Regulator of G-protein Signaling 3 (RGS3) Inhibits Gβ1γ2-induced Inositol Phosphate Production, Mitogen-activated Protein Kinase Activation, and Akt Activation*

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Regulator of G-protein signaling 3 (RGS3) enhances the intrinsic rate at which Gaq and Gαi hydrolyze GTP to GDP, thereby limiting the duration in which GTP-Gαi and GTP-Gαq can activate effectors. Since GDP-Gα subunits rapidly combine with free Gβγ subunits to reform inactive heterotrimeric G-proteins, RGS3 and other RGS proteins may also reduce the amount of Gβγ subunits available for effector interactions. Although RGS6, RGS proteins may also reduce the amount of Gαi inactive heterotrimeric G-proteins, RGS3 and other RGS proteins rapidly combine with free Gβγ subunits to reform Gαi and GDP-Gαq, which facilitates its dissociation from the receptor and Gβγ subunits. GTP-bound Gαq then binds Gβγ subunits and limits their ability to trigger the production of inositol phosphates and the activation of Akt and mitogen-activated protein kinase. Co-expression of RGS3 with Gβ1γ2 inhibits Gβ1γ2-induced inositol phosphate production and Akt activation in COS-7 cells and mitogen-activated protein kinase activation in HEK 293 cells. The inhibition of Gβ1γ2 signaling does not require an intact RGS domain but depends upon two regions in RGS3 located between acids 313 and 390 and between acids 391 and 458. Several other RGS proteins do not affect Gβ1γ2 signaling in these assays. Consistent with the in vitro results, RGS3 inhibits Gβγ-mediated activation of phospholipase Cβ in vitro. Thus, RGS3 may limit Gβγ signaling not only by virtue of its GTPase-activating protein activity for Gαi subunits, but also by directly interfering with the activation of effectors.

Heterotrimeric G-proteins link seven transmembrane receptors to downstream signaling pathways. Receptor activation triggers the exchange of GTP for GDP by the Ga subunit of the heterotrimeric G-protein, causing a conformational change in the Ga subunit, which facilitates its dissociation from the receptor and Gβγ subunits. GTP-bound Ga and free Gβγ subunits then bind and activate downstream effectors. However, Ga subunits possess an intrinsic GTPase activity, which returns Ga to its GDP bound state and thereby limits the duration of Ga signaling. Because GDP-Ga possesses a high affinity for Gβγ subunits, the heterotrimeric G-protein rapidly reforms, effectively ending Gβγ-mediated signaling as well (reviewed in Refs. 1 and 2).

Cells possess another important mechanism that curtails the duration in which a Ga subunit remains GDP bound. Members of a family of proteins termed regulators of G-protein signaling (RGSs) dramatically accelerate the intrinsic rate that certain Ga subunits hydrolyze GTP, a property that identifies them as GTPase-activating proteins (GAPs). The mammalian RGS proteins have a 120-amino acid region, RGS domain, or RGS box, which binds Gaq and Gαi subfamily members in a transition state of the GTP hydrolysis reaction, thereby lowering the free energy of the reaction (reviewed in Refs. 3 and 4). In addition, Rho guanine nucleotide exchange factors have a divergent RGS domain, which accelerates the intrinsic GTPase activity of Ga12 and Ga13 (5, 6). RGS proteins with GAP activity for members of the Gαi subfamily remain enigmatic.

The mammalian RGS proteins can be broadly divided into two groups (7): those composed predominantly of an RGS domain such as RGS1, RGS2, RGS4, and RGS5 and those that contain an RGS domain but also other domains. The second group includes RGS6, RGS7, RGS9, RGS11, RGS12, and RGS14. The smaller RGS proteins likely function solely as Gaq GAPs, whereas some of the larger RGS proteins are undoubtedly Gαi effectors such as p115 Rho guanine nucleotide exchange factors. RGS3 exists as two isoforms, thus falling into both groups (8, 9): a shorter version that encodes largely an RGS domain (RGS3C) and a larger isoform that has a strongly acidic region and an unusual region that contains a hexapeptide repeat enriched for proline, glutamine, and acidic residues (8, 9). Both versions possess GAP activity for Gaq and Gαi and can impair signaling through Gaq and certain Gaq-linked signaling pathways (10). The function of the N-terminal domain of RGS3 is unknown, although the N-terminal fragment shifts to membranes after a calcium signal (11). Transient expression of RGS3 potently inhibits the chemotaxis of a pre-B cell line, even better than does RGS1, which has excellent Gaq GAP activity (12, 13). The known importance of Gβγ signaling in chemokine-directed migration (14, 15) led us to test whether RGS3 employs another mechanism besides its Gaq GAP activity to impair Gβγ signaling. In three in vivo models of Gβ1γ2 signaling, inositol phosphate generation through the stimulation of phospholipase Cβ, the activation of mitogen-

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The abbreviations used are: RGS, regulator of G-protein signaling; GAP, GTPase-activating protein; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; PLC, phospholipase C; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; TTBS, Tween 20 Tris-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; IP3, inositol 1,4,5-trisphosphate.
activated protein kinase (MAPK), and the activation of Akt, we find that RGS3 potently inhibits the activation of these signaling pathways even when it lacks a functional RGS domain. Supporting the in vivo data, purified RGS3 blocks Gβγ-induced inositol phosphate production by phospholipase Cβ2 in vitro.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—To make pET15b His₆-RGS3, PCR fragments generated from pCMV-Plc (8) were inserted into the NdeI/XhoI sites of the bacterial expression vector pET15b (Novagen, Madison, WI) in frame with the hexahistidine tag. To make FLAG-RGS3, FLAG-RGS3NT (RGS3 1–313), FLAG-RGS3CT (RGS3 314–520), FLAG-RGS3NT1 (RGS3 1–390), and FLAG-RGS3NT2 (RGS3 1–458), the appropriate PCR products were subcloned into pFLAGCMV2. FLAG-RGS3/4 was formed by fusing the coding region of the first 58 amino acids of RGS4 with the coding region of amino acids 390–519 of RGS3 via PCR using PCR primers that generate the appropriate overlapping fragments. The PCR products were denatured, annealed, and extended before subcloning into pFLAGCMV2. FLAG-RGS3 E419A was made by site-directed mutagenesis of the FLAG-RGS3 construct (Stratagene, La Jolla, CA). To make GST-RGS3, GST-RGS3CT, and RGS3NT, PCR products encompassing the coding regions of RGS3, RGS3 (1–313), and RGS3 (314–520) were cloned in-frame with the GST-coding region using the GST expression vector pEBG. The veracity of all the DNA constructs was verified by nucleotide sequencing. The RGS2, RGS4, and RGS10 constructs have been previously described (8, 16, 17). A schematic showing the expected RGS domain and acidic region are indicated.

The bacterial expression vector pET15b (Novagen, Madison, WI) was used to express RGS proteins. The His₆-RGS3 construct was expressed in Escherichia coli BL21(DE3) and purified as described by the manufacturer (Novagen). The purified protein was dialyzed against the wash buffer and stored at −70 °C. The recombinant proteins were purified by nickel-nitrotriacetic acid beads (Qiagen, Santa Clara, CA) and eluted with an imidazole gradient. The purified protein fractions were dialyzed against the wash buffer and stored at −70 °C. In some instances RGS3 was further purified over a monoQ column (Amersham Pharmacia Biotech). The recombinant His-tagged Jun kinase was purchased from Santa Cruz (Santa Cruz, CA). GST-RGS3 fusion proteins were prepared from lysates of HEK 293 cells previously transfected with the appropriate expression vector. The fusion proteins were partially purified on glutathione-Sepharose 4B as described by the manufacturer (Amersham Pharmacia Biotech). The eluted GST-RGS3 fusion proteins were concentrated and dialyzed using Centricon 30 (Millipore, Bedford, MA) with the equilibration buffer (20 mM Hepes, 1 mM EDTA, and 1% Triton X-100 along with a mixture of protease inhibitors) for 60 min. Fractions (1 ml) were collected, and 20 μl of each fraction were separated on 10% gel by SDS-PAGE. The fusion proteins were visualized by Coomassie blue staining. All the GST-RGS3 fusion proteins were eluted around 0.4 M NaCl. Peak fractions (three fractions) were pooled, concentrated to ~0.1 ml by Centricon 30, divided into portions, and stored at −70 °C. The recombinant Gβγ was prepared from S9 cells infected with baculovirus constructs encoding Gβγ, GγTc, and His-tagged Gαi3 according to the published procedure (19).

Immunoblotting and Immunoprecipitations—Cell lysates were prepared using a solution containing 150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, and 1% Triton X-100 along with a mixture of protease inhibitors for 20 min on ice. The detergent-insoluble material was removed by microcentrifugation for 10 min at 4 °C. Equal amounts of protein from each sample were separated using SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with 10% milk in TBS for 1 h and then incubated with an appropriate dilution of the primary antibody in 5% milk and 0.05% sodium azide in TBS overnight. The blots were washed twice with TBS before the addition of a biotinylated goat-anti rabbit immunoglobulin (DAKO, Carpinteria, CA) diluted 1:1500 in TBS containing 5% fetal calf serum. After a 1-h incubation, the blot was washed twice with TTBS and then incubated with streptavidin conjugated to horseradish peroxidase (DAKO). The signal was detected by enhanced chemiluminescence (ECL) following the recommendations of the manufacturer (Amersham Pharmacia Biotech). The co-immunoprecipitations were performed using lysates (20
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mm Tris, pH 8.0, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, plus protease inhibitors) prepared from COS-7 cells or HEK 293 cells co-expressing Gβγ2 and various RGS proteins. Anti-FLAG monoclonal antibody or anti-Gβγ polyclonal antisera was added, and the immunoprecipitates were collected with the appropriate secondary antibody-coupled magnetic beads (Dynal Corp., Lake Success, NY). They were washed three times in lysis buffer, twice in lysis buffer with 0.5 mM NaCl, fractionated by SDS-PAGE, and analyzed by immunoblots with the appropriate antibody.

In Vivo Inositol Phosphate Production—Inositol phosphate production was measured as previously described (20). Briefly, COS-7 cells were transfected using LipofectAMINE (1:8). Twenty-four hours after transfection, the culture media was replaced with inositol-free Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum and 1 mM sodium orthovanadate, and the transfected Gβγ subunits (0.5 µg) in a 25-µl volume for 10 min at 30 °C. After incubation, reaction tubes were transferred on ice and prepared for the PLC assay. PLC activity was measured as previously described (18). Briefer, substrate was prepared as sonicated micelles of 75 µM [3H]phosphatidylinositol 4,5-bisphosphate (10,000–12,000 cpm/µg) and 750 µM phosphatidylethanolamine. 10 µg of PLCβ2 was added to each tube, and CaCl2 was added to the assay mixture to give 200 nM free Ca2+. Assays were performed in 60-µl volume for 10 min at 30 °C. Under these conditions, [3H]PIP2 formation was linear with respect to time and enzyme concentration.

RESULTS

RGS3 Impairs the Generation of Inositol Phosphates by Gβγ2—Since RGS3 possesses GAP activity for both Gαq and Gαi, analyzing whether RGS3 modulates Gβγ signaling through either Gαq- or Gαi-coupled receptors is not feasible. However, the intracellular expression of Gβqγ1, Gβqγ2, or Gβqγ5 is known to activate PLCβ2, resulting in the production of intracellular inositol phosphates (23). The newly synthesized Gβγ subunits target to the plasma membrane, where insufficient Gα subunits exist to form inactive heterotrimers. The free Gβγ subunits can activate PLCβ enzymatic activity, which hydrolyzes phosphatidylinositol 4,5-bisphosphate, releasing the second messengers inositol 1,4,5-trisphosphate and diacylglycerol. Therefore, by concomitantly introducing a construct that expresses RGS3, we could test whether RGS3 modulates the production of inositol phosphates by free Gβγ subunits. We found that expression of Gβqγ1 enhanced inositol phosphate production by COS-7 cells, co-expressing PLCβ2 7–9-fold over background levels. The introduction of RGS3 inhibited inositol phosphate production by nearly 70%, whereas two other RGS proteins, RGS4 and RGS10, did not impair inositol phosphate production in similar experiments (Fig. 2). The expression of RGS3 did not alter the levels of Gβ3 subunits or PLCβ2, although the transfected Gβ3 subunits co-migrated with the endogenous Gβ3 subunits. Thus, although RGS4 has better GAP activity for Gαq than does RGS3 and equivalent Gαi activity (10), RGS4 does not impair Gβγ signaling to phospholipase Cβ like RGS3 does.

RGS3 Inhibits the Activation of MAPK and Akt by Gβγ—Gβγ subunits directly activate other effectors beside PLCβ. Distinct sets of contacts along the edge of the β propeller of Gβ subunits are thought to establish the specificity of Gβγ for its effectors (24, 25). Through yet unknown effectors Gβγ subunits stimulate MAPK and Akt activation. Expression of Gβqγ5 subunits in COS-7 cells enhances the activity of the MAPK ERK2 (26). Therefore, by concomitantly expressing RGS3 we could
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**Fig. 3.** RGS3 impairs MAPK activation by G\(\beta_1\gamma_2\), but not by an active form of Ras. HEK 293 cells were transfected with constructs expressing G\(\beta_1\), G\(\gamma_2\), and HA-ERK1 in the presence of various RGS3 constructs, RGS4, RGS10, or a control vector. Similarly, HEK293 cells were transfected with constructs directing the expression of RasV12 and HA-MAPK, and RGS3 or a control. HA immunoprecipitates were subjected to an \textit{in vitro} kinase assay using myelin basic protein (MBP) as a substrate. Scanning the autoradiographs assessed the amounts of \(^{32}\)P incorporated into substrate. The fold induction compared with the culture without G\(\beta_1\gamma_2\) is indicated. The levels of HA-ERK1, G\(\beta\), and RGS proteins in the cell lysates are shown. The results are representative of one of four experiments performed.

**Mapping the Regions in RGS3 Important for Inhibiting G\(\beta_1\gamma_2\)-induced Inositol Phosphate Production**—We also mapped the regions of RGS3 necessary for inhibiting G\(\beta_1\gamma_2\)-mediated phospholipase C\(\beta\) activation. We transfected COS-7 cells with constructs that expressed G\(\beta_1\gamma_2\) and PLC\(\beta_2\) in the presence or absence of constructs that expressed RGS3, RGS3CT, RGS3NT, RGS3NT1, RGS3NT2, or RGS4/3 and measured inositol phosphate production. The RGS4/3 construct encoded a fusion protein that included the first 58 amino acids of RGS4 along with amino acids 390–519 of RGS3. Similar to the MAPK experiments, RGS3CT inhibited inositol phosphate production by G\(\beta_1\gamma_2\) even better than did RGS3. RGS3NT2, which lacked the C-terminal 61 amino acids of RGS3, behaved like wild RGS3 did. Comparison of RGS3, RGS3NT, RGS3NT1, and RGS3NT2 indicated that amino acids 314–390 and 391–458 both contributed to the inhibitory effect of RGS3 on G\(\beta_1\gamma_2\) signaling to phospholipase C\(\beta\) (Fig. 5). This differed slightly from the findings with Akt activation, where the RGS3NT1 construct did not impair activation. Transferring amino acids 390–519 to the N terminus of RGS4 conferred upon RGS4 the ability to inhibit G\(\beta_1\gamma_2\) signaling equivalent to that of wild type RGS3, although not equivalent to RGS3CT. Overall these results show that RGS3 requires the residues between 314–390 and 391–458 to fully inhibit G\(\beta_1\gamma_2\) signaling.

**RGS3 Co-Immunoprecipitates with G\(\beta\gamma\) Subunits**—We determined whether RGS3 associated with G\(\beta\gamma\) subunits \textit{in vivo} by co-transfecting HEK 293T cells with constructs directing the expression of full-length RGS3 and G\(\beta_1\gamma_2\) and analyzing immunoprecipitates for the respective proteins. The FLAG antibody readily immunoprecipitated the FLAG-tagged RGS3 and in addition co-immunoprecipitated G\(\beta\gamma\) subunits (Fig. 6A). Conversely, a polyclonal antibody directed against G\(\beta\gamma\) subunits immunoprecipitated both endogenous G\(\beta\gamma\) subunits and the expressed G\(\beta_1\gamma_2\) subunits and co-immunoprecipitated the FLAG-tagged RGS3 (Fig. 6A). Control ERK and Myc antibodies failed to immunoprecipitate either FLAG-RGS3 or G\(\beta\gamma\) subunits. Next, we examined whether RGS3 directly bound G\(\beta\gamma\) subunits by combining recombinant His-tagged RGS3 with purified G\(\beta\gamma\) subunits and then examined anti-His immunoprecipitates for RGS3 and G\(\beta\gamma\) subunits. Anti-His immunoprecipi-
RGS3 can directly bind G bg precipitated with an HA antibody. These results indicate that RGS3 is constitutively associated with G bg or with G bg in the cytosol and can be shifted to membranes by G-protein signaling. In contrast, His-tagged Jun kinase failed to co-immunoprecipitate G bg subunits, and neither RGS3 nor G bg subunits immunoprecipitated with an HA antibody. These results indicate that RGS3 can directly bind G bg subunits.

RGS3 expressed in cell lines localizes predominantly in the cytosol and can be shifted to membranes by G-protein signaling (11). A previous study indicates that endogenous RGS3 localizes predominantly in a membrane fraction rather than the cytosolic fraction of αT3−1 cells (31). The RGS3 antisera used in these experiments, while raised against recombinant RGS3, reacted only with RGS3 and not with RGS3CT. Here we used an anti-peptide antisera prepared against a peptide from the N-terminal portion of RGS3. This antisera recognized recombinant and transfected full-length RGS3 and detected a band that co-migrated with recombinant RGS3 in COS cells (10). Fractionation of COS-7 cells into a membrane-enriched and -depleted fractions revealed that the membrane-enriched fraction contained the majority of the RGS3, although some RGS3 also localized in the cytosolic fraction (data not shown). When we examined G bg immunoprecipitates using the broadly reactive G bg antisera, we found that they contained small amounts of RGS3 (Fig. 6C). Conversely, RGS3 immunoprecipitates also contained small amounts of G bg subunits. Thus, in COS-7 cells, endogenous RGS3 localizes predominantly at intracellular or plasma membranes, and a small portion is constitutively associated with G bg subunits.

Both the N-Terminal Portion of RGS3 and RGS3CT Co-Immunoprecipitate with βγ Subunits—Based on the signaling experiments we expected to co-immunoprecipitate RGS3CT with G bg subunits but not RGS3NT. In contrast, we found that RGS3NT, RGS3NT1, RGS3NT2, and RGS3CT all co-immunoprecipitated with G bg subunits when we co-expressed them along with G bg1,2. RGS3NT very readily co-immunoprecipitated with a polyclonal antibody against G bg subunits but failed to immunoprecipitate when we used a polyclonal antibody against the HA epitope (Fig. 7A). When we co-expressed G bg1,2 with RGS2, RGS3, or RGS4, G bg subunits did not co-immunoprecipitate with either RGS2 or RGS4, but they did with RGS3 (Fig. 7B). Thus, although both RGS3CT and RGS3NT associate with G bg1,2 subunits, only RGS3CT inhibits G bg signaling to PLC bg and the MAPK and AKT pathways.

RGS3 and RGS3CT Inhibit G bg1,2-induced PLCbg2 Activation in an in Vitro Reconstitution Assay—To test whether RGS3 can directly inhibit the activation of phospholipase C bg by G bg1,2, we added recombinant PLCbg2 to sonicated micelles of [3H]phosphatidylinositol 4,5-bisphosphate and phosphatidylylthanolamine and then measured [3H]IP2 formation triggered by G bg1,2 subunits that had been preincubated with RGS3 or not. The initial results indicated that RGS3 may not directly affect the activation of PLC bg by G bg subunits as E. coli-expressed recombinant RGS3 minimally affected it (data not shown). However, when instead of using E. coli-expressed RGS3, we used RGS3, RGS3NT, and RGS3CT prepared as GST fusion proteins from HEK 293 cells, we obtained a very different result. The purified GST-RGS3 reduced the G bg1,2-induced...
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**DISCUSSION**

RGS3 inhibits G\(\beta\)\(\gamma\)-mediated Akt activation and inositol phosphate production in COS-7 cells and MAPK activation in HEK 293 cells. The inhibition of these pathways by RGS3 does not depend upon an intact RGS domain or on its N-terminal 390 amino acids but requires a region in RGS3 that overlaps the RGS domain. These data are best explained by RGS3 blocking G\(\beta\)\(\gamma\)-mediated activation of its effectors. Less likely, RGS3 could directly inhibit the effectors per se or impair downstream elements in the signaling pathways. Arguing for RGS3 acting at the level of G\(\beta\)\(\gamma\) signaling to its effectors, RGS3 does not reduce phorbol ester-induced ERK activation (8) or Ras-mediated ERK or Akt activation (this study).

How might RGS3 block G\(\beta\)\(\gamma\)-mediated effector signaling? The failure of RGS4 and the success of RGS3EN and the truncated RGS3 proteins in inhibiting G\(\beta\)\(\gamma\) signaling indicates that the G\(\alpha\) and G\(\alpha\)\(q\) GAP activity of RGS3 cannot explain its success in inhibiting G\(\beta\)\(\gamma\)\(\alpha\)2-triggered ERK activation. Also the N-terminal portion of RGS3 does not assist in the inhibition of G\(\beta\)\(\gamma\)\(\alpha\)2 signaling since RGS3CT also more potently inhibits. Since RGS3 binds G\(\beta\)\(\gamma\)\(\alpha\)2 subunits both in vitro and in vivo and since overexpressed RGS3 localizes in part in the cytosol, the transiently expressed RGS3 could sequester G\(\beta\)\(\gamma\)\(\alpha\)2 away from its usual microenvironment, thereby inhibiting access of G\(\beta\)\(\gamma\)\(\alpha\)2 to its effectors. However, although both RGS3NT and RGS3CT bind G\(\beta\)\(\gamma\) subunits, only RGS3CT potently inhibits G\(\beta\)\(\gamma\)\(\alpha\)2 signaling. Furthermore, RGS3 and RGS3CT block the activation of PLC\(\beta\)2 in vitro, whereas RGS3NT does not. This last result indicates that RGS3 can directly inhibit PLC\(\beta\)2 activation of G\(\beta\)\(\gamma\) subunits. Thus, we favor the possibility that, when localized in the vicinity of heterotrimeric G-proteins undergoing GDP-GTP exchange, RGS3 can interact with GTP-bound G\(\alpha\) subunits, promoting G\(\alpha\) GTP hydrolysis, and with G\(\beta\)\(\gamma\) subunits, reducing their availability for effector activation. Since the presence of G\(\beta\)\(\gamma\) subunits does not impair RGS3 GAP activity,\(^2\) perhaps RGS3 can simultaneously act as a G\(\alpha\) GAP

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\(^2\) C. Dessauer, unpublished observation.
and a Gβγ inhibitor. Mapping the site or sites in Gβ1γ2 that interact with RGS3 and testing the effects of RGS3 on signaling by other Gβγ isoforms should provide additional insights into the importance of RGS3 as a regulator of Gβγ signaling.

Among the known RGS proteins, is RGS3 in its ability to inhibit Gβγ signaling? Neither RGS4 nor RGS10 inhibits Gβ1γ2-mediated MAPK activation or the generation of inositol phosphates, indicating that not all RGS proteins share this property. Furthermore, neither RGS2 nor Gαi interacting protein inhibits the induction of inositol phosphates after Gβ1γ2 expression in COS-7 cells. Thus among five RGS proteins, only RGS3 reduces Gβ1γ2-mediated signal transduction, suggesting that the inhibition of Gβγ signaling may be a unique property of RGS3 or, if not, only possessed by a minority of the RGS proteins. Alignment of RGS3CT with RGS2, RGS4, RGS10, and Gαi interacting protein reveals considerable differences between the five proteins in the regions N-terminal to the RGS domains. Within the RGS domains these proteins share ~25% of their amino acids, and RGS3 possesses 22 unique residues not found in the other four proteins. To delineate critical residues in RGS3 needed for its inhibition of Gβ1γ2-mediated signaling, we created 5 constructs with triple-residue point mutations in the region that encodes amino acids 350–415 of FLAG-RGS3. Four or the five proteins expressed in COS-7 cells; however, all of the expressed mutant RGS3 proteins lost their ability to inhibit Gβ1γ2 signaling. This suggested that we had introduced conformational changes in this region rather than identifying specific residues needed for the inhibition. Further mapping with RGS3 proteins containing a single point mutation is in progress.

The structural basis of RGS3 interaction with Gβ1γ2 is not revealed by comparing the primary amino acid sequence of RGS3 to those of proteins known to directly interact with Gβγ. A motif, QXXER, present in the Gβγ effectors adenyl cyclase II and Ca2+ and Na+ channels may specify their interaction with Gβ subunits (32, 33). However, no such motif occurs in RGS3. Phosducin, a phosducin like protein (PhLP), and beta disruption mimic factor-1 (BDM-1) all share a highly conserved 11-amino acid region needed for Gβγ binding (34–36). However, again no such stretch of amino acids exists in RGS3. Interestingly, like full-length RGS3, phosducin has two separate domains that bind Gβγ. The N-terminal domain of phosducin binds loops on the “top” of the Gβ4 surface, overlapping the Gαi binding surface, whereas the N-terminal domain binds the outer strands of Gβ4 seventh and first blades, which may disrupt the normal orientation of Gβγ relative to the membrane and receptor (37). Suggesting a regulatory role for phosphorylation, phosphophosducin no longer competes with Gαi for binding to Gβγ (38). Similar structural studies of the RGS3/Gβγ complex should provide insights into the biologic role of the interaction. Finally, RGS3 does not possess a PH domain such as PLC-β2 or β-adrenergic receptor kinase through which it could interact with Gβγ (39, 40).

Why did the E. coli and mammalian-expressed RGS3 differ in their ability to block Gβγ-mediated activation PLCβ in vitro? Despite binding purified Gβγ subunits in vitro, E. coli-expressed recombinant RGS3 minimally affected the ability of Gβγ subunits to activate recombinant PLCβ, whereas the GST-RGS3CT-purified from mammalian cells markedly inhibited the ability of Gβγ subunits to activate recombinant PLCβ. One attractive explanation is that an in vitro post-translational modification of RGS3 controls whether it inhibits Gβγ effector activation. Phosducin undergoes such a regulation. Phosducin and phosphophosducin possess similar affinities for Gβγ, yet phosducin competes with Gαi for binding to Gβγi, whereas phosphophosducin does not (38). Since RGS3 undergoes extensive phosphorylation in vitro, perhaps the mammalian-expressed RGS3 is phosphorylated, allowing it to inhibit Gβγ-induced PLCβ activity. Another possibility is that the bacterially expressed RGS3 is mis-folded. Although still able to bind Gβγ in vitro, it is unable to inhibit Gβγ signaling, because a portion of the molecule is disordered. Further studies with truncated RGS3-GST fusion proteins should allow a more precise mapping of the regions in RGS3 necessary for inhibiting Gβγ-induced PLCβ activity.

Does RGS3 interact with heterotrimeric G-proteins? Although we show that Gβ immunoprecipitates from COS-7 cell contain RGS3, neither Gαq nor Gαi immunoprecipitates from the same cells do. The Gαq and Gαi immunoprecipitates contain as much if not more Gβ than does the Gβ immunoprecipitates. These data argue that RGS3 does not constitutively associate with Gβγ and is consistent with previous studies where RGS3 co-immunoprecipitates with Gαq or Gαi only after exposure of the cell lysates to AlF4 (11), which dissociates the heterotrimeric G-proteins and activates the Gα subunits. Together these observations suggest that endogenous RGS3 may associate with a pool of Gβγ, which is not bound Gα. Several studies indicate the presence of an intracellular pool of Gβγ not associated with Gα (41, 42). A pool of free Gβγ subunits has been postulated to function in receptor-mediated endocytosis (42).

In summary, elevating the levels of RGS3 potently inhibits

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3 C. Shi, unpublished observation.
4 S. B. Lee, unpublished observation.
5 J. Kehrl, unpublished observation.
the activation of several distinct signaling pathways stimulated by Gβγ subunits. A portion of RGS3 located between amino acids 314–458, which partially overlaps the RGS3 Gβγ domain, is required to observe the inhibition. RGS3 co-immunoprecipitates with transfected Gγb1γ2 subunits, binds Gβγ subunits in vitro, and inhibits the activation of PLCβ by Gβγ subunits. Overall, these results indicate that RGS3 can modulate Gβγ signaling not only by virtue of its Go GAP activity but also by directly interfering with Gβγ signaling. Such a mechanism may contribute to the efficacy of RGS3 as an inhibitor of chemokine-directed cell migration.

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