, M. I. (1997) J. Biol. Chem. 272, 8853–8856

39. Nateschin, M., Lipkin, V. M. & Artemyev, N. O. (1997) FEBS Lett. 411, 179–182

40. Lan, K. L., Sarvazyan, N. A., Taussig, R., Mackenzie, R. G., DiBello, P. R., Dohlman, H. G. & Neubig, R. R. (1998) J. Biol. Chem. 273, 12794–12797

41. Shuey, D. J., Betty, M., Jones, P. G., Khawaja, X. Z. & Cockett, M. I. (1998) J. Neurochem. 70, 1984–1987

42. Wess, J., B. N., Mutschler, E. & Blum, K. (1995) Life Sci. 51, 915–922

43. Nahorski, S. R., Tobin, A. B. & Willars, G. B. (1997) Life Sci. 60, 1039–1045

44. Offermanns, S., Wieland, T., Hossmann, D., Sandmann, J., Bombe, E., Spicher, K. & Schultz, G. & Jakobs, K. H. (1994) Mol. Pharmacol. 45, 80–88

45. Simonds, W. F., Butynski, J. E., Gautam, N., Unson, C. G. & Spiegel, A. M. (1991) J. Biol. Chem. 266, 5363–5366

46. Wildman, D. E., Tamir, H., Leberer, E., Northup, J. K. & Dennis, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 798–802

47. Northup, J. K., Smigel, M. D., Sternweis, P. C. & Gilman, A. G. (1983) J. Biol. Chem. 258, 11369–11376

48. Benzing, T., Brandes, R., Sellin, L., Schermer, B., Lecker, S., Walz, G. & Kim, E. (1999) Nat. Med. 5, 913–918

49. Chen, C. K., Burns, M. E., He, W., Wensel, T. G., Baylor, D. A. & Simon, M. I. (2000) Nature 403, 557–560

50. Kovoor, A., Chen, C. K., He, W., Wensel, T. G., Simon, M. I. & Lester, H. A. (2000) J. Biol. Chem. 275, 3397–402

51. Schmidt, C. J. & Neer, E. J. (1991) J. Biol. Chem. 266, 4538–4544

52. Pronin, A. N. & Gautam, N. (1993) FEBS Lett. 329, 89–93

53. Hirschman, J. E., De Zutter, G. S., Simonds, W. F. & Jenny, D. D. (1997) J. Biol. Chem. 272, 240–248

54. Wang, Q., Mullah, B. K. & Robishaw, J. D. (1999) J. Biol. Chem. 274, 17365–17371

55. Saitoh, O., Kuba, Y., Odagiri, M., Ichikawa, M., Yamagata, K. & Sekine, T. (1999) J. Biol. Chem. 274, 9899–9904

56. Zeng, W., Xu, X., Popov, S., Mukhopadhyay, S., Chidiac, P., Swistok, J., Danho, W., Yagil, K., Fisher, S. L., Ross, E. M., Mualem, S. & Wilkie, T. M. (1998) J. Biol. Chem. 273, 34687–34690

57. Xu, X., Zeng, W., Popov, S., Berman, D. M., Davignon, I., Yu, K., Yowe, D., Offermanns, S., Mualem, S. & Wilkie, T. M. (1999) J. Biol. Chem. 274, 3549–3556

58. Euler, T. & Wessl, H. (1995) J. Comp. Neurol. 361, 461–478

59. Liang, J. J., Chen, H. H., Jones, P. G. & Khawaja, X. Z. (2000) J. Neurosci. Res. 60, 58–64

60. Maier, U., Babich, A., Macrez, N., Leopold, D., Gierschik, P., Illenberger, D. & Nurnberg, B. (2000) J. Biol. Chem. 275, 13746–13754