A Truncated Form of RGS3 Negatively Regulates G Protein-coupled Receptor Stimulation of Adenylyl Cyclase and Phosphoinositide Phospholipase C*

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Identification of a new family of proteins (RGS proteins) that function as negative regulators of G protein signaling has sparked new understanding of desensitization of this signaling process. Recent studies with several mammalian RGS proteins have delineated their ability to interact with and function as GTPase-activating proteins specifically for G proteins in the Gi family. Here, we investigated the functional activity of RGS3 and a truncated form of RGS3 on G protein-coupled receptor-mediated activation of adenylyl cyclase, phosphoinositide phospholipase C, and mitogen-activated protein kinase in intact cells. Polymerase chain reaction and 5′-rapid amplification of cDNA ends analyses revealed the tissue-specific expression of a short form of the RGS3 transcript that encodes the approximate carboxy-terminal half of RGS3. This truncated form of RGS3 (RGS3T) was shown recently to function as a negative regulator of pheromone signaling in yeast (Druey, K. M., Blumer, K. J., Kang, V. R., and Kehrl, J. H. (1996) Nature 379, 742–746). Baby hamster kidney cells transiently transfected with RGS3T cDNA exhibited a pronounced impairment in platelet-activating factor receptor-stimulated inositol phosphate production, a pertussis toxin-insensitive response. Similarly, calcitonin gene-related peptide receptor-stimulated increases in intracellular cAMP and pituitary adenylate-cyclase activating polypeptide receptor-stimulated increases in both cAMP and inositol phosphates were reduced significantly in RGS3T transfectants compared with vector-transfected control cells. In contrast, baby hamster kidney cells transfected with the full-length RGS3 cDNA showed no impairment in cAMP and inositol phosphate production mediated by these G protein-coupled receptors. However, lysophosphatic acid receptor-stimulated phosphorylation of endogenous ERK1 and ERK2 was impaired markedly in both RGS3 and RGS3T transfectants, demonstrating the functional ability of both RGS forms to modulate Gβγ-mediated signaling. These results provide the first evidence for regulatory effects of an RGS protein on Gβγ and Gαi-mediated signaling in intact cells and document that the carboxy-terminal region of RGS3 comprises the structural domain for this activity.

A myriad of hormones, neurotransmitters, olfactory and taste molecules, and light produce their cellular effects by interacting with seven transmembrane-spanning receptors. In addition to their heptahelical structure, this superfamily of receptors shares a common mode of signal transduction involving interaction with and activation of various heterotrimeric GTP-binding proteins (G proteins). Ligand binding to these receptors produces activation of G proteins by stimulating exchange of GTP for GDP on the α subunit of the inactive heterotrimer to promote dissociation into α-GTP and βγ subunits, both of which function as signal transducing molecules by regulating the activities of various cellular effector systems including enzymes and ion channels (1). Termination of G protein signaling is mediated by the intrinsic GTPase activity of the α subunits resulting in their reassembly with βγ subunits to form the inactive α-GDPβγ heterotrimer.

Recent genetic studies have documented the existence of a new gene family encoding proteins that have been named RGS proteins based upon their ability to function as regulators of G protein signaling. Initial studies in yeast showed that mutations in the sst2 gene (encoding Sst2p) produced supersensitive pheromone signaling, a response mediated by a G protein-signaling pathway. Dominant gain of function mutations in Sst2p inhibited this G protein signaling pathway showing that Sst2p functioned as a pheromone desensitization factor in yeast (2). Similar genetic analysis of the Caenorhabditis elegans Sat2p homolog EGL-10, a protein involved in egg laying and certain periodic behaviors, suggested that it functioned to negatively regulate signaling mediated by the C. elegans G protein GOA-1 (3). Based upon a semiconserved region of approximately 120 amino acids (the “RGS domain”) of Sst2p, EGL-10, and two previously unidentified human gene products, Koelle and Horvitz (3) used PCR to identify a family of mammalian genes encoding RGS proteins. Southern analysis predicted the existence of at least 15 human RGS genes, all of which share the conserved RGS domain. The functional ability of several RGS proteins to reverse the supersensitive phenotype of yeast sst2Δ mutants and to impair mitogen-activated protein kinase activation by the G protein-coupled IL-8 and PAF receptors recently was demonstrated by Druey et al. (4).

The negative regulatory effects of these novel proteins on G protein signaling appears to result from direct interactions

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* The abbreviations used are: G protein, guanine nucleotide-binding protein; bp, base pair(s); GPR, calcitonin gene-related peptide; DMEM, Dulbecco's modified Eagle's medium; Gbg, a subunit of G protein; GAP, GTPase-activating protein; IP, inositol phosphate; kb, kilobase(s); LPA, lysophosphatidic acid; MAP, mitogen-activated protein; PACAP, pituitary adenylate cyclase activating polypeptide; PACAPR, PACAP receptor; rPACAPR, rat PACAPR; PAF, platelet-activating factor; PAFR, PAF receptor; rPAFR, rat PAFR; PI-PLC, phosphoinositide phospholipase C; RACE, rapid amplification of cDNA ends; RGS3T, truncated form of RGS3; BHK, baby hamster kidney; IL, interleukin.
with G protein α subunits resulting in an enhancement in their GTPase activity (5–9). Thus, RGS proteins are thought to function as GTPase-activating proteins (GAPs) for α subunits of heterotrimeric G proteins in a manner analogous to the action of RasGAP of Ras. Interestingly, the specificity of this GAP activity of the RGS proteins (RGS1, RGS4, RGS10, and GAIP or “Gα-interacting protein”) examined in these biochemical studies was limited to G proteins in the G1 family, a finding consistent with evidence demonstrating their selectivity of interaction with these G protein α subunits (6–9). These observations have led to the belief that RGS proteins function as GAPs for proteins in the G1 family of G proteins. This raises an interesting dilemma regarding the physiological significance of such a large gene family for a particular subfamily of G proteins. Surprisingly, no studies have evaluated the ability of any RGS protein to regulate activation of adenylyl cyclase and PI-PLC by G protein-coupled receptors in intact cells. Moreover, the study by Druey et al. (4) represents the only study in which RGS protein regulation of mammalian G protein signaling has been studied in cells.

The present study was initiated to investigate the functional activity of RGS3, an RGS protein whose G protein specificity has not been studied, on G protein-coupled receptor-mediated activation of adenylyl cyclase and PI-PLC in intact cells. Preliminary examination of the tissue-specific expression of RGS3 transcripts revealed the existence of a transcript encoding a truncated form of RGS3 (RGSST) encoding the approximate carboxyl-terminal half of RGS3. Cells transfected with RGS3 full-length cDNA demonstrated a pronounced impairment in G protein-coupled receptor stimulation of adenylyl cyclase and PI-PLC, an effect not observed in RGS3 transfectants. However, LPA receptor-stimulated phosphorylation of endogenous ERK1 and ERK2 was markedly impaired in both RGS3 and RGSST transfectants, demonstrating the functional ability of both RGS forms to modulate Gα-mediated signaling. These findings are the first to document regulatory influences of an RGS protein on Gα in any experimental system and on Gα in intact cells. The finding that this functional activity is encoded by the carboxyl-terminal half of RGS3 indicates that this region comprises a domain of RGS3 that is capable of negatively regulating Gα and Gα-mediated signaling.

EXPERIMENTAL PROCEDURES

Materials—PAP (1-O-hexadecyl-2-acetyl-sn-glycerol-3-phosphocho- line), CGRP and PACAP-27 were purchased from Bachem, and LPA was from Sigma. Myo-[2-3H]inositol (16.5 Ci/mmol) was from Amer- sham Corp. LipofectAMINE, OptiMEM, inositol-free DMEM, and Elongase were obtained from Life Technologies, Inc. Perfect Preps for plas-mid DNA preparation were obtained from 5 Prime → 3 Prime, Inc. (Boulder, CO), and pCR3.1 vector was obtained from Invitrogen. Cell culture medium and serum were obtained from the Diabetes Endocri-nology Research Center of the University of Iowa. Oligonucleotide primers and other molecular biological reagents were obtained from the University of Iowa DNA Core. BHK-21 cells (ATCC) were a gift from Dr. Jeffrey Pessin (University of Iowa). Full-length cDNAs encoding the rPAFR and rPACAPR (0.25 μg/well) were cotransfected with cDNAs encoding pCR3.1 vector or pCR3.1 vector carrying the truncated RGS3 (RGS3T) encoding the approximate carboxyl-terminal half of RGS3. Cells transfected with RGS3T cDNA demonstrated a pronounced impairment in G protein-coupled receptor stimulation of adenylyl cyclase and PI-PLC, an effect not observed in RGS3 transfectants. However, LPA receptor-stimulated phosphorylation of endogenous ERK1 and ERK2 was markedly impaired in both RGS3 and RGSST transfectants, demonstrating the functional ability of both RGS forms to modulate Gα-mediated signaling. These findings are the first to document regulatory influences of an RGS protein on Gα in any experimental system and on Gα in intact cells. The finding that this functional activity is encoded by the carboxyl-terminal half of RGS3 indicates that this region comprises a domain of RGS3 that is capable of negatively regulating Gα and Gα-mediated signaling.

PCR Analysis of Tissue Distribution of mRNAs Encoding RGS3—A panel of human tissue cDNA libraries in Agt10 (CLONTECH) and oligo(dT)-primed cDNA synthesized from human glioma cell mRNA using avian myeloblastosis virus reverse transcriptase was used to examine the tissue distribution of RGS3 mRNAs by PCR. PCR was performed using Elongase and forward primers to nucleotides 1–24 or 937–965 and a reverse primer to nucleotides 1564–1590 of RGS3 cDNA. The outer primer pairs (1–24 and 1564–1590) encompass the entire coding sequence of RGS3 cDNA (1–1557), and the inner primer pairs (937–965 and 1564–1590) encompass approximately the 3′ half of RGS3 cDNA. The resulting products were resolved by 1.5% agarose gel electrohoresis using 100-bp and 1-kb DNA ladders (Life Technologies, Inc.) as molecular size markers. In some experiments, the forward primer was phosphorylated at its 5′ end to aid in unidirectional cloning of the amplified product in pCR3.1 vector. In those studies, individual clones were isolated, and mini-prep DNAs from several clones were sequenced to confirm the identity of the amplified products.

Isolation of the 5′ cDNA End of a Truncated Form of RGS3 Using 5′-RACE—Oligo(dT)-primed human brain cDNA with a synthetic oligonucleotide (anchor) ligated to its 5′ end (RACE ready cDNA, CLON-TECH) was used as template for 5′-RACE PCR. PCR was performed using a forward primer complementary to the anchor sequence and reverse primers specific to sequences of human RGS3 cDNA. A single PCR product of approximately 300 bp was amplified using two rounds of seminested PCR, i.e. using the same forward primer and nested reverse primers (see Fig. 2). The reverse primer used in the final round of PCR corresponded to nucleotides 747–773 of human RGS3 cDNA, suggesting that the 5′ end of RGS3 expressed in brain corresponded approximately to nucleotide 521 of the full-length RGS3 cDNA (i.e. subtracting the 48-nucleotide anchor primer from the 300-bp 5′-RACE product). Double stranded sequencing of the amplified product showed that the 5′-RACE product corresponded to nucleotides 527–773 of the full-length RGS3 cDNA.

Cell Culture and Transfections—BHK cells were grown in DMEM supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml) in a 5% CO2 humidified atmosphere at 37 °C. Cells were plated in 24-well culture dishes at a density of 5 × 104 cells/well and were allowed to grow for 24 h prior to transfection.

BHK cells were transiently transfected with pCR3.1 containing cDNAs for RGS3 or RGS3T (1 μg/well) using LipofectAMINE (5 μg/l) DNA) according to the manufacturer’s protocol. In some experiments, RGS3 and RGSST cDNAs (0.75 μg/well) were cotransfected with cDNAs encoding rPAFR or rPACAPR (0.25 μg/well). Empty vector was used in place of pCR3.1 containing RGS3 or RGSST cDNAs as a vector control. Lipofection was performed for 16 h at 37 °C and was terminated by replacing the transfection mixture with culture medium.

Inositol Phosphate and cAMP Measurements—All experiments in which cAMP or inositol phosphate (IP) accumulation was measured were performed 48 h following termination of transfections. For measurements, BHK cells transfected with each RGS3 expression plasmid were allowed to recover for 24 h following termination of transfections and then were labeled for 16 h with [3H]inositol (2 μCi/ml) in inositol-free DMEM containing 10% dialyzed fetal bovine serum (Sigma). Labeled cells were rinsed with Earle’s balanced salt solution, preincubated in Earle’s balanced salt solution containing 10 mM LiCl for 20 min at 37 °C, and stimulated with vehicle or agonists for 20 min. Incubations were terminated by removing the medium and adding 1.0 ml of methanol. Total IPs were extracted and subjected to Dowex chromatography as we described previously (10). IP accumulation was expressed as dpm of total IPs/10^6 dpm in the lipid fraction.

Measurements of cAMP in transfected cells were performed essentially as we described previously (12). Briefly, transfected BHK cells were preincubated in DMEM containing 1% bovine serum albumin and 0.5 mM isobutyl methylxanthine for 20 min and then stimulated with vehicle or agonists for 5 min. Incubations were terminated by removing the medium and adding 1.0 ml of methanol. Total IPs were extracted and subjected to Dowex chromatography as we described otherwise.

Dual Phosphorylation Analysis of MAP Kinase Phosphorylation—MAP kinase studies were performed in BHK cells cultured in 6-well dishes using the same transfection protocol described above, adjusting the amount of DNA and lipofectamine for four times as many cell. Experiments were performed 48 h following termination of transfections, and the cells were incubated in serum-free DMEM for at least 5 h prior to beginning experiments. Cells transfected with RGS3 cDNA, RGS3T cDNA or empty pCR3.1 vector were stimulated with vehicle or LPA (10 μM) for 5
min at 37 °C in serum-free DMEM. Reactions were terminated by removal of medium, rinsing twice with ice-cold Dulbecco’s phosphate-buffered saline, and adding 0.15 ml of ice-cold lysis buffer (40 mM Hepes, pH 7.4, 4 mM EDTA, 2% Triton X-100, 200 mM sodium fluoride, 20 mM sodium pyrophosphate, 0.2 mM sodium vanadate, 1 mM phenylmethylsulfonflouride, 5 μg/ml leupeptin, and 1 μg/ml pepstatin). The cell lysate was centrifuged at 15,000 x g for 15 min at 4 °C, and the resulting supernatant was assayed for protein. Equal amounts of cell protein (30–40 μg) from each treatment condition were suspended in sample buffer and subjected to SDS-polyacrylamide electrophoresis. The separated proteins were transferred to polyvinylidene difluoride membrane and subjected to immunoblotting with antibodies specific for ERK2 (Santa Cruz) or phosphoERK1 and phosphoERK2 (New England Biolabs) followed by ECL detection (Amersham Corp.). The resulting autoradiogram was scanned using a Bio-Rad imaging densitometer to quantify the intensity of the signals.

Data shown represent the means ± S.E. of 3–9 separate transfections. Significance of differences between conditions was determined using analysis of variance followed by Fisher’s post hoc analysis. The Western blot data summarized in Fig. 6 (n = 5) were analyzed by analysis of variance followed by Student’s modified t test with the Bonferoni correction for multiple comparisons.

RESULTS

Koelle and Horvitz (3) recently identified RGS3 as a member of the mammalian family of RGS proteins by amplifying its partial sequence from rat brain cDNA using degenerate primers to the conserved RGS domains of EGL-10 and two human genes (RGS1 and RGS2) whose function was unknown. At about the same time, Druey et al. (4) reported isolation of a human RGS3 cDNA encoding a 519-amino acid RGS3 protein. Northern analysis revealed a rather wide distribution of RGS3 mRNAs in human tissues and, in addition, the existence of multiple hybridizing bands in many tissues. Because the identity of these hybridizing transcripts is unknown, it is unclear whether the multiple hybridizing bands represent multiple forms of RGS3 mRNA or cross-reactivity of the full-length RGS3 probe with other RGS family members due to the conservation of sequence encoding the RGS domain in this family of proteins.

Therefore, we initiated our studies of human RGS3 by examining the tissue expression of its transcript(s) by PCR using primers specific for RGS3 and by sequencing the amplified products to determine their identity(s). Fig. 1 shows the results of amplification using primer pairs encompassing the entire coding sequence of RGS3 and using human tissue cDNA or cDNA libraries as template (Fig. 1, A and B). As shown, the expected 1.6-kb PCR product was amplified from glioma, heart, smooth muscle, and lung but not from brain, liver, or kidney. Sequencing of each of the 1.6-kb products confirmed their identity as cDNAs encoding RGS3. Although PCR is a sensitive technique to detect even a rare transcript, we persistently failed to amplify the 1.6-kb RGS3 cDNA in brain, liver, or kidney using two different cDNA libraries. Although our analysis supports the Northern blotting data of Druey et al. (4) showing the presence of strong hybridization signals in heart, lung, and muscle with low or no detectable signals in brain, our inability to amplify RGS3 cDNAs from liver and kidney is quite at odds with the presence of strong hybridization signals in those tissues in that study. Although Druey et al. (4) found that many tissues expressed either a predominant transcript of 3 kb or comparable levels of the 3-kb transcript and a smaller (~2 kb) transcript, both kidney and liver expressed high levels of the small transcript and little or none of the 3.0-kb transcript. Thus, our inability to amplify RGS3 cDNAs from kidney, liver, and perhaps brain could result from expression in these tissues of an RGS3 transcript that has a deletion or truncation in either of the regions targeted by our PCR primers.

To consider this possibility, we performed PCR using primers to a variety of RGS3 cDNA sequences in an attempt to detect RGS3 transcripts in brain, kidney, and liver. Fig. 1C shows a typical result from such an experiment. As shown, PCR products of the expected size were amplified from brain, kidney, and liver using primer pairs encompassing nucleotides 937–1590 of RGS3 but not with primers encompassing the entire coding sequence of RGS3. In contrast, both primer pairs readily amplified the expected sized products from heart, lung, smooth muscle, and glioma. Sequencing of the 650–700-bp products amplified from brain, liver, and kidney showed that they were identical to the RGS3 cDNA sequence over this region. These results suggest that RGS3 transcripts in brain, liver, and kidney are truncated in the 5′ region or that the truncated cDNAs detected in this study arise artifactually from failure of reverse transcriptase to synthesize a complete cDNA copy of the mRNA. It is difficult to understand how this latter possibility could be tissue-specific as would be required to explain
the present results. This possibility seems unlikely also due to our inability to amplify a full-length RGS3 cDNA using two different human cDNA libraries as well as a preparation of human brain cDNA.

To examine further the possibility that transcripts encoding RGS3 may be truncated at the 5′ end in some tissues, we performed 5′-RACE to identify the 5′ cDNA end of RGS3 in human brain. The results shown in Fig. 1B suggested that the 5′ end of RGS3 transcripts in human brain extended at least to nucleotide 937. Therefore, we performed seminested PCR with the final round employing the forward anchor primer and a reverse primer to nucleotides 747–773 of RGS3 (Fig. 2, A and B). As shown, a single product of 300 bp was amplified using this strategy, indicating that the 5′ end of the RGS3 transcript in human brain is approximately 521 nucleotides downstream of the translation start site of the full-length transcript (i.e. accounting for the size of the anchor primer). Sequencing of the 300-bp PCR product confirmed that the 5′-RACE product corresponded to nucleotides 527–773 of the RGS3 transcript. These results suggest the existence of a truncated form (RGS3T) of the human RGS3 transcript whose 5′ end is located 527 nucleotides downstream of the translation start site identified for the RGS3 cDNA cloned from human B cells. Such a transcript could arise by alternative splicing; however, there are no introns at appropriate locations to account for such a transcript within a 14.7-kb segment encoding the entire coding sequence of human RGS3 that we isolated recently.² Alternatively, the truncated form could arise from incomplete gene duplication or by use of an alternate promoter site within the RGS3 gene. Examination of the sequence of the truncated form of RGS3 revealed the existence of three in-frame translation start sites located at nucleotides 715–717, 889–891, and 940–942, respectively, that could encode proteins of 281, 223, and 206 amino acids each. These three potential start sites encode RGS proteins whose amino termini are located 151, 93, and 76 amino acids from the start of the conserved RGS domain of RGS3.

In view of the present findings, it is of particular interest that Druey et al. (4) reported that expression of a truncated human RGS3 cDNA in yeast cell set2Δ mutants (i.e. lacking the yeast RGS protein Setp2) partially complemented the pheromone-supersensitive phenotype of the mutants. The truncated RGS3 cDNA used by these investigators was identical to the 206-amino acid form described above. It is unclear how this clone was obtained and/or why this particular truncation was tested. Nonetheless, Druey et al. (4) found that its ability to reverse the supersensitive phenotype of set2Δ mutants was 3–10-fold greater than that of RGS3 itself. Although effects of RGS3T(206) were not evaluated on mammalian cell signaling, RGS3 was shown to attenuate IL-8 receptor-mediated activation of MAP kinase (4). Because IL-8 receptors couple specifically to Gαi in human embryonic kidney cells, IL-8 receptor activation of MAP kinase is likely mediated by Gαi (13). These findings are consistent with evidence demonstrating the specificity of RGS proteins for G proteins in the Gα family (5–9). However, the functional evaluation of RGS3 on mammalian cell signaling has not been investigated beyond this study.

Therefore, we examined whether RGS3 had any effects on signaling by G proteins other than Gαi and whether RGS3T had functional activities on G protein-coupled receptor signaling in mammalian cells. The yeast complementation study by Druey et al. (4) prompted us to select the 206-amino acid form of RGS3T for this study. First, we examined PAFR-mediated activation of PI-PLC in BHK cells cotransfected with pCR3.1 containing cDNAs encoding the rat PAFR and RGS3 or RGS3T. BHK cells transfected with empty pCR3.1 in place of pCR3.1 containing RGS3 and RGS3T cDNAs served as a vector control in these studies. PAF-stimulated IP accumulation in PAFR transfectants was unaffected by overnight treatment with pertussis toxin (100 ng/ml), demonstrating that pertussis toxin-insensitive G proteins mediate activation of PI-PLC by the PAFR. As shown in Fig. 3A, PAF-mediated IP accumulation in response to a maximally effective concentration of PAF (100 nM) was reduced significantly in cells transfected with RGS3T cDNA but not in cells transfected with RGS3 cDNA. PAFR activity was reduced to approximately 30% of control values in RGS3T transfectants. Dose-response studies demonstrated that PAF stimulated IP accumulation with the same EC₅₀ value in cells transfected with vector, RGS3, or RGS3T, although the maximal response was reduced significantly in RGS3T transfectants (Fig. 3B). These results show that the efficacy but not the potency of PAF for stimulation of IP production is reduced in RGS3T transfectants.

To show further the unique ability of RGS3T to negatively regulate signaling pathways other than those mediated by members of the Gαi family of G proteins, we examined the ability of RGS3 and RGS3T to influence CGRP-stimulated cAMP production in BHK cells. CGRP receptors couple to Gαs and are expressed endogenously in BHK cells. Therefore, CGRP-stimulated cAMP increases were determined in BHK cells transfected with pCR3.1 containing cDNAs encoding RGS3 or RGS3T as well as with empty vector. Fig. 4A shows that CGRP-stimulated cAMP production was reduced significantly in cells transfected with RGS3T cDNA but not in cells transfected with RGS3 cDNA. CGRP receptor-mediated cAMP production was routinely reduced to 50% or less of control values in RGS3T transfectants with no change in EC₅₀ value for CGRP (Fig. 4B).

To determine whether effects of RGS3T on G protein-coupled receptor signaling via adenyl cyclase and PI-PLC pathways are receptor-specific, we evaluated PACAP-stimulated cAMP
and IP accumulation in BHK cells cotransfected with cDNAs encoding the PACAPR and RGS3 or RGS3T. The PACAPR is a member of the multifunctional group III family of G protein-coupled receptors, receptors that stimulate both adenylyl cyclase and PI-PLC. It is presently unclear whether this dual signaling results from receptor coupling to Gs (adenylyl cyclase) and a Gq (PI-PLC) G protein or by receptor coupling to other G proteins that stimulate adenylyl cyclase (requires concomitant Gs activation) and PI-PLC by their dissociated β subunits (14, 15). Fig. 5A shows that PACAPR-mediated IP accumulation was reduced by more than 60% (*p < 0.05) in cells transfected with RGS3T but not in cells transfected with RGS3. PACAP-stimulated cAMP accumulation was also significantly reduced in cells transfected with RGS3T but not in cells transfected with RGS3 (Fig. 5B). Dose-response experiments (not shown) showed that the maximal response but not the EC50 value for stimulation of IP and cAMP accumulation by PACAP was reduced in RGS3T transfectants compared with vector controls.

To determine whether the full-length RGS3 is expressed in BHK cells and is functionally able to negatively regulate Gi-mediated signaling, we examined whether LPA receptor-mediated phosphorylation of MAP kinase was attenuated in RGS3 transfectants. LPA receptors activate MAP kinase by coupling to Gi (16) and are expressed endogenously in BHK cells. Therefore, LPA-stimulated tyrosine phosphorylation of endogenous ERK1 and ERK2, required for their activation (17), was examined in BHK cells transiently transfected with empty pCR3.1 vector or pCR3.1 containing RGS3 or RGS3T cDNAs. Transfected cells were challenged with vehicle or LPA (100 nM), and phosphorylation of ERK1 and ERK2 was measured as described under "Experimental Procedures." Fig. 6A shows that LPA stimulated a dramatic increase in phosphorylation of ERK1 and ERK2, which was reduced significantly in RGS3 and RGS3T transfectants compared with vector control (Fig. 6, A and B). The finding that LPA receptor-stimulated phosphorylation of ERK1 and ERK2 was reduced nearly 50% in RGS3 and RGS3T transfectants suggests that RGS3 and RGS3T are extremely effective at inhibiting this response, because all cells express endogenous LPA receptors, ERK1 and
ERK2, whereas the efficiency of transfecting RGS3 and RGS3T is probably far less than 100%. These results show that RGS3 and RGS3T are functionally active in attenuating Gi-mediated phosphorylation of MAP kinase in BHK cells and support the findings of Druey et al. (4) stating that RGS3 inhibits MAP kinase activation mediated by the G\textsubscript{i}-coupled IL-8 receptor.

**DISCUSSION**

The major finding of the present study is the demonstration that a truncated form of RGS3 negatively regulates signaling mediated by G proteins other than those in the G\textsubscript{i} family. Here, we show that G protein-coupled receptor activation of adenylyl cyclase and PI-PLC can be inhibited substantially by expression of a truncated form of RGS3, an effect not reproduced by RGS3 itself. Surprisingly, no previous studies have evaluated the ability of any RGS protein to regulate signaling pathways mediated by G proteins other than G\textsubscript{i} in intact cells. Recent biochemical studies suggest that RGS proteins selectively interact with and negatively regulate members of the G\textsubscript{i} family of G proteins. De Vries et al. (5) used the yeast two-hybrid system to show that the RGS protein GAIP interacts strongly with G\textsubscript{ai3}, interacts weakly with G\textsubscript{ai2}, and does not interact with G\textsubscript{aq}. Hunt et al. (6) showed specific interaction of RGS10 with activated forms of G\textsubscript{ai3} and G\textsubscript{az} but not G\textsubscript{as}, whereas Watson et al. (7) found that RGS1, RGS4, and GAIP interacted with activated forms of G protein \alpha subunits identified as a mixture of G\textsubscript{ai} and G\textsubscript{ao} but not with G\textsubscript{as}. These latter studies and those of Berman et al. (8, 9) showed additionally that GAIP, RGS1, RGS4, and RGS10 function as GAPs specifically for \alpha subunits in the Gi family of G proteins but not for G\textsubscript{as}. No studies have examined the ability of RGS3 or RGS3T to interact with or regulate individual G proteins by genetic or in vitro analysis.

Although the precise mechanism by which RGS3T negatively regulates G protein-coupled receptor signaling cannot be determined from our experiments, the present results are consistent with negative regulatory effects of RGS3T on G\textsubscript{i} and pertussis toxin-insensitive G proteins that activate PI-PLC (i.e. G\textsubscript{i} family). First, the lack of receptor specificity in the effects of RGS3T reported here suggests that RGS3T does not act at the level of the receptor. Second, the absence of reduced levels of
cAMP or IPs in cells expressing RGS3T is not consistent with direct inhibitory effects of RGS3T on adenyl cyclase or PI-PLC. Our finding that CGRP and PACAP receptor-mediated cAMP increases were attenuated in RGS3T transfectants implicates Gα as a target for the negative regulatory effects of RGS3T. Similarly, the dramatic reduction in the pertussis toxin-insensitive activation of PI-PLC mediated by the PAFR in RGS3T transfectants implicates G proteins in the Gi family as a target for negative regulation by RGS3T. Although the identity of the G protein(s) mediating PAFR activation of PI-PLC in BHK cells is not known, previous studies have demonstrated coupling of the PAFR to Gα11 and Gα13 to stimulate PI-PLC (18). The present findings are the first to document regulatory influences of an RGS protein on Gα in any experimental system and on Gα in intact cells. Recently, Berman et al. (9) demonstrated a weak interaction of Gαq with RGS4 by assessing the ability of the α,βγGTP-AlF4 transition state complex to competitively inhibit the GAP activity of RGS4 on Gαq, Gαq interacted with RGS4 with an affinity 10–100-fold lower than that of Gαi proteins, and Gαi was shown to have no interactions with RGS4. However, as these authors pointed out, Gαq must be reconstituted with an appropriate receptor to determine whether RGS4 functions as its GAP due to the low intrinsic GTPase activity of Gαq. These observations are of particular interest in view of the present results that implicate Gα proteins as targets for the negative regulatory actions of RGS3T on G protein-coupled receptor signaling. Indeed, while the present work was under review, Hepler et al. (19) provided in vitro evidence that RGS4 and GAIP are GTPases for purified Gα and also can inhibit Gα-mediated activation of PI-PLC by a mechanism independent of their GAP activity. Our results in intact cells and those of Hepler et al. (19) using purified proteins and membranes fractions are in agreement with the contention that RGS proteins can interact with G proteins other than those in the Gi family.

Although LPA receptor-mediated phosphorylation of MAP kinase was impaired in RGS3 and RGS3T transfectants, G protein-coupled receptor activation of adenyl cyclase and PI-PLC was not impaired in these cells. This may reflect a specificity of RGS3 for G proteins in the Gi family, to which the LPA receptor couples to activate MAP kinase (16). This suggestion is consistent with the finding that RGS3 inhibited MAP kinase activation by the Gα13-coupled IL-8 receptor and reverses the pheromone supersensitivity phenotype of yeast sst2Δ mutants by actions on GPA1 (2), a yeast G protein highly homologous to Gαq and Gαi (20). What is clear from the present studies is that G-protein-coupled receptor activation of adenyl cyclase and PI-PLC is impaired considerably in cells transfected with a cdNA encoding the 206-amino acid truncated form of RGS3. These results show that the carboxyl-terminal 206 amino acids of RGS3 are sufficient for negative regulation of Gαq and Gαi-stimulated signaling. Our results also show that this region of RGS3 retains the functional activity of RGS3 to negatively regulate Gαq-mediated MAP kinase phosphorylation. This region encompasses the entire RGS domain of RGS3 and includes 59 amino acids amino-terminal to the RGS domain. Interestingly, RGS3 is by far the largest RGS family member described to date, whereas RGS3T compares favorably in size to other RGS family members, e.g. RGS4 is 205 amino acids, and RGS1, RGS2, RGS10, and GAIP range in size from 173 to 217 amino acids (5, 6, 21, 22). Despite these similarities in size, the sequence identities of these proteins range only from 15 to 33%. RGS3T lacks the amino-terminal 313 amino acids of RGS3 that encodes a hexapeptide repeat motif that overlaps four PEST sequences and an acidic region predicted to assume a coiled-coil configuration. An immediate question that must be raised is whether the amino-terminal domain of RGS3 imparts regulatory influences on the ability of the carboxyl-terminal domain to negatively regulate G protein signaling. Alternatively, it is possible that the lack of an effect of RGS3 on Gαq-mediated signaling could result from a reduced level of expression of RGS3 compared with RGS3T. This seems unlikely in part because LPA receptor-mediated phosphorylation of MAP kinase was impaired as efficiently in RGS3 transfectants as in RGS3T transfectants. However, it is certainly possible that the level of RGS3 expression required to inhibit Gα is less than that needed to inhibit Gαi or Gαq. Regardless of the ultimate conclusion concerning the functional significance of the amino-terminal portion of RGS3, it is clear that the carboxyl-terminal portion of RGS3 that includes the RGS domain comprises a protein domain that is capable of negatively regulating Gαq and Gαi-mediated signaling.

The preferential interaction of RGS proteins with members of the Gi family of G proteins is surprising in view of the diversity of RGS proteins. Whether a separate RGS protein family specific for other types of G proteins exists or whether the currently identified RGS proteins contain specificity determinants for other G proteins is a question of considerable interest. Here we provide the first evidence for regulatory effects of an RGS protein on signaling mediated by proteins other than Gi in intact cells. The finding that this functional activity is encoded by the approximate carboxyl-terminal half of RGS3 suggests that this domain of RGS3 constitutes both a G protein-interacting and negatively regulating domain of RGS3 for G proteins. Hopefully, these results will facilitate identification of the structural features involved in the specificity of RGS protein interactions with G proteins.

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