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RGS14, a GTPase-Activating Protein for Giα, Attenuates Giα- and G13α-Mediated Signaling Pathways

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ABSTRACT

Regulator of G protein signaling (RGS) proteins are a family of approximately 20 proteins that negatively regulate signaling through heterotrimeric G protein-coupled receptors. The RGS proteins act as GTPase-activating proteins (GAPs) for certain Gα subunits and as effector antagonists for Gqα. Mouse *RGS14* encodes a 547-amino-acid protein with an N-terminal RGS domain, which is highly expressed in lymphoid tissues. In this study, we demonstrate that RGS14 is a GAP for Giα subfamily members and it attenuates interleukin-8 receptor-mediated mitogen-activated protein kinase activation. However, RGS14 does not exhibit GAP activity toward Gsα or Gqα nor does it regulate Gsα- or Gqα-mediated signaling pathways. Although RGS14 does not act as a GAP for G12/13α, it impairs *c-fos* serum response element activation induced by either a constitutively active mutant of G13α (G13αQ226L) or by carbachol stimulation of muscarinic type 1 receptors. An RGS14 mutant (EN92/93AA), which does not block Giα-linked signaling, also inhibits serum response element activation. RGS14 localizes predominantly in the cytosol, but it can be recruited to membranes by expression of G13αQ226L. Although RGS14 is constitutively expressed in lymphoid cells, agents that activate B or T lymphocytes further enhance its levels. Taken together, our results suggest that signals generated after lymphocyte activation may via RGS14 directly impinge on Giα- or G13α-mediated cellular processes in lymphocytes, such as adhesion and migration.

Extracellular signals such as hormones, neurotransmitters, and chemokines that stimulate heptahelical receptor are transmitted via heterotrimeric G proteins, signal transducers, resulting in regulation of a variety of enzymes and ion channels (Hamm and Gilchrist, 1996). One way to control the duration and sensitivity of the G protein-mediated signaling is to alter the intrinsic GTPase activity of Gα subunits. Regulator of G protein signaling (RGS) proteins are a newly described family of approximately 20 proteins that can act as GTPase-activating proteins (GAPs) for certain Gα subunits, thereby negatively regulating signaling through G protein-coupled receptors (GPCR). They were originally identified as functional homologs of yeast Sst2p and EGL10 of *Caenorhabditis elegans*, and subsequently shown to impair signaling mediated via GPCRs in mammalian systems (Druey et al., 1996, for reviews, see Berman and Gilman, 1998; Kehrl, 1998).

RGS proteins have a highly conserved, 120-amino-acid core region called "RGS domain". Solution of a cocrystal structure of RGS4 and Giα1 revealed that critical residues in the RGS domain stabilize the flexible switch regions of Gα proteins in the transition state of GTP hydrolysis, thus lowering the activation energy barrier (Tesmer et al., 1997). The RGS domain contains all of the crucial elements necessary for the GAP activity. Furthermore, alteration of critical residues in RGS4 located at the contact sites between RGS4 and Giα1 completely abolished its GAP activity and ability to bind to Giα (Druey and Kehrl, 1997; Srinivasa et al., 1998).

Although it seems redundant that 20 or so RGS proteins should all act as GAPs for Giα and Gqα, clear differences among the family members are emerging. RGS proteins differ in their molecular masses (~20 to 150 kDa), their specificities for various Gα subfamily members, their tissue- or cell-specific expression patterns, their subcellular localization, and their types of post-translational modifications (Zerangue and Jan, 1998; Druey et al., 1998). Furthermore, a variety of proteins that interact with specific RGS family members has been identified. For example, RAP1/2, GIPC, Rho, and Gβ5 interact with RGS14, GAIP, p115 RhoGEF, and RGS7, respectively (Cabrera et al., 1998; De Vries et al., 1998; Hart et al., 1998; Traver et al., 2000). Finally, RGSr (RGS16) is induced by the tumor suppressor protein p53,

ABBREVIATIONS: RGS, regulator of G protein signaling; GAP, GTPase-activating protein; GPCR, G protein-coupled receptor; FCS, fetal calf serum; IL, interleukin; HA, hemagglutinin; ERK, extracellular signal-related kinase; MAP, mitogen-activated protein; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; M1, muscarinic type 1; PLC, phospholipase C; CREB, cAMP response element-binding protein; TCR, T cell receptor; BCR, B cell receptor; PAF, platelet-activating factor; SRE, serum response element.

suggesting an involvement in its role in regulating apoptosis or cell cycle arrest (Buckbinder et al., 1997). There are four salient questions in studying the RGS proteins: 1) What specificities do RGS proteins exhibit for various G proteins? 2) What other signaling molecules do RGS proteins interact with? What is the significance of that interaction? 3) How are the RGS proteins regulated? and 4) What are the in vivo roles of different RGS proteins?

In this report, we characterized the RGS14 protein to address the above-mentioned questions. RGS14 was originally identified as RAP1/2-interacting protein in yeast 2-hybrid screen (Traver et al., 2000) and by degenerate polymerase chain reaction cloning (Snow et al., 1997). We find that the GAP activity of RGS14 is directed at members of Giα subfamily, although RGS14 inhibits both Giα- and G13α-linked signaling pathways. To understand the physiological function(s) of RGS14 protein, we studied tissue- and cell-specific expression patterns, and subcellular localization of RGS14. In addition, because of the expression of RGS14 in lymphocytes, we studied the effects on RGS14 expression of signals that trigger either B or T cells.

Materials and Methods

Cell Culture, Transfection, and Lymphocyte Purification. All lymphoid cells were maintained in RPMI 1640 (Life Technologies Inc., Gaithersburg MD) supplemented with 10% fetal calf serum (FCS). Human embryonic kidney 293T and monkey kidney COS-7 cells were grown in Dulbecco's modified Eagle's medium containing 10% FCS. Transfection of the 293T and COS-7 cells was performed by using calcium-phosphate precipitation method or by using Lipofectamine (Life Technologies Inc.). The total amount of plasmid DNA for each transfection was always normalized with vector DNA. Peripheral leukocytes were isolated from blood of healthy human donors by ficoll hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. T cells were separated by adsorption to sheep red blood cells. B cells were purified from the remaining cells by the removal of CD14-positive cells with a CD14 mouse monoclonal antibody (Pharmingen, San Diego, CA) and goat anti-mouse dynabeads (Dynal, Oslo, Norway). The purity of the T and B fractions was verified by a fluorescence-activated cell sorter Calibur flow cytometer after staining with monoclonal antibodies directed against CD3 and CD19 (Pharmingen). Purified T cells were stimulated with CD3 (0.1 μg/ml; Pharmingen) and interleukin (IL)-2 (20%; Hemagen Diagnostics, Inc., Waltham, MA) every 3 days to maintain cell viability and purified B cells stimulated with anti-IgM F(ab′)$_2$ fragment (20 μg/ml; ICN Pharmaceuticals, Inc., Costa Mesa, CA) in conjunction with CD40 (1 μg/ml; Pharmingen). Forskolin and ionomycin were purchased from Sigma (St. Louis, MO)

Production of Recombinant RGS14 Protein. We generated hexa-histidine-tagged RGS14 protein by subcloning a cDNA fragment that would encode either full-length RGS14 or the RGS14 RGS domain (W64 to E187) into *Nde*I and *Bam*HI restriction sites of pET15b vector (Novagen, Inc., Madison, WI). The resulting constructs were used to overexpress RGS14 proteins in an *Escherichia coli* strain BL21 (DE3) by induction with 1 mM isopropyl β-D-thiogalactoside for 1 h. Histidine-tagged RGS14 recombinant proteins were purified with nickel-nitrilotriacetic acid resin (Qiagen, Chatsworth CA) as described in manufacturer's protocol (Novagen, Inc.).

RGS14 Antiserum, Immunoblotting, and Immunofluorescence. Full-length mouse recombinant RGS14 was used to generate anti-RGS14 antiserum in rabbit and immunoblotting (1:1000 dilution) was performed as previously described (Druey et al., 1998). For immunofluorescent cytochemistry, 293 cells were transfected with hemagglutinin (HA)-epitope tagged RGS14 (0.5 μg) and grown in culture dishes containing glass coverslips overnight. Cells were washed in PBS once and then fixed in 50% methanol/50% acetone for 1 h at 4°C. The cover slips were washed twice with PBS and incubated in 10% goat serum plus 2% BSA in PBS for 1 h. Each coverslip was then placed in 2% BSA in PBS containing anti-RGS14 antiserum (1:800 dilution) for 2 h at room temperature. The coverslips were washed, incubated with Cy3-conjugated goat anti-rabbit immunoglobulins (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) for 1 h. The coverslips were washed again with PBS, mounted on silanized glass slides, and examined with a fluorescence microscope.

Cell Fractionation. Cells were homogenized briefly in the hypotonic buffer containing 10 mM Tris (pH 7.4), 10 mM KCl, 1 mM EGTA, 0.5 mM $MgCl_2$, PefablocSC (Boehringer Mannheim, Indianapolis, IN), and protease inhibitor cocktail tablets (Boehringer Mannheim) with a Dounce pestle. Homogenates were cleared of debris by centrifugation (3000*g*, 5 min) and the postnuclear supernatants were subjected to ultracentrifugation (100,000*g*, 30 min) to separate membrane from cytosol. The fractions were analyzed by immunoblotting.

Giα Signaling Assay. The 293T cells were cotransfected with IL-8 receptor (a kind gift of Dr. Philip Murphy, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD) and HA-tagged extracellular signal-related kinase (ERK)-1 construct in the absence or presence of RGS14. After 24 h, the cells were serum starved overnight and then stimulated with IL-8 (50 ng/ml; Genzyme, Cambridge, MA) for 3 min at 37°C. The stimulated cells were then washed in cold PBS and lysed in the kinase assay buffer containing 20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na_3VO_4, 1% Triton X-100, and 10% glycerol. Cell lysates were subjected to immunoprecipitation with anti-HA antibody (Babco, Richmond, CA) and anti-mouse dynabeads for 90 min. The beads were extensively washed and used for mitogen-activated protein (MAP) kinase assays with myelin basic protein (MBP) as substrate as described previously (Druey et al., 1996). The immunoprecipitates were separated on SDS-polyacrylamide gel electrophoresis (PAGE) and top half of the gel was transferred to a membrane and subjected to immunoblotting with anti-ERK-1 antiserum (Santa Cruz Biotechnology, Santa Cruz, CA). The bottom half was dried and subjected to autoradiography. The cell lysates also were immunoblotted to determine RGS14 expression.

Measurement of Inositol Phosphates in COS-7 Cells. To determine the effect of RGS14 on Gqα-mediated signaling, we cotransfected COS-7 cells with constructs directing the expression of the muscarinic type 1 (M1) receptor (a kind gift from Dr. Silvio Gutkind, National Heart, Lung, and Blood Institute, NIH) and phospholipase Cβ_2 (PLCβ_2) (a kind gift from Dr. Sue Goo Rhee, National Institute on Dental Research, NIH) in the absence or presence of RGS14. Cells were labeled 24 h after transfection with myo-[2-^{3}H]inositol (Amersham, Piscataway, NJ) and simultaneously stimulated with 1 mM carbachol for 18 h. We measured the generation of inositol phosphates as previously described (Panchenko et al., 19989). To test the effect of RGS14 expression on generation of inositol phosphates induced by a constitutively active mutant of Gqα, Gqα-Q209L, we performed similar experiments as described above except the construct directing M1 receptor was replaced with that of Gqα-Q209L (Dr. Silvio Gutkind).

Reporter Gene Assays. For the Gsα signaling assay, 293T cells were cotransfected with constructs directing the expression of β2-adrenergic receptor (Dr. Silvio Gutkind) pCREB-β-Gal (a kind gift from Dr. R. Cone, Vollum Institute, OR) in the absence or presence of RGS14. Additionally, simian virus 40-luciferase (pGL2 promoter, Promega) was transfected to normalize transfection efficiencies. After 48 h, we stimulated the cells with 10 mM isoproterenol, washed them in cold PBS, and lysed them in reporter lysis buffer (Promega). We cleared the lysates of cellular debris and assayed them for β-galatosidase and luciferase activities with a luminometer (Analytical Luminescence Laboratory, San Diego, CA).

For G12/13α signaling assay, constructs directing the expression

of a constitutively active mutant of G12α (G12α-Q229L) or G13α (G13α-Q226L) (both constructs were provided by Dr. Silvio Gutkind) were used to activate *c-fos* SRE-luciferase, a reporter construct (Stratagene, San Diego, CA). pCMV-β-Gal was used to normalize transfection efficiencies. The next day, cells were washed with PBS, serum starved for 6 h in Dulbecco's modified Eagle's medium supplemented with 0.5% FCS, and lysed in the reporter lysis buffer. Cell lysates were assayed for luciferase and β-galactosidase activities as described above. The expression of RGS14 and G13α (dilution 1:200; Santa Cruz Biotechnology) was examined by immunoblotting.

GAP Assays. We performed measurements of single-cycle GTPase rates of Giα, G12/13α, and Gsα as previously described (Druey and Kehrl, 1997; Kozasa et al., 1998). Various recombinant Gα subunits were expressed in and purified from *E. coli* or Sf9 cells as described (Kozasa and Gilman, 1995). The Gα proteins were loaded with [γ^{32}-P]GTP (5–10 μM; Amersham) and hydrolysis of GTP was then measured in the absence or presence of His6RGS14 containing only the RGS domain (200 nM). The RGS domain of RGS14 was used because the full-length RGS14 was highly prone to degradation. The RGS domain contained all of the crucial elements necessary for the GAP activity (Berman and Gilman, 1998). Aliquots were removed at the indicated times and counted by liquid scintillation spectrometry. For Gqα GAP assay, a mutant of Gqα, GqαR183C, was used (Chidiac and Ross, 1999).

Results

RGS14 Is Highly Expressed in Human Lymphoid Cells. Expression patterns of RGS proteins vary greatly from being expressed only in a narrow range of tissues to being expressed almost ubiquitously. Rat *RGS14* is expressed at high levels in brain and spleen, at a modest level in lung, and at very low levels in various other tissues (Snow et al., 1997). Tissue distribution of human *RGS14* was determined by Northern blot analysis with poly(A)$^+$ RNA isolated from various organs (Fig. 1A). One major and one minor transcript with the sizes of approximately 2.5 and 3.0 kilobases were readily detected in lymphoid organs such as spleen, thymus, and peripheral blood leukocytes. To facilitate further examination of RGS14 expression an anti-RGS14 rabbit polyclonal antiserum was generated against hexahistidine-tagged recombinant mouse RGS14. The resulting antiserum readily detected mouse RGS14 as well as that of human origin, and did not show any cross-reactivities with any other RGS proteins tested (data not shown). Next, the expression pattern of RGS14 in various human hematopoietic cells was examined by immunoblotting with the anti-RGS14 antiserum (Fig. 1B). RGS14 was expressed at modest-to-high levels in most B and T cell lines tested with the exception of the pre-B cell line Nalm6 and monocytic cell line HL-60. Longer exposure of the same immunoblot revealed a very low expression in these two cell lines. In addition, a high level of RGS14 expression was observed in primary lymphoid cells. Electrophoretic mobility of RGS14 (~75 kDa) in SDS-PAGE analyses differed considerably from its calculated molecular mass (~59 kDa). Mouse RGS14 expressed in a human cell line, 293T also was detected as a ~75-KDa protein that was clearly not present in immunoblots performed with preimmune serum (Fig. 1B, lanes 14 and 15). In addition, RGS14 was detected as either a single band or a doublet under different experimental conditions. These discrepancies may be due to aberrant migration and/or post-translational modifications.

RGS14 Impairs Giα-Mediated ERK-1 Activation by Acting as a GAP. To examine the involvement of RGS14 in a Giα-linked signaling pathway the activation of ERK-1 in response to IL-8 (Damaj et al., 1996) was monitored in 293T cells transiently expressing the IL-8 receptor (Fig. 2A). IL-8 induced 4- to 6-fold increases in ERK-1 activity and coexpression of RGS14 reduced ERK-1 activity. The inhibition on ERK-1 activation by RGS14 was in a dose-dependent manner showing approximately 35 and 55% reduction with 4 and 8 μg of RGS14 plasmid, respectively. Glutamic acid (E) 92 and asparagine (N) 93 of RGS14 correspond to E87 and N88 in RGS4 and are highly conserved residues in RGS proteins. They reside in the contact region between RGS4 and Giα1 (Druey and Kehrl, 1997; Tesmer et al., 1997). Substitution of these two residues in RGS14 with alanines (EN mutant) resulted in loss of inhibition on IL-8-induced ERK-1 activation (Fig. 2B) as similarly observed in the E87A/N88A double mutant of RGS4 retaining only 3 to 4% of wild-type GAP activity in vitro (Srinivasa et al., 1998).

Next, we determined whether RGS14 regulated Gsα- or Gqα-linked signaling pathways. We used 293T cells transiently expressing the β2-adrenergic receptor for the Gsα

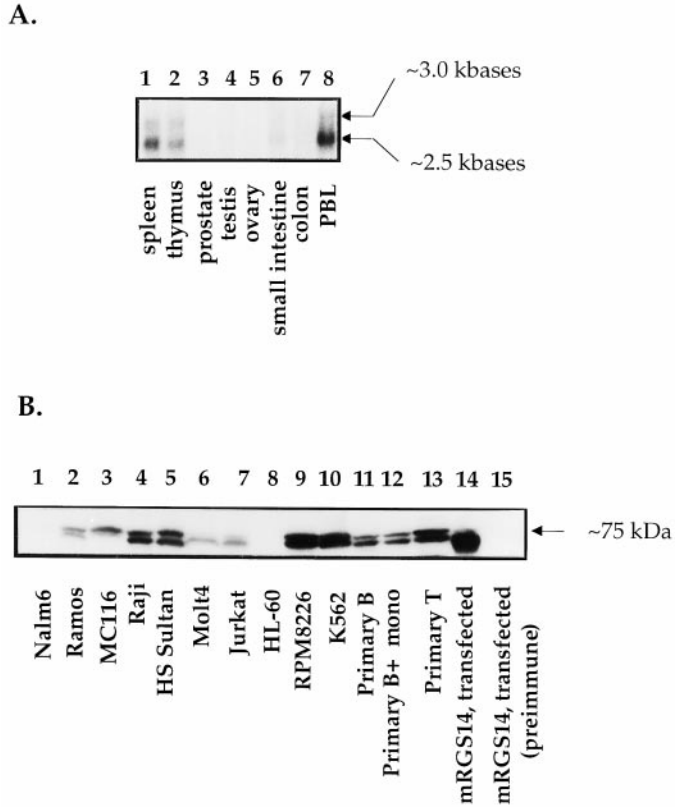

Fig. 1. Expression of RGS14 in various human tissues and hematopoietic cell lines. A, Northern blot analysis of RGS14 expression. A membrane containing 2 μg of poly(A)$^+$ RNA from various human tissues was hybridized at 50°C with a [γ-^{32}P] dCTP-labeled human RGS14 cDNA probe, washed twice in 2× standard saline citrate at room temperature, and once for 1 h at 50°C in 0.1× standard saline citrate. The membrane was then subjected to autoradiography. Human RGS14 cDNA clone was identified in human expressed sequence tag library and ordered from Genome Systems, Inc. (St. Louis, MO). B, immunoblot of RGS14 expression. Protein (100 μg) from various hematopoietic cell lysates was analyzed for RGS14 expression by immunoblotting with an anti-RGS14 antiserum. The immunoblots were detected by enhanced chemiluminescence. Lane 14 shows a lysate prepared from 293T cells transfected with mouse RGS14 cDNA as a positive control. Lane 15 shows a result of immunoblotting performed with a preimmune serum with the same lysate that was used for lane 14.

signaling assay and activation of a reporter gene, cAMP response element-binding protein (CREB)-β-galactosidase, was monitored after stimulation with an agonist, isoproterenol (Fig. 2C). Concomitant expression of RGS14 showed little effect on the activation of CREB-β-galactosidase induced by isoproterenol, whereas a known inhibitor of Gsα signaling, RGS3 (full length), attenuated CREB activation. Immunoblotting the cell lysates used for the signaling assay showed that RGS14 was expressed at a high level (data not shown). For the Gqα signaling assay the generation of inositol phosphates was measured in COS-7 cells transiently expressing the M1 receptor and PLCβ_2 (Fig. 2D). Stimulation with an agonist, carbachol, resulted in an approximately 14-fold increase in inositol phosphates. In contrast to RGS3, which significantly reduced the generation of inositol phosphates, RGS14 exhibited little effect. We also examined the effect of RGS14 expression on a GTPase-deficient mutant, Gqα-Q209L-induced generation of inositol phosphates (Fig. 2D). Concomitant expression of RGS14 did not affect the generation of inositol phosphates, whereas other RGS proteins potently do so (Scheschonka et al., 2000).

To determine whether RGS14 exhibited any GAP activities toward various Gα subunits in vitro single turnover GTPase assays were performed with purified recombinant Gα proteins and a truncated recombinant RGS14 protein that contained the RGS domain. We found that RGS14 enhanced the GTPase activity of Giα and Goα as efficiently as did RGS4

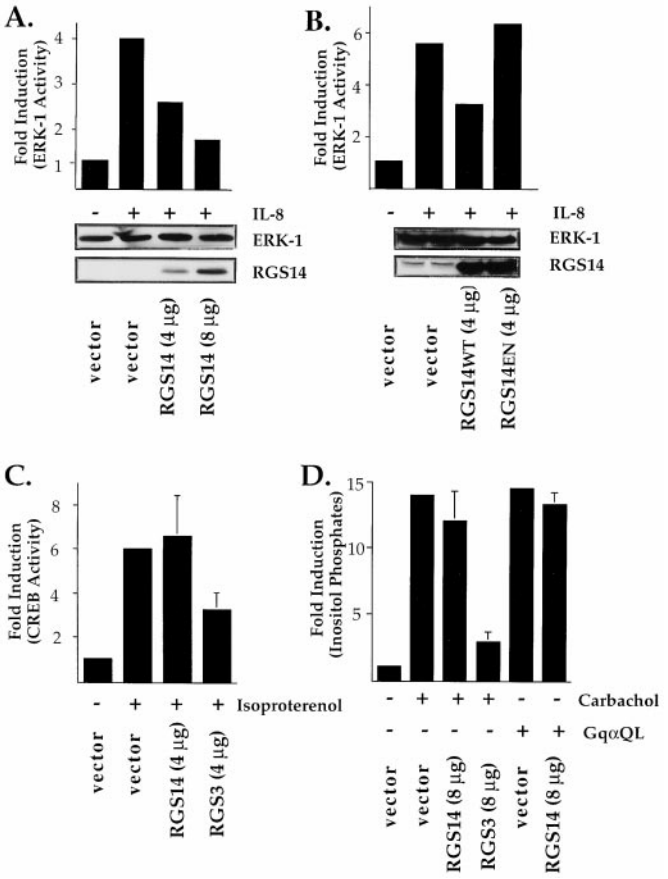

Fig. 2. Effect of RGS14 on Giα-mediated MAP kinase activation, and Gsα- or Gqα-mediated CREB activation. A, RGS14 attenuates IL-8-induced ERK-1 activation. The 293T cells were cotransfected with constructs that direct the expression of HA-ERK-1 and the IL-8 receptor in the absence or presence of wild-type RGS14. The micrograms shown in the parentheses indicate the amount of DNA used for 10 ml of transfection medium. Activation of HA-ERK-1 by IL-8 was monitored as described under *Materials and Methods*. Incorporation of [γ^{32}-P]ATP into MBP by ERK-1 was quantitated by autoradiography and NIH Image. The data represent fold induction in [γ^{32}-P]ATP incorporation into MBP with respect to control cells untreated with IL-8. The amount of EKR-1 in the immunoprecipitates was determined by immunoblotting with anti-ERK-1 antiserum. In addition, RGS14 expression in the cell lysates was examined by immunoblotting. B, RGS14EN does not attenuate IL-8-induced ERK-1 activation. Similar experiment to that shown in part A except a construct directing the expression of the RGS14 EN mutant also was used. C, RGS14 does not down-regulate Gsα-mediated CREB activation. The 293T cells were cotransfected with constructs that direct the expression of the β-adrenergic receptor, pCREB β-gal, and pSV40 Luc in the presence or absence of RGS14 (or RGS3). CREB activity was measured 6 h after stimulation with isoproterenol as described under *Materials and Methods*. The data represent fold induction in β-galatosidase activity normalized by the luciferase activity in each cell lysate with respect to control cells untreated with isoproterenol. The data are the mean ± S.E. of three independent assays performed in duplicate. D, RGS14 does not down-regulate Gqα-mediated generation of inositol phosphates. COS-7 cells were cotransfected with constructs that direct the expression of the M1 receptor (or Gqα-Q209L) and PLCβ_2 in the presence or absence of RGS14 (or RGS3). Inositol phosphates were measured after stimulation with carbachol as described under *Materials and Methods*. When Gqα-Q209L was used, cells were not stimulated with carbachol. The data represent fold induction in each cell lysate with respect to control cells untreated with carbachol. The data are the mean ± S.E. of two independent assays performed in duplicate.

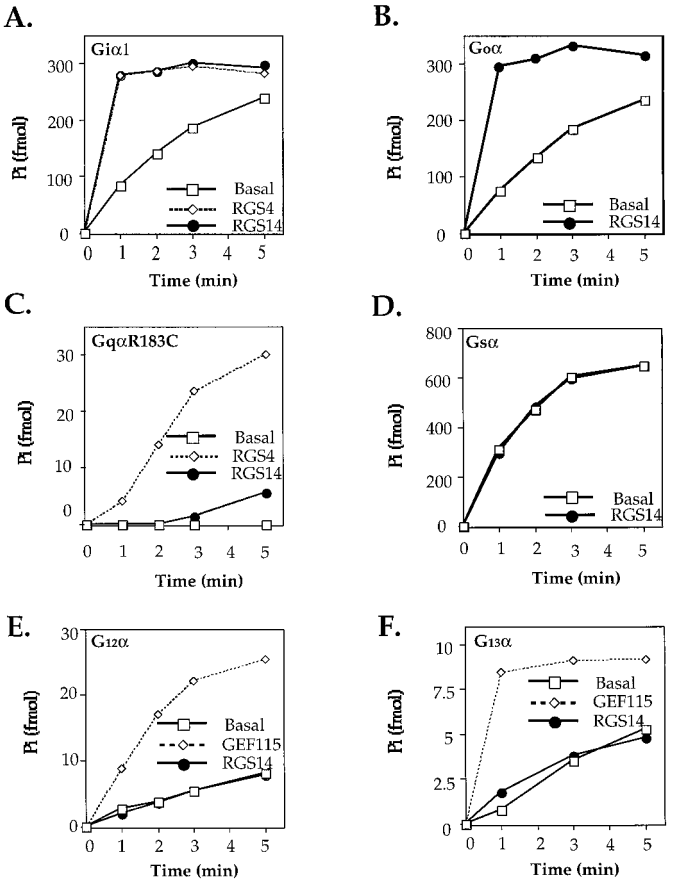

Fig. 3. Enhancement of GTPase activity of Giα subfamily by RGS14. The ability of RGS14 to accelerate the GTPase activities of Giα1 (A), Goα (B), Gqα (C), Gsα (D), G12α (E), and G13α (F) was monitored. GTP hydrolysis reaction was started by addition of various Gα subunits loaded with [γ-^{32}P]GTP to a reaction buffer in the absence or presence of RGS4 (100 nM), RGS14 (200 nM), or p115 RhoGEF (20 nM). "Basal" stands for the basal GTPase activity of each Gα subunit. Aliquots were removed from the reaction mixture at the specified intervals and the amount of released $^{32}P_i$ was measured by liquid scintillation spectrometry.

(Fig. 3, A and B; Berman et al., 1996). However, the intrinsic GTPase activities of Gqα, Gsα, G12α, and G13α were unaltered by RGS14 (Fig. 3, C–F). The Gqα GAP assay was performed three times, observing the release of P_i up to 15 min. The small increase in P_i release by RGS14 at 5 min (Fig. 3C) is an experimental variation due to low cpm counts. Therefore, it appears that the GAP activity of RGS14 is restricted to Giα subfamily.

RGS14 Impairs G13α-Mediated SRE Activation. Because little is known about receptors that exclusively couple to G12α or G13α we activated G12/13α signaling pathways by expressing GTPase-deficient mutants of G12α (G12α-Q229L) and G13α (G13α-Q226L). Expression of these G proteins potently increases the SRE-dependent transcription of *c-fos* (Fromm et al., 1997). We assessed the possible involvement of RGS14 in G12/13α-linked signaling pathways by monitoring the activation of a reporter gene, *c-fos* SRE-luciferase (Fig. 4, A and B). The transient expression of G12α-Q229L and G13α-Q226L in 293T cells resulted in a 20- and 10-fold increase in luciferase activity, respectively. Concomitant expression of RGS14 exhibited little effect on G12α-Q229L-mediated SRE activation despite high levels of RGS14. However, RGS14 attenuated SRE activation induced by G13α-Q226L, despite its lack of GAP activities toward the G12α subfamily members. This attenuation was not due to a decrease in the expression levels of G13α-Q226L by RGS14 as shown in anti-G13α immunoblot. The RGS14 EN mutant, which did not attenuate Giα-mediated ERK-1 activation, impaired G13α-Q226L-induced SRE activation as efficiently as did the wild-type RGS14 (Fig. 4B). RGS1 and RGS4, two other members of the RGS family, showed little inhibition on G13α-Q226L-induced SRE activation (Fig. 4C). We further examined whether RGS14 wild type and the EN mutant exerted any inhibition on M1 receptor-mediated activation of SRE by stimulation with carbachol (Fig. 4D). Both wild-type RGS14 and the EN mutant inhibited M1-receptor mediated SRE activation. Next, we tested the effect of RGS14 expression on SRE activation induced by constitutively active forms of the small GTPases RhoA or Ras (Fig. 4, E and F). RGS14 did not reduce RhoAQL or RasV12-induced SRE activation, indicating that RGS14 inhibits SRE activation at a level upstream of RhoA or Ras activation.

Cytoplasmic RGS14 Is Recruited to a Membrane Fraction after Expression of G13α-Q226L. To determine subcellular location of RGS14 protein, we preformed a cell fractionation experiment with the lymphoid cell lines HS-Sultan and Jurkat (as well as primary lymphoid cells). RGS14 immunoblotting of SDS-PAGE fractionated cytoplasmic and membrane fractions revealed that the cytoplasmic fractions contained more RGS14 (approximately 5-fold) than did the membrane fractions (Fig. 5A), thereby demonstrating a predominantly cytosolic location of RGS14. To verify the integrity of fractions, we reprobed the immunoblot membrane with antiserum against Gi/o/t/zα, which recognizes several Gα subunits that localize at the membrane. In addition, Cy-3 immunofluorescent staining of 293 cells transfected with HA-RGS14 by using anti-RGS14 antiserum showed a diffused staining of cytoplasm (Fig. 5B, left), confirming the cell fractionation result. The same Cy-3 staining of endogenous RGS14 in nontransfected 293 cells revealed faint cytoplasmic staining with a stronger Golgi staining (Fig. 5B, right). Preimmune serum resulted in no staining. Next, we tested whether coexpression of G13α-Q226L recruited cytoplasmic RGS14 to the plasma membrane to block G13α-mediated signaling. We transfected 293T cells with constructs directing expression of HA-RGS14 in the absence or presence of varying amount of G13α-Q226L (0.5–4 μg) and then fractionated the lysates by differential centrifugation (Fig. 6). Coexpression of 0.5 μg of G13α-Q226L resulted in an approximately 4-fold increase in the amount of RGS14 in the membrane fraction. Although increasing the amount of G13α-Q226L DNA resulted in a higher expression of G13α (data not shown) it did not further increase the amount of RGS14 in the membranes fraction, suggesting that there is a limited capacity to translocate RGS14. Exposing cells to a phorbol ester phorbol-12-myristate-13-acetate did not shift RGS14 to the membrane nor did coexpression of GTPase-

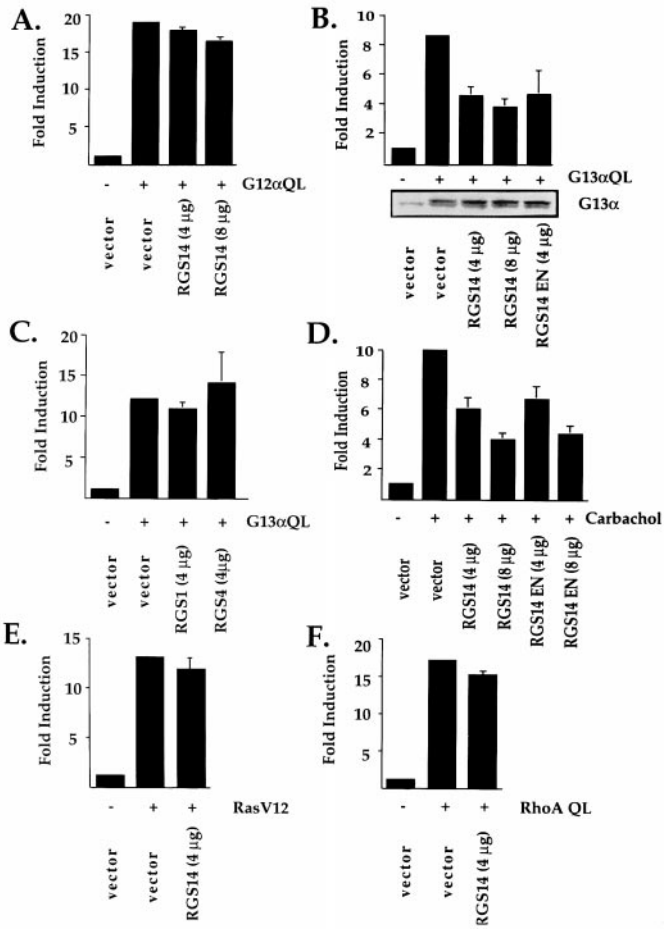

Fig. 4. RGS14 inhibits G13α-Q226L-induced SRE activation. The 293T cells were transfected with constructs that direct the expression of pSRE Luc and pCMV β-gal in the absence or presence of wild-type RGS14 (or RGS14EN mutant), RGS1, or RGS4. The micrograms shown in the parentheses indicate the amount of DNA used for 10 ml of transfection medium. SRE reporter gene activity was induced by cotransfection of constructs that direct the expression of G12α-Q229L (A), G13α-Q226L (B, C), M1 receptor followed by carbachol stimulation (D), RasV12 (E), or RhoAQL (F). The levels of reporter gene activity were monitored as described under *Materials and Methods*. The data represent fold induction in luciferase activity normalized by the β-galatosidase activity in each cell lysate with respect to control cells. The data are the mean ± S.E. of two or three independent assays performed in duplicate. To show expression levels of G13α-Q226L immunoblotting with anti-G13α-antiserum was performed (B). The lower band present in all lanes is endogenous G13α. Transfected G13α-Q226L runs as a slightly larger protein in SDS-PAGE due to HA tagging.

deficient mutants of other G proteins, Gsα-Q227L, Gqα-Q209L, or G12α-Q229L. As mentioned, the Giα proteins were found in the membrane fractions.

RGS14 Expression Is Enhanced in Lymphoid Cells Exposed to Activation Stimuli. Because RGS14 is constitutively expressed at modest to high levels in various lymphoid cells, we examined whether RGS14 expression would be down-regulated by stimuli that trigger lymphocyte activation. Contrary to our expectation, lymphocyte activation resulted in a further increase in RGS14 expression (Fig. 7A). In B cells activated with anti-CD40/anti-IgM, the level of RGS14 began to increase 5 h after stimulation and peaked around 24 h, showing an approximately 3-fold increase. By 48-h postactivation the level of RGS14 protein had returned to nearly that of unstimulated cells. A modest induction of endogenous RGS14 protein also was observed in T cells activated with anti-CD3/IL-2. However, a recognizable increase was seen 24 h after the activation and RGS14 levels remained elevated for up to 9 days. The reduction in RGS14 level at the time point, day 3, was not consistently observed. Thus, RGS14 expression in B and T cells seems to be differentially regulated.

In addition, we tested whether RGS14 expression would be affected by forskolin or ionomycin treatment, stimuli that increase the intracellular level of cAMP or Ca^{2+}, respectively, and that mimic responses triggered by effectors of G protein signaling (Fig. 7B). We stimulated the B and T cell lines HS-Sultan and Jurkat as well as primary lymphoid cells with either ionomycin or forskolin and analyzed cell lysates by immunoblotting with the anti-RGS14 antiserum. Treatment with forskolin or ionomycin resulted in a modest increase of endogenous RGS14 in T cells, whereas it did not affect RGS14 expression in primary B cells or the B cell line HS-Sultan (data not shown).

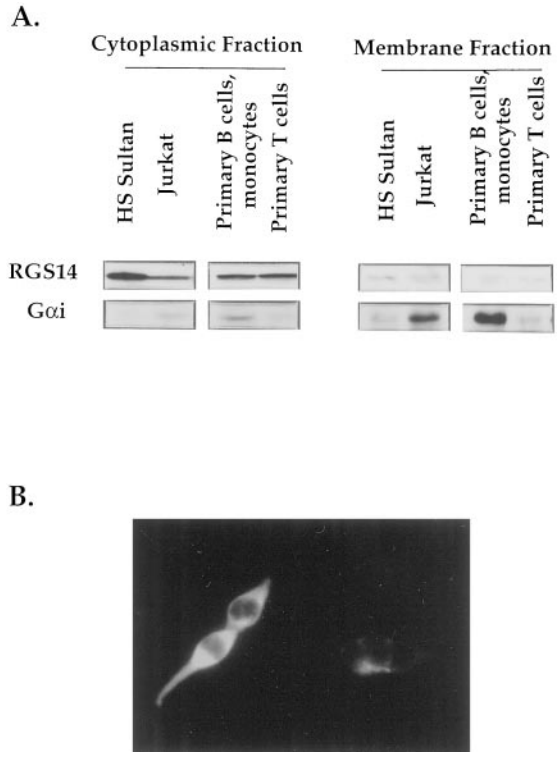

Fig. 5. Subcellular localization of RGS14. (A) RGS14 is predominantly localized in the cytoplasm. Various lymphoid cells were homogenized in the hypotonic buffer using a Dounce pestle, and fractionated by differential centrifugation. Equal proportions of cytoplasmic and membrane fractions were used for SDS PAGE followed by immunoblottings with anti-RGS14 and anti-Giα antisera. (B) Indirect immunofluorescence staining of RGS14. 293 cells were transfected with HA-RGS14, incubated with anti-RGS14 antibody, and stained with Cy3-conjugated secondary antibodies.

Discussion

In this study we extend our knowledge of the RGS family by characterizing one of the members that possesses a larger molecular mass, RGS14. Based on expression and signaling experiments RGS14 is likely to be involved in lymphocyte functions via its ability to regulate Giα- and G13α-mediated signaling pathways. In addition, lymphocyte activation further enhances RGS14 levels, suggesting a possible cross talk between the TCR- or BCR-initiated signaling pathways and G protein-linked signaling pathways.

Whereas numerous RGS proteins have been reported to be GAPs for Gαi and Gαq subfamily members, no RGS proteins has been shown to accelerate the GTPase activity of Gsα (Zerangue and Jan, 1998). Although two studies have suggested that RGS proteins may regulate Gsα-mediated signaling the mechanism by which they accomplish this is unclear (Chatterjee et al., 1997; Tseng and Zhang, 1998). p115 RhoGEF is a distant member of the RGS family and the only member shown to have GAP activity directed toward the G12/13α subfamily (Kozasa et al., 1998). Our in vitro GAP assays revealed that GAP activity of RGS14 is restricted to Giα subfamily members. This is in contrast to the previously

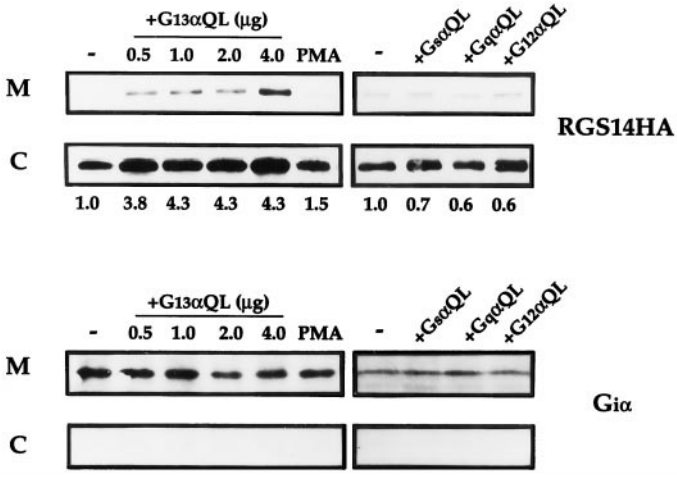

Fig. 6. Translocation of cytoplasmic RGS14 to the plasma membrane by coexpression of G13α-Q226L. The 293T cells were transfected with constructs that direct the expression of RGS14-HA in the absence or presence of various amount of G13α-Q226L (or 0.5 μg of Gsα-Q227L, Gqα-Q209L, or G12α-Q226L). Cells expressing only RGS14 also were exposed to phorbol-12-myristate-13-acetate (50 ng/ml). The cells were homogenized in the hypotonic buffer and fractionated by differential centrifugation as described under *Materials and Methods*. Equal proportions of cytoplasmic and membrane fractions were used for SDS-PAGE and subsequently immunoblotted with anti-HA antibody (top). The immunoblot was detected by enhanced chemiluminescence and quantitated by NIH Image. The numbers shown below represent fold increase in the amount of RGS14 in the membrane fraction normalized by that of RGS14 in the cytoplasmic fraction with respect to control cells, which were not expressing any GTPase-deficient mutants of Gα subunits. M and C stand for membrane and cytoplasmic fractions, respectively. The anti-HA immunoblot was reprobed with an anti-Giα antiserum to determine the level of Giα expression (bottom).

tested RGS (RGS1, RGS2, RGS3, RGS4, and GAIP), which are GAPs for both Giα and Gqα (Zerangue and Jan, 1998; Scheschonka et al., 2000). The GAP activities of RGS14 for Giα1 and Goα are comparable to those of RGS4, which is an excellent Giα GAP. Consistent with the GAP data, RGS14 impaired an IL-8 receptor-coupled Giα signaling pathway, whereas it did not inhibit signaling through Gsα- or Gqα-coupled receptors. Substitution of two residues conserved with other RGS proteins, E92 and N93 of RGS14 to alanine (RGS14EN mutant), resulted in a loss of its ability to impair Giα-coupled signaling as previously observed with the equivalent residues in RGS4 (Druey and Kehrl, 1997; Srinivasa et al., 1998).

RGS14 inhibited SRE activation induced by a GTPase-deficient mutant of G13α, G13α-Q226L, even though it failed to act as a GAP for the G12/13α subfamily in a standard GAP assay. The inhibition on G13α-Q226L-induced SRE activation is specific for RGS14 at least among the RGS proteins tested. The inhibition of M1 receptor-induced SRE activation by RGS14 is likely via G13α in 293T cells, although the M1 receptor can couple to Gqα and G12/13α subfamily members to activate downstream effectors (Luthin et al., 1997; Fromm et al., 1997). RGS14 is not a GAP for Gqα and did not attenuate M1 receptor-triggered inositol phosphate formation, a Gqα-linked pathway. Furthermore, when the EN mutation in RGS14, which crippled its ability to inhibit Giα signaling is introduced to RGS3, it renders RGS3 incapable of reducing the induction of inositol phosphates by a GTPase deficient form of Gqα (Scheschonka et al., 2000). Thus, if RGS14 had any capacity to interfere with Gqα-mediated signaling, the EN mutation would have been expected to impair it, yet RGS14 EN was effective as RGS14 in inhibiting M1 receptor-induced SRE activation. Interestingly, the RGS14 EN mutant inhibited the SRE activation induced by G13α-Q226L or by carbachol stimulation via M1 receptor. Taken together, our results suggest that the mechanism for RGS14 to inhibit G13α-mediated SRE activation is different from that necessary to attenuate Giα-linked pathways.

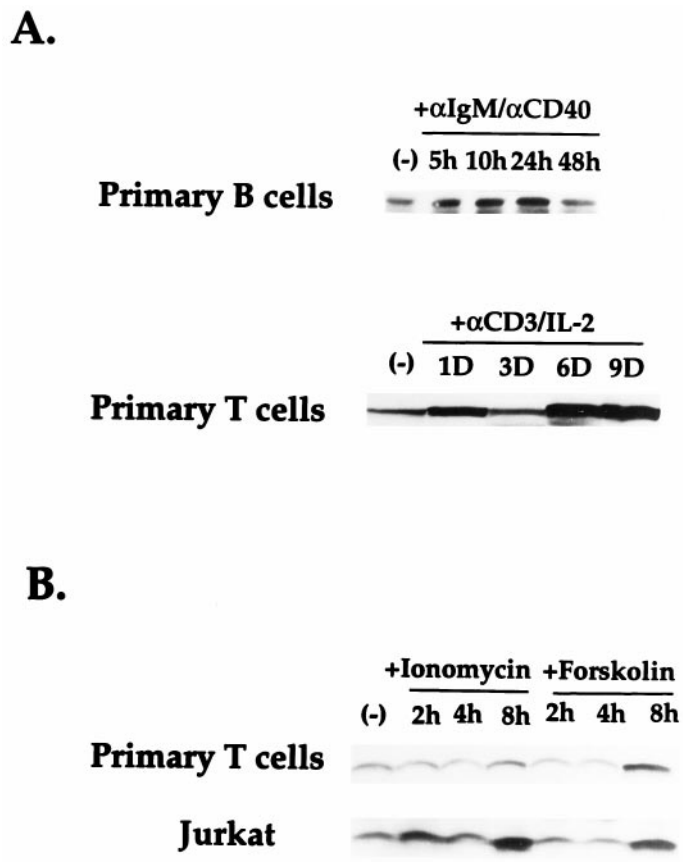

Fig. 7. Induction of RGS14 in lymphoid cells. A, effect of signals that activate T and B cells via their antigen receptors on RGS14 expression. Induction of RGS14 in enriched T and B cells from human blood stimulated with CD3 (0.1 μg/ml) in the presence of IL-2 or anti-IgM F(ab')$_2$ fragment (20 μg/ml) in conjunction with CD40 (1 μg/ml), respectively. B, effects of calcium ionophore ionomycin or the adenylyl cyclase activator forskolin on RGS14 expression in lymphocytes. Primary T cells and the T cell line Jurkat were stimulated with ionomycin (1 μM) or forskolin (10 μM). Aliquots were taken at times indicated and analyzed by immunoblotting with the anti-RGS14 antiserum.

Because previous studies suggested that some RGS proteins could act as effector antagonists for Gqα subunits (Hunt et al., 1996; Hepler et al., 1997), we examined whether RGS14 could act as an effector antagonist for G13α. First, we looked for an interaction between G13α and RGS14 by performing coimmunoprecipitation experiments with lysates prepared from 293T cells transfected with both G13α-Q226L and RGS14 or with those of prepared from COS-7 cells transfected with RGS14 followed by AlF_4^- stimulation. However, despite performing multiple experiments with a variety of conditions we were unable to detect a coimmunoprecipitating band (data not shown). This suggests either a transient and low-affinity interaction or no interaction between RGS14 and G13α. We then tested whether RGS14 could interfere with the GAP activity of p115RhoGEF toward G13α performing in vitro GAP assays. Even in the presence of 20-fold molar excess of RGS14 the GAP activity of p115RhoGEF toward G13α was not affected (data not shown). Therefore, it seems plausible that RGS14 inhibits the activation of Rho mediated by G13α by using a novel mechanism. Recently, RGS12 was shown to inhibit G12/13α-mediated signaling (Mao et al., 1998a,b), however the mechanism by which it accomplished this was not reported.

Considering that RGS proteins act as GAPs or effector antagonists for Gα proteins, it would be reasonable to assume either that RGS proteins localize in the membrane or that they can be recruited to the membrane if they localize in the cytoplasm. RGS-GAIP and Sst2p are shown to be the former, being predominantly present at the membrane, whereas RGS3, RGS4, and RGS14 are predominantly cytoplasmic (Druey et al., 1998; Dulin et al., 1999; present study). Coexpression of a GTPase-deficient G13α mutant, G13α-Q226L (not Gsα-Q227L, Gqα-Q209L, or G12α-Q226L) shifted a portion of RGS14 from cytoplasm to the plasma membrane as observed previously with RGS4 being recruited to the membrane by coexpression of Gi2α-Q204L (Druey et al., 1998). Some RGS proteins contain transmembrane domains or motives known to promote membrane association such as cysteine-string motif, PDZ (PSD95/Dlg/ZO1 homology) domain, or DEP (Dishevelled/EGL-10/pleckstrin homology) domain (De Vries and Farquhar, 1999). However, RGS14 does not contain any domains or motives known to promote membrane association. In addition, the mechanism of translocation of cytoplasmic RGS proteins to the membrane is not known. The interaction between Gα subunits and RGS proteins is not likely to be necessary for translocation of cytoplasmic RGS proteins to the membrane as demonstrated by Druey et al. (1998) with an RGS4 mutant that can no longer

bind to Giα. Therefore, it seems likely that activation of G13α signaling pathway but not the interaction between G13α and RGS14 is necessary for translocation of RGS14 to the membrane. In addition, an agonist, endothelin-1 or the calcium ionophore ionomycin could induce translocation of RGS3 to the plasma membrane (Dulin et al., 1999). Therefore, recruitment of an RGS protein from cytoplasmic pool in response to relevant signals may be a common mechanism to regulate multiple RGS proteins within a given cell.

Although considerable information has been accumulated with respect to the GAP functions of RGS proteins in G protein-linked signaling, little is known about the physiological regulation of the RGS proteins. Induction of RGS1 in HS-Sultan by PAF and the inhibition of PAF-induced activation of MAP kinase by RGS1 suggested presence of a negative feedback loop to decrease signal transduction via the PAF receptor (Druey et al., 1996). The enhanced RGS14 expression in T cells triggered by ionomycin suggests that either antigen receptor or a GPCR-induced calcium flux may trigger a negative feedback loop, which may inhibit activation of Giα or a G13α-coupled signaling pathway. The enhancement of RGS14 expression by forskolin implies that RGS14 may participate in a positive feedback loop to enhance Gsα-mediated signaling. The up-regulation of RGS14 may inhibit the inhibitory activity of Giα on adenylyl cyclases, thereby augmenting Gsα-induced adenylyl cyclase activation, which results in increased cAMP accumulation.

Recently, certain members of the RGS family of proteins have been shown to modulate chemoattractant-stimulated cell migration and adhesion in culture systems (Bowman et al., 1998). Chemoattractants that bind to heptahelical receptors trigger downstream signaling pathways by activating heterotrimeric G proteins of mainly the Giα subclass. In addition, a downstream effector of G13α, Rho, has been shown to participate in signaling from chemoattractant receptors to trigger rapid adhesion in leukocytes (Laudanna et al., 1996). Therefore, it is plausible that RGS14 plays a role in relaying TCR- or BCR-coupled signals generated during development of lymphoid organs or normal immune surveillance to G proteins to modulate processes such as lymphocyte adhesion and migration. To delineate physiological role(s) of RGS14 protein with respect to lymphocyte function, we are generating *RGS14* transgenic and RGS14−/− mice. The prediction is that the constitutive high expression or the absence of RGS14 will interfere with the relay of TCR- or BCR-coupled signals to Giα or G13α, thus impairing the development of lymphoid organs or compromising the ability to orchestrate a normal immune response.

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