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Amino-terminal Cysteine Residues of RGS16 Are Required for Palmitoylation and Modulation of G_i- and G_q-mediated Signaling*

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RGS proteins (Regulators of G protein Signaling) are a recently discovered family of proteins that accelerate the GTPase activity of heterotrimeric G protein α subunits of the i, q, and 12 classes. The proteins share a homologous core domain but have divergent amino-terminal sequences that are the site of palmitoylation for RGS-GAIP and RGS4. We investigated the function of palmitoylation for RGS16, which shares conserved amino-terminal cysteines with RGS4 and RGS5. Mutation of cysteine residues at residues 2 and 12 blocked the incorporation of [^{3}H]palmitate into RGS16 in metabolic labeling studies of transfected cells or into purified RGS proteins in a cell-free palmitoylation assay. The purified RGS16 proteins with the cysteine mutations were still able to act as GTPase-activating protein for $G_i\alpha$. Inhibition or a decrease in palmitoylation did not significantly change the amount of protein that was membrane-associated. However, palmitoylation-defective RGS16 mutants demonstrated impaired ability to inhibit both G_i- and G_q-linked signaling pathways when expressed in HEK293T cells. These findings suggest that the amino-terminal region of RGS16 may affect the affinity of these proteins for Gα subunits *in vivo* or that palmitoylation localizes the RGS protein in close proximity to Gα subunits on cellular membranes.

RGS proteins[1] enhance the GTPase activity of heterotrimeric G protein α subunits to turn off signaling between cell-surface receptors and intracellular effectors (1). More than 20 mammalian RGS proteins have been identified by virtue of a common stretch of 125 amino acids termed the RGS box (2–3), which is a site of interaction of RGS proteins and $G_i\alpha$ subunits. RGS proteins bind and stabilize $G_i\alpha$ in its transition state (between GTP- and GDP-bound), thus lowering the energy barrier for GTP hydrolysis (4–6). Recently a group of proteins with a divergent RGS domain that act exclusively on $G_{12}\alpha$ and $G_{13}\alpha$ to accelerate their GTPase activity has been identified (7). Studies of reconstituted signaling pathways in cell lines, consisting of overexpressed or endogenous receptors, RGS proteins, effectors (in some cases), and endogenous G proteins, have shown that transiently expressed RGS proteins inhibit G protein-mediated signaling in these systems (3, 8–9).

A perplexing question is the regulation of these 20 RGS proteins because almost all of them have powerful enzymatic activity toward the same substrates, and many may be expressed in the same cell type. Temporal or stimulus-specific transcriptional regulation, protein turnover, or post-translational modifications represent possible means of controlling the availability of a particular RGS protein to preside over a G protein-coupled signaling event at the plasma membrane. One such modification, addressed in this report, is palmitoylation.

Palmitoylation, the reversible thioacylation of cysteine residues, occurs on several molecules involved in G protein-linked signaling pathways. G protein-coupled receptors incorporate [^{3}H]palmitate upon addition of ligand (10–11). Gα proteins of all four classes undergo palmitoylation at their amino termini (12–13). Receptor activation leads to palmitate turnover on $G_s\alpha$ and regulates its signaling (14–16). Palmitoylation may also be involved in targeting Gα subunits to the plasma membrane (17).

At least two RGS proteins, RGS4 and RGS-GAIP, undergo palmitoylation *in vivo.* RGS-GAIP, which contains an amino-terminal cluster of cysteines, was labeled with [^{3}H]palmitic acid in the membrane fraction of transfected COS-7 cells, but no functional significance of this finding was determined (18). RGS-GAIP was subsequently localized to the membranes of clathrin-coated vesicles and not the plasma membrane (19). RGS4, which shares conserved amino-terminal cysteines with RGS5 and RGS16 (Cys-2 and Cys-12), was palmitoylated on these residues in baculovirus-infected Sf9 insect cells (20). However, palmitoylation was not necessary for membrane attachment in yeast, and an RGS4 double cysteine mutant (Cys-2/Cys-12) was relatively functional in yeast cells as measured by its ability to inhibit pheromone-induced signaling (20).

RGS4 and RGS16 are highly related RGS proteins (55% identical in their RGS domains and 42% identical in their amino-terminal 30 amino acids) with similar GAP activity (21–22). RGS16 was originally termed RGSr because of its abundance in the murine retina (23). However, the human orthologue was renamed RGS16 as it is found in many tissues (24).

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[1] The abbreviations used are: RGS protein, Regulator of G protein Signaling; GAIP, Gα interacting protein; G protein, guanine nucleotide-binding protein; $G_i\alpha$ and $G_s\alpha$, G protein α subunits associated with adenylyl cyclase inhibition and stimulation, respectively; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; GAP, GTPase-activating protein; ERK, extracellular signal regulated kinase; CREB, cAMP response element-binding protein; HPLC, high performance liquid chromatography; MAP, mitogen-activated protein.

In this study, we show that RGS16 underwent palmitoylation *in vitro* and in mammalian cells. Mutation of putatively palmitoylated cysteine residues in RGS16 did not substantially alter their affinity for G proteins *in vitro* as measured by GAP activity or their membrane attachment, but mutants showed quantitative defects in their ability to inhibit G_i- and G_q-coupled signaling pathways *in vivo*. These results suggest that palmitoylation may be an important means of regulation of RGS activity in mammalian cells.

EXPERIMENTAL PROCEDURES

Plasmids, Proteins, and Antisera—For site-directed mutagenesis of murine RGS16, QuikChange (Stratagene), a polymerase chain reaction-based method, was performed except that *Pwo* polymerase (Roche Molecular Biochemicals) was used. The following primers were used: C2A, GCGTCCGCAACCATGGCCCGCACCCTAGCCAC, and C12A, ACCTTCCCCAACACCGCCCTGGAGAGAGCCAA. Mutations were confirmed using Applied Biosystems PRISM dye terminator cycle sequencing (Perkin-Elmer). Production of recombinant RGS16 was performed by inducing *Escherichia coli* strain BL21(DE3) containing pET15B-RGS16 with isopropyl β-D-thiogalactopyranoside as described (23). Following sonication, RGS16 was purified by affinity chromatography over a Ni^{2+}-nitrilotriacetic acid superflow column (Qiagen) and subsequent gel filtration over a Superose column (23). For production of recombinant RGS16 and mutants for GAP assays, mutations in the RGS16-coding region were generated by polymerase chain reaction as described previously (5) using pcDNA3-RGS16 as a template. These polymerase chain reaction fragments were digested with *Eco*RI and *Xho*I and subcloned in frame with glutathione *S*-transferase into the vector pGEX4T1 (Amersham Pharmacia Biotech). Plasmids were transformed into the BL21(DE3)pLysS bacterial strain, and protein production was induced from a 1-liter mid-log phase culture with 0.1 mM isopropyl β-D-thiogalactopyranoside for 3 h. Bacteria were pelleted and lysed by freeze-thaw and sonication. Clarified supernatants were purified by affinity chromatography on glutathione-agarose. The protein yield was >4 mg/liter culture. Of note, we did not detect any difference in the GAP activity of RGS16 with an amino-terminal His tag compared with an amino-terminal glutathione *S*-transferase tag at a concentration as low as 25 nM (data not shown). RGS16 antiserum (the generous gift of Carol Beadling and Kendall Smith, Cornell University Medical College) was raised against recombinant His-tagged human RGS16 (85% homology to mouse). Antiserum to murine RGS16 was generated by injecting RGS16 protein and complete Freund's adjuvant into New Zealand White rabbits (Cocalico Biologicals). Crude antisera (CT-265) were affinity purified with RGS16-coupled CNBr-agarose. Antibody elution was performed with 100 mM glycine at pH 2.5 and was kept at 4 °C in 50% $(NH_4)_2SO_4$ in phosphate-buffered saline, pH 7.4. As shown in Figs. 1*C*, 4, and 5, these antisera detected only transfected RGS16 (as opposed to other similarly sized, endogenous RGSs such as GAIP or RGS2) in HEK293T cells used for signaling assays. The plasmid encoding rat $G_i\alpha_1$ and the affinity purified antibody that recognizes $G_i\alpha_1$ have been described previously (25).

Transfection, Metabolic Labeling with [^{3}H]Palmitate, and Cell Fractionation—COS-7 cells were grown to subconfluency in 75-cm^2 tissue culture flasks in complete DMEM supplemented with 10% fetal bovine serum and transfected using 5 μg/flask plasmid DNA and the DEAE-dextran method, as described (26). HEK293T cells were maintained in complete DMEM and transfected using the calcium phosphate method. Two days after transfection, cells were incubated for 2 h in serum-free DMEM and then incubated in serum-free media containing 1% dimethyl sulfoxide and 500 μCi/ml [^{3}H]palmitic acid (American Radiolabeled Chemicals, specific activity 60 Ci/mmol) for 1 h. Cells were harvested by scraping in 45 ml of ice-cold phosphate-buffered saline and centrifuged at 500 × *g* for 10 min. The cell pellets were stored at −70 °C and then lysed, homogenized, and fractionated into particulate and soluble fractions by centrifugation at 125,000 × *g* for 1 h as described before (27).

Immunoprecipitation, Immunoblotting, and Hydroxylamine Treatment—400 μg of tritium-labeled protein from the particulate fraction was immunoprecipitated with RGS16 antiserum in 500 μl of solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100 (v/v), 0.2% SDS (w/v) and 1 mM EDTA) overnight at 4 °C. The immune complexes were purified by using protein A-Sepharose, as described previously (25). The proteins were separated by SDS-PAGE using 12% and gradient 8–16% Tris glycine polyacrylamide gels (Novex). Duplicate gels containing the tritium-labeled immunoprecipitates were treated either in 1 M Tris-HCl, pH 7.0, or 1 M hydroxylamine, pH 7.0, for 1 h at room temperature with gentle shaking. The gels were then prepared for indirect fluorography (25). For immunoblots, 40 μg of protein from each particulate and soluble fraction was separated by SDS-PAGE and transferred to nitrocellulose paper. The proteins were detected with the CT-265 antisera (1:4000 dilution) and enhanced chemiluminescence (Amersham Pharmacia Biotech) as described by the manufacturer.

Identification of [^{3}H]Palmitate Incorporation by HPLC—Immunoprecipitates of tritium-labeled proteins were separated by SDS-PAGE, and a band was excised at 17–22 kDa for RGS16 and 30–42 kDa for $G_i\alpha_1$. Fatty acids were cleaved from the proteins in the gel slices by base treatment and were extracted into chloroform/methanol, using the method of Linder *et al*. (28). The extracted fatty acids were dried under nitrogen and dissolved in chloroform/methanol (2:1, v/v) with unlabeled palmitic acid as carrier. The released fatty acids were separated by reversed phase HPLC, using a C_8 column (Bakerbond wide pore octyl, 4.6 × 250 mm). The column was equilibrated with water, 0.1% trifluoroacetic acid, 60% acetonitrile at a flow rate of 1 ml/min, and the sample was applied. After 2 min, the column was eluted in a linear gradient from 60 to 80% acetonitrile in 30 min. Fatty acid standards (Sigma) were monitored by absorbance at 210 nm. Fractions were collected every 30 s, and the radioactivity was determined by scintillation counting.

Cell-free Palmitoylation Assay—Cell-free palmitoylation was performed as described by Caron (29) with modifications to optimize for palmitoylation of RGS proteins. RGS protein (0.28 mg/ml) was incubated at 37 °C in incubation buffer (20 mM Tris, pH 8.0, 0.3 M NaCl, 1 mM EGTA, 10 mM β-mercaptoethanol) with rat liver membrane extract (0.35 mg/ml), 0.2 mM CoA, 2 mM ATP, and [9,10-^{3}H]palmitate (180 μCi/ml, NEN Life Science Products, specific activity 52 Ci/mmol), in the presence of protease inhibitors (pepstatin, 2 μg/ml; leupeptin, 2 μg/ml). At different times, samples were frozen in a dry ice/ethanol bath and stored at −80 °C for further analysis. Alternatively, the reaction was stopped by addition of Laemmli sample buffer, and proteins were subjected to one-dimensional PAGE using 10 mM dithiothreitol as the reducing agent. Gels were stained with Coomassie Blue, photographed, and prepared for fluorography with Amplify (Amersham Pharmacia Biotech) and exposed to Kodak X-Omat AR film at −70 °C.

GAP Assays—Single turnover GTPase assays were performed as described previously (5). The amount of labeled inorganic phosphate released by 200 nM myristoylated $G_i\alpha_1$ (Calbiochem) loaded with 1 μM [^{32}P]GTP (40–60 cpm/fmol) for 20 min at 30 °C was monitored after equilibration at 4 °C and subsequent addition of $MgSO_4$ (final 10 mM), unlabeled substrate (100 μM GTP), and RGS protein. Ten aliquots were withdrawn over 2 min, added to activated charcoal, and centrifuged, and the radioactivity in the supernatants was determined by liquid scintillation counting. The first point was set arbitrarily at 0, and the amount of inorganic phosphate in the supernatant was calculated by subtracting the zero time point value. The initial rate of GTP hydrolysis was determined by a linear regression curve fitting these time points. Rates in the absence of RGS protein averaged 0.3/min, and in the presence of RGS16 averaged 1.5–6/min (depending on RGS concentration).

Signaling Assays—To determine the activity of a G_i-linked pathway, cAMP was measured in response to stimulation of endogenous β-adrenergic and somatostatin receptors in transfected HEK293T cells. One day after transfection, cells were transferred to 12-well plates and incubated overnight in media containing 2 μCi/ml [^{3}H]adenine. The cells were incubated for 30 min in HEPES/DMEM and then incubated in HEPES/DMEM containing 1 mM 1-methyl-3-isobutylxanthine alone or with 5 μM isoproterenol or 5 μM isoproterenol and 1 μM somatostatin (Sigma) for 20 min. The reaction was stopped by aspirating the medium and adding 1 ml of stopping buffer (0.2% (w/v) SDS, 50 mM Tris, pH 7.4, and 1 mM cAMP). The cAMP was then separated by sequential chromatography over Dowex and alumina columns as described previously (30). The cAMP accumulation was calculated as ([^{3}H]cAMP cpm/([^{3}H]cAMP cpm + [^{3}H]ATP cpm)) × 10^3.

For evaluation of G_q-mediated signaling, two methods were employed. First, hormone-stimulated CREB activity of transfected m1 muscarinic receptors (1 μg, the gift of J. Silvio Gutkind, NIDR, National Institutes of Health) was measured in HEK293T cells transfected with a CREB-β-galactosidase reporter plasmid (1 μg, the gift of Roger Cone, Oregon Health Sciences University) and 4 μg of empty vector or RGS16 expression plasmids for 12 h. Cells were serum-starved in DMEM, 0.1% bovine serum albumin for an additional 24 h before the addition of 1 mM carbachol (Sigma) for 6 h. Cell extracts were prepared by lysis in 50 μl of reagent lysis buffer (Promega), a freeze-thaw cycle, and centrifuga-

tion. 10 μl of the supernatant was incubated in diluted β-galactosidase substrate (Galacton, Tropix). Luminescence was measured using a Monolight 3010 Luminometer (Analytical Luminescence Laboratory). Second, endogenous MAP kinase activity was measured in these cells after carbachol stimulation. The cells were transfected with 1 μg of a plasmid containing the cDNA for the m1 muscarinic receptor and 5 μg vector or RGS16 expression plasmids using LipofectAMINE for 12 h (Life Technologies, Inc.). After an additional 24 h of serum starvation, cells were stimulated with 1 mM carbachol for 7 min and lysed in kinase lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1% Triton X-100). Following centrifugation, equal amounts of supernatant protein (50 μg) were resolved on 12% SDS gels and transferred to nitrocellulose. Endogenous mitogen-activated protein (MAP) kinase activity was detected with an antibody specific for the tyrosine 204-phosphorylated form of ERK1 and -2 (Santa Cruz Biotechnology) diluted 1:300. Parallel lysates were run in the same manner, and the blot was probed with an antibody reactive to ERK1 and -2 (Santa Cruz Biotechnology) at a 1:500 dilution, to ensure that equal levels of ERK protein were present in each sample. The relative activity of MAP kinase compared with unstimulated cells was determined by densitometry of bands using IP Lab Spectrum software (Eastman Kodak Co.).

For evaluation of $G_q\alpha$(Q209L) activity, 1 μg of this expression plasmid (the gift of J. Silvio Gutkind) was co-transfected with 1 μg of pLuc-CRE reporter plasmid (Stratagene) with or without RGS16 expression plasmids for 48 h. Cells were incubated in serum-free media for 24 h before extraction. Luciferase activity was determined by adding 50 μl of luciferase substrate (Promega) to 10 μl of cell extract and measuring luminescence as before. To normalize for transfection variations, 250 ng of cytomegalovirus-β-galactosidase plasmid was co-transfected in each sample and the β-galactosidase activity determined.

Miscellaneous—Statisical analysis was performed using the SigmaPlot software. The HelicalWheel, PeptideStructure, and LineUp programs of GCG Software (55) were used for analysis of protein secondary structure and homology.

RESULTS

Palmitoylation of RGS16—We investigated whether RGS16 underwent palmitoylation by expressing this protein in COS cells followed by metabolic labeling with [^{3}H]palmitate and immunoprecipitation of the particulate fraction with affinity purified CT-265, raised against RGS16. Tritium incorporation was seen in a 23-kDa band that corresponds to the predicted molecular weight of RGS16 (Fig. 1*A*). The incorporation of tritium was analyzed further by treating gels that contain immunoprecipitates of tritium-labeled RGS16 with either 1 M Tris-HCl, pH 7, or 1 M hydroxylamine, pH 7, which breaks thioester bonds. Hydroxylamine treatment released the tritium signal, indicating that the modification was through a thioester linkage (data not shown). We also analyzed the tritium-labeled compound by treating immunoprecipitates of tritium-labeled RGS16 under alkaline conditions to release lipids followed by separation by high performance liquid chromatography (HPLC) with a reverse phase C_8 column. A protein that is known to undergo palmitoylation, $G_i\alpha_1$, was used as a control. For both proteins, the tritium counts eluted with a peak at 13 min, near the palmitate standard elution time of 13.3 min (Fig. 2). A small peak at 19.5 min eluted after our last standard of stearic acid (C18) and may represent a longer chain fatty acid. These results show that RGS16 undergoes palmitoylation.

We also analyzed palmitoylation of RGS16 by performing an *in vitro* palmitoylation assay on purified proteins (Fig. 1*B*). Incubation of a rat liver membrane extract, [^{3}H]palmitate, coenzyme A, and ATP with purified RGS16 led to incorporation of tritium into proteins with a relative molecular mass of 23 kDa.

We mutated two cysteine residues in the amino terminus either singly or together because they were the likely sites of palmitoylation for this protein (Table I). Tritium incorporation was decreased in both of the single mutants, C2A and C12A, with a greater decrease for the C12A mutant (Fig. 1*A*). The double mutant (C2A/C12A) mutant showed only an insignificant amount of tritium incorporation that might have been due to intracellular conversion of palmitate to tritium-labeled amino acids. This result indicates that Cys-2 and Cys-12 are critical for palmitoylation of RGS16 and are the likely sites of the modification.

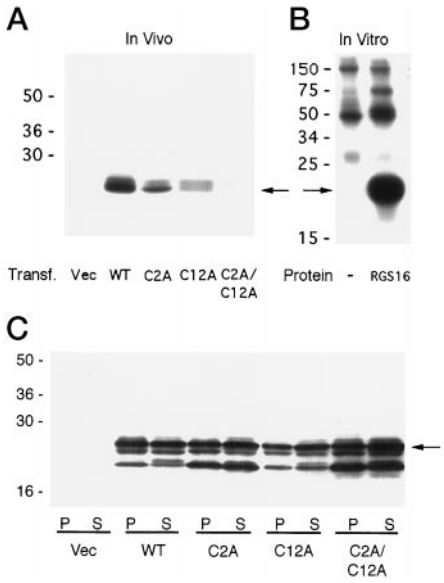

FIG. 1. **Palmitoylation and membrane localization of RGS16.** *A,* for *in vivo* studies, COS cells transfected with vector alone or with cDNAs for the wild-type RGS16 or the indicated cysteine mutants were metabolically labeled with [^{3}H]palmitate and then homogenized and separated into particulate and soluble fractions by centrifugation. The particulate fraction was immunoprecipitated with a polyclonal antibody to RGS16, followed by separation on SDS-PAGE on 12% acrylamide gels and fluorography. The gels were exposed to Kodak MS film for 13 days at −70 °C. *B,* for *in vitro* palmitoylation, the purified RGS16 protein was incubated with [^{3}H]palmitate, ATP, CoA, and a rat liver membrane extract at 30 °C for 4 h. Aliquots were separated by SDS-PAGE and analyzed by fluorography and exposed to Kodak X-Omat AR film for 1 day. *C,* HEK293T cells were transfected with vector alone or cDNAs for wild-type RGS16 and the cysteine mutants and separated into particulate and soluble fractions. 40 μg of protein from each fraction was separated on by SDS-PAGE and transferred to nitrocellulose paper. Immunoblotting was performed with CT-265 and the Amersham Pharmacia Biotech ECL kit for detection. Molecular mass markers in kDa are shown to the *left* of each figure. The *arrows* indicate RGS16.

Membrane Localization of Palmitoylation-defective Mutants—RGS16 was found in both the particulate and soluble fractions after expression in HEK293 cells with about one-half of the protein in the particulate fraction (Fig. 1*C*). Mutation of Cys-2 and Cys-12 altered this distribution so that slightly more of the protein was distributed to the soluble fraction. However, a significant amount of protein was still found in the particulate fraction after a mutation that prevented palmitoylation. This result indicates that palmitoylation was not critical for the membrane attachment of this protein.

GAP Activity—The stoichiometry of palmitate incorporation into recombinant RGS16 by the *in vitro* palmitoylation method was too low to determine differences in enzymatic activity between the palmitoylated and nonpalmitoylated forms of the protein. Therefore, we tested whether the cysteine mutations themselves, rather than palmitoylation, might affect the GAP activity of RGS16 by determining the ability of the cysteine mutants to accelerate GTP hydrolysis by $G_i\alpha$ (Fig. 3). The GAP activity of RGS proteins is also the most sensitive measure of their affinity for Gα subunits because they bind with high affinity to the transition state conformation of $G_i\alpha$ subunits and stabilize it during GTP hydrolysis rather than contributing catalytic residues directly (6). The enzymatic activity of various RGS4 mutants was directly proportional to the degree of sub-

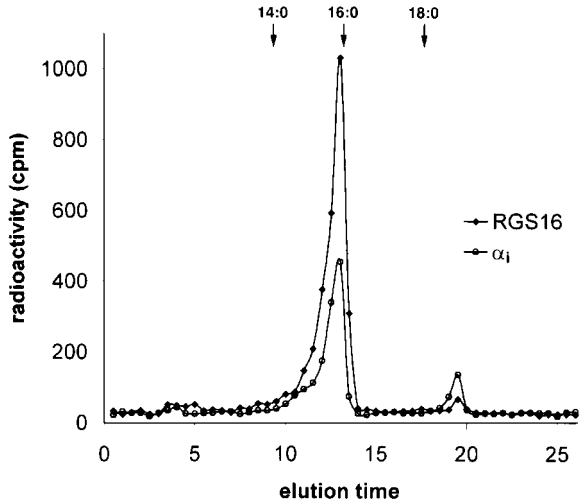

FIG. 2. **HPLC analysis of the released ^{3}H-labeled fatty acid.** COS cells were transfected with wild-type RGS16 or $G_i\alpha_1$ and metabolically labeled with [^{3}H]palmitate. The cells were homogenized and separated into particulate and soluble fractions, and immunoprecipitation was performed on the particulate fractions. The samples were separated by SDS-PAGE, and bands corresponding to the molecular weight region of the proteins were excised from the gel. Gel bands were treated with base to release the fatty acids, and extracted fatty acids were applied to a reverse phase C_8 column for HPLC. Fractions were collected every 30 s, and radioactivity was determined by scintillation counting. Elution times of myristate, C14:0, palmitate, C16:0, and stearate, C18:0 standards are shown.

strate binding in two previous studies (4, 5). Rates of single turnover hydrolysis by $G_i\alpha_1$ in the presence of a range of RGS protein concentrations revealed no significant differences in GAP activity between the wild-type protein and cysteine mutants (Fig. 3). The rate of GTP hydrolysis by $G_i\alpha_1$ in the presence of the double cysteine RGS16 mutant at various concentrations (Fig. 3*B*) varied from 88 to 102% of the control rate (wild-type RGS16). Although we cannot rule out subtle effects of the cysteine mutations themselves, no significant trend in the defects was present. At least for this assay with soluble RGS16 proteins, cysteines 2 and 12 did not contribute significant contact points to the RGS-$G_i\alpha_1$ interface.

Amino-terminal Cysteines Were Essential for Full in Vivo Activity of RGS16—We tested a G_i-linked pathway to evaluate the function of RGS16 palmitoylation. HEK293T cells that contain endogenous somatostatin and β-adrenergic receptors were transiently transfected with plasmids containing the wild-type and palmitoylation-defective RGS16 proteins. The somatostatin receptor activates $G_i\alpha$, leading to inhibition of adenylyl cyclase. Isoproterenol, acting through the β-adrenergic receptor and $G_s\alpha$, results in stimulation of adenylyl cyclase and cAMP accumulation within the cell. Addition of somatostatin and isoproterenol leads to a lower cAMP response than treatment with isoproterenol alone (Fig. 4). Somatostatin was less effective in decreasing the cAMP accumulation in response to isoproterenol in cells transfected with the wild-type RGS16 compared with the vector-transfected cells. We saw some decrease in the cAMP accumulation because with transient transfection not all cells are expressing the RGS protein. This result is consistent with previous findings (8) and biochemical data that show that RGS16 can increase the GTP hydrolysis of $G_i\alpha$ family proteins (22). cAMP accumulation in response to isoproterenol and somatostatin in cells expressing the nonpalmitoylated C2A/C12A mutant was similar to the vector-transfected cells. The single cysteine mutants showed an intermediate response. Immunoblotting performed on the detergent-soluble fraction of whole cell lysates from these transfections demonstrated that wild-type and cysteine mutants were expressed at similar levels (and thus were not likely to be aggregated as a result of the various mutations) (Fig. 4, *inset*). In these cells, the palmitoylation-deficient mutants were defective in their ability to turn off a G_i-linked signaling pathway.

We investigated the ability of the wild-type RGS16 and its cysteine mutants to inhibit a G_q-linked signaling pathway using two different methods. First, we measured the activity of endogenous MAP kinase as reflected by phosphorylation of ERK1–2 after carbachol stimulation in HEK293T cells transfected with the m1 muscarinic receptor and RGS plasmids (32). Phosphorylated ERK was detected by an antibody specific for that form of the protein (Fig. 5*A*). We observed a nearly 5-fold increase in MAP kinase activity in hormone-stimulated cells (Fig. 5, *A* and *B*). Expression of wild-type RGS16 blocked activation to almost 25% of the activity of the vector-transfected cells. In contrast, MAP kinase activity in the presence of the RGS16 cysteine mutants was significantly different than with wild-type RGS16, with activities 60–80% of vector-transfected cells. The samples contained similar amounts of ERK1 and -2 and RGS proteins as detected by immunoblotting with the appropriate antibodies (Fig. 5*A, middle* and *bottom panels*).

In a second assay of G_q-mediated signaling, we measured the activity of a CREB-β-galactosidase reporter gene after stimulation with carbachol in cells expressing the m1 muscarinic receptor and the RGS16 proteins. The CREB-β-galactosidase construct reflects activation of CREB resulting from an increase in intracellular Ca^{2+} or cAMP (33). We observed an approximately 6.5-fold increase in reporter gene expression in cells stimulated with carbachol, whereas expression of wild-type RGS16 blunted the response by about 50% (Fig. 5*C*). In contrast, β-galactosidase activity in cells transfected with RGS16 single or double cysteine mutants was similar to vector-transfected cells exposed to carbachol. Similar results were seen with a double cysteine RGS4 mutant, but disparate expression levels compared with the wild-type protein made quantitative analysis difficult. Thus, two independent assays revealed that amino-terminal cysteine residues on RGS16 were critical for its regulation of G_q-mediated signaling.

We tested the function of the acylation-deficient RGS16 mutants, independent of GAP activity, by co-expression with a constitutively active $G_q\alpha$ mutant (Q209L). The Gln to Leu mutation leads to a constant GTP-bound state by virtue of its deficient intrinsic GTPase activity (34). In HEK293T cells transfected with $G_q\alpha$(Q209L) and a reporter gene (a CREB-dependent luciferase gene in these assays), we observed an almost 20-fold increase in activity compared with cells transfected with reporter alone (Fig. 5*D*). In cells expressing $G_q\alpha$(Q209L) and wild-type RGS16, however, the response was inhibited some 66%. Surprisingly, mutation of Cys-2 had little effect on the ability of RGS16 to inhibit $G_q\alpha$(Q209L)-induced CREB activation, whereas the Cys-12 mutant-transfected cells responded similarly to vector-transfected cells. Cells transfected with the double cysteine mutant showed an intermediate defect. This result suggests that palmitoylation may also affect the ability of RGS proteins to act as effector antagonists for $G_q\alpha$.

DISCUSSION

RGS proteins regulate G protein signaling by increasing the GTPase activity of heterotrimeric G protein α subunits, thus augmenting deactivation. Amino-terminal cysteines of RGS16 were critical for its palmitoylation and regulation of signaling in mammalian cells. Mutations that prevented palmitoylation did not significantly alter GAP activity *in vitro* or subcellular localization but did substantially decrease the RGS activity on G_q- and G_i-linked pathways *in vivo*.

TABLE I
Amino termini of RGS4 and RGS16

Protein	1	10	20	30	40	50
RGS16	M**C**RTLATFP	NT**C**LERAKEF	KTRLGIFLHK	SELSSDTGGI	SKFEWASKHN	KER[a]
RGS4	MCKGLAGLP	ASCLRSAKDM	KHRLGFLLQK	SDSCEHSSSH	SKKDKV VT	CQR
RGS5	MCKGLAALP	HSCLERAKEI	KIKLGILLQK	PDSAVDLVIP	YNEKPEKPAN	GHK
Consensus	MCKGLA-LP	-SCLERAKE-	K-RLGILLQK	SDS--D----	SK-------N	--R[b]

[a] The cysteine residues that were mutated to alanines are in boldface.
[b] The consensus sequence was determined using the GCG software Lineup program for rat RGS4, mouse RGS16, and mouse RGS5. The dashes indicate no conserved residues at that position.

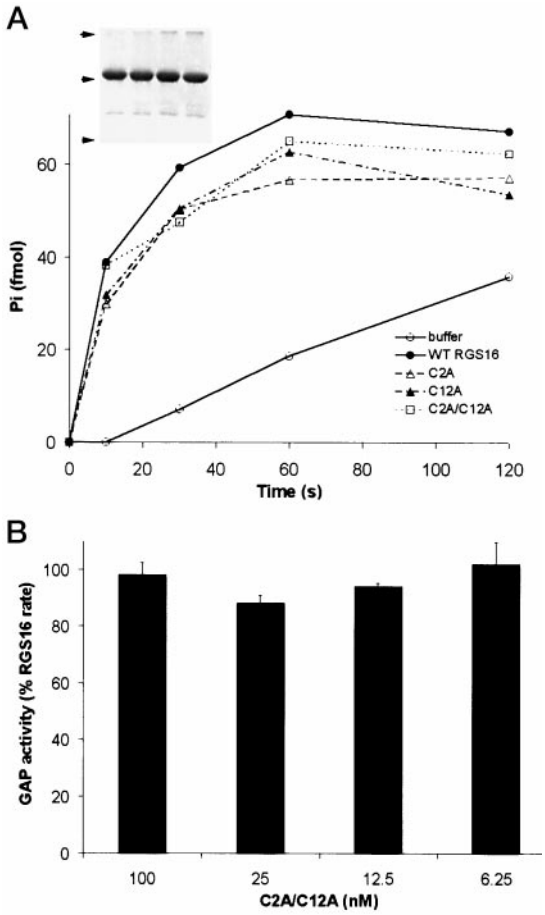

FIG. 3. **GAP activity of RGS16 mutants.** GAP activity of RGS16 was monitored over 2 min as described under "Experimental Procedures." *A,* typical time course of GTP hydrolysis by $G_i\alpha_1$ alone (*open circles*) or in the presence of 6.25 nM RGS16 (wild-type, *closed circles*; C2A, *open triangles*; C12A, *closed triangles*; C2A/C12A, *rectangles*). The *graph* is representative of two such experiments. The *inset* shows a Coomassie Blue-stained SDS gel of glutathione *S*-transferase-RGS16 proteins used in these studies. Molecular mass markers (kDa) are indicated on the *left* (from *top* 64, 52, and 26). *B,* GAP activity of the C2A/C12A mutant relative to wild-type activity at various concentrations. The initial rate of GTP hydrolysis by $G_i\alpha_1$ was 1.5–6/min depending on the RGS16 concentration. *Bar graph* shows the mean ± S.E. of three determinations expressed as the percentage of the wild-type rate.

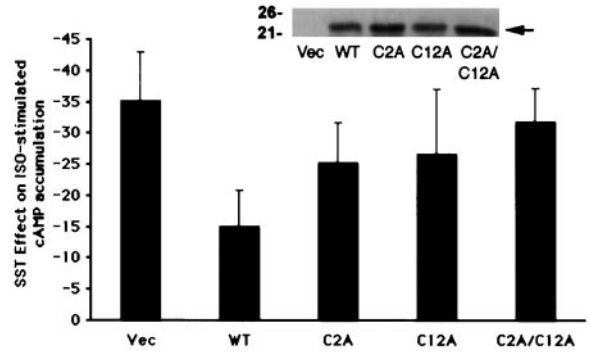

FIG. 4. **Effects of palmitoylation-defective RGS mutants on G_i-mediated signaling.** HEK293T cells expressing RGS16 mutants after transient transfection were transferred to 12-well plates and then incubated with [^{3}H]adenine. Cells were treated with 1 mM 1-methyl-3-isobutylxanthine alone or with 5 μM isoproterenol (*ISO*) or 5 μM isoproterenol and 1 μM somatostatin (*SST*) for 20 min, and cAMP accumulation was determined. The decrease in cAMP accumulation in the presence of somatostatin was calculated from the cAMP accumulation with isoproterenol and somatostatin compared with cAMP accumulation of isoproterenol alone expressed as a percentage. The cAMP accumulation with isoproterenol alone was about 10-fold greater than the control condition for each of the transfectants. The values shown are the mean ± S.E. of five experiments performed in triplicate ($p = 0.008$ for wild-type (*WT*) *versus* vector (*Vec*) control; $p < 0.05$ for wild-type *versus* C2A/C12A mutant using Student's *t* test). *Inset,* an immunoblot of 40 μg of protein from a detergent extract (1% v/v Triton X-100) of whole cells using an antibody raised against human RGS16.

Palmitoylation has previously been found on RGS-GAIP and RGS4 (18, 20). These two proteins may represent prototypes of distinct forms of RGS palmitoylation. RGS-GAIP and two other RGS proteins, Ret-RGS1 and RGSZ1, contain a cluster of cysteines analogous to cysteine string proteins (35–37). Palmitoylation occurs on the cysteine clusters of cysteine string proteins, and this modification may be involved in attaching these proteins to synaptic vesicle membranes (38–39). RGS-GAIP incorporates [^{3}H]palmitate and displays properties of tight membrane association consistent with palmitoylation (18). Although RGS-GAIP has been localized on clathrin-coated vesicles, the role of palmitoylation in the targeting or function of this protein is not known (19).

In contrast to RGS proteins with a cysteine string, RGS16, RGS4, and RGS5 have only 2–3 cysteine residues outside the RGS box, at their amino terminus (20). The amino termini of these proteins are conserved with cysteines at residues 2 and 12 for all three (Table I). Structural modeling of a consensus sequence of the amino terminus predicts that it forms an amphipathic α helix with Cys-2 and Cys-12 on the hydrophobic face. Palmitoylation may aid the membrane attachment of the protein, but other regions of the protein must be involved because the absence of palmitoylation on RGS16 does not preclude its retention at the membrane. Palmitoylation may orient an α helix or possibly another conformation toward the membrane and facilitate interactions between this region of the RGS protein and another membrane-bound protein that recognizes the conserved residues. Without palmitate, the protein interactions may be adequate for attachment but inadequate for functional interactions. The possibility of another protein that targets RGS proteins to the membrane is suggested by a recent study (40). We found that expression of a constitutively active mutant, $G_i\alpha_2$(Q205L), results in translocation of cytosolic RGS4 to the plasma membrane (40). Paradoxically, RGS proteins do not directly interact with a similar $G_i\alpha_1$ mutant (Q204L) (31). We also found that an RGS4 mutant (L159F), which does not bind $G_i\alpha$ in any nucleotide-binding state, translocated as well as the wild-type RGS4 protein. Thus, the mechanism whereby a protein-protein interaction may target an

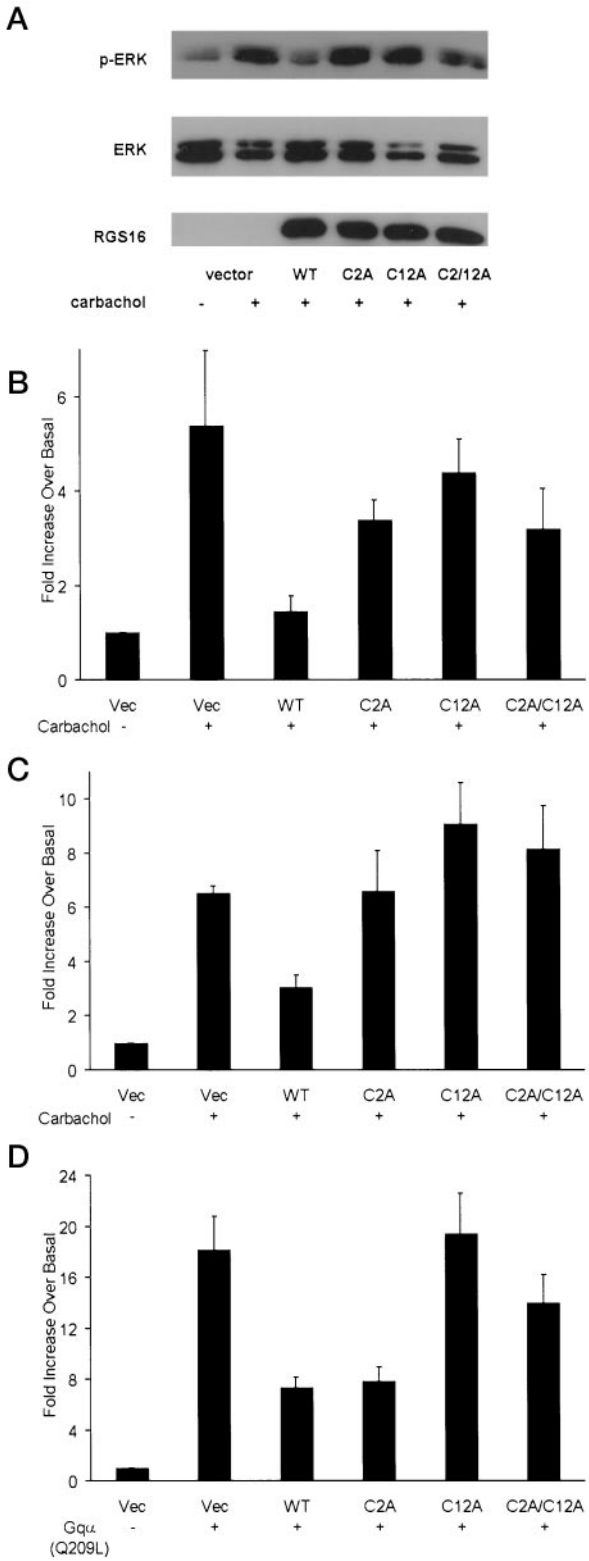

FIG. 5. **RGS16 cysteine mutants are defective in modulating G_q-coupled signaling.** *A* and *B,* endogenous MAP kinase activity. HEK293T cells transfected with an m1 muscarinic receptor plasmid and vector alone or with the cDNA for the wild-type RGS16 or the cysteine mutants. After 24 h of serum starvation, the cells were stimulated with 1 mM carbachol for 7 min and lysed. *A,* equal amounts of protein were separated on SDS-PAGE gels and immunoblotted for phospho-ERK1–2 (*top*), total ERK1–2 (*middle*), and RGS16 (*bottom*). *B, bar graph* showing mean ± S.E. from three independent experiments of the fold increase in MAP kinase activity of stimulated cells over unstimulated cells (*lane 1*) as determined by densitometry. *C,* CREB activity. HEK293T cells were transiently transfected with the m1 receptor, CREB-β-galactosidase and wild-type or mutant RGS16 plasmids, or empty vector as indicated. 48 h after transfection and serum starvation, cells were stimulated with 1 mM carbachol for an additional 6 h. Cell extracts were prepared and β-galactosidase activity measured as described under "Experimental Procedures." *Bar graphs* represent the fold increase in activity over unstimulated cells (mean ± S.E. of three independent experiments). *D,* CREB activity with expression of a constitutively active $G_q\alpha$. HEK293T cells were transfected with plasmids encoding a CREB-Luciferase reporter, $G_q\alpha$(Q209L) (all except *lane 1*), and cDNAs for RGS16 (wild-type or mutants) or empty vector as shown. After 48 h of serum starvation, cell extracts were prepared, and the luciferase activity therein was measured as described. *Bar graphs* represent the fold increase in activity over vector-transfected cells (mean ± S.E. of three independent experiments).

RGS to the membrane is unclear at present.

The conserved RGS box alone is sufficient for GAP activity *in vitro* (4, 41), but it is not sufficient for *in vivo* function (42, 43). Although the RGS box is the primary site of RGS4 interaction with $G_i\alpha_1$ based on the crystal structure, this structure included neither the 50 amino-terminal residues of RGS4 nor the 5 amino-terminal residues of $G_i\alpha_1$, so it is not known if there are other sites of interaction (6). Palmitoylation of Gα subunits, which occurs on cysteine 3 for $G_i\alpha$ family members, inhibited the GAP activity of RGS proteins (44), indicating the importance of regions not seen in the crystal structure for the interaction between these proteins. Similarly, De Vries *et al.* (45) found that deletion of the 10 most carboxyl-terminal residues of $G_i\alpha_3$ markedly diminished its interaction with RGS-GAIP in a yeast two-hybrid assay. Recently published studies (43, 46) show that RGS proteins may work not only through RGS and G protein interactions but also through interactions with the receptor complex, with the amino terminus of RGS proteins imparting the receptor selectivity. A peptide consisting of the first 33 residues of RGS4 blocked carbachol-dependent but not cholecystokinin-dependent signaling, both of which are mediated by G_q proteins (43). Interestingly, a peptide with the cysteines at positions 2, 12, and 33 replaced by alanines was 100-fold less effective (43). Palmitoylation and tethering of the RGS protein to the membrane could establish a protein conformation critical for interactions with both G proteins and receptors.

Our results with the constitutively active $G_q\alpha$(Q209L) mutant suggest that palmitoylation may alter another function of RGS proteins. Inhibition of CREB activation induced by $G_q\alpha$(Q209L) is probably through effector antagonism because RGS proteins do not interact with or stimulate the GTPase activity of a similar $G_i\alpha$ mutant ($G_i\alpha_1$Q204L) (31). Both RGS4 and RGS-GAIP have been shown to block the interaction between $G_q\alpha$-GTP and its effector, phospholipase Cβ (47). In addition, a peptide with the amino-terminal sequence of RGS4 could block the interactions of $G_q\alpha$ and phospholipase Cβ (45). The reason for the partial function of the double mutant in our assays is unclear; perhaps the Cys-2 mutation actually enhances interaction with the $G_q\alpha$ protein in this GTP-bound conformation and serves to counteract the effect of the Cys-12 mutation. In fact, we cannot exclude structural differences of these mutants *in vivo* (independent of lipid modification) even though the cysteine mutations did not affect the catalytic power of RGS16 *in vitro.* For $G_q\alpha$, amino-terminal cysteine residues are important for coupling to the effector, phospholipase Cβ, independent of their palmitoylation (48). For RGS proteins, these cysteine residues may directly interact with Gα proteins because neither the stoichiometry of palmitoylated to unpalmitoylated protein nor the kinetics of palmitate turnover on RGS proteins during the GTPase cycle is known.

Palmitoylation of RGS may also regulate Gα interactions independently of its effect on the protein-protein interface simply by targeting RGS proteins to membrane microdomains that

also hold G_{α} subunits and possibly other signaling components. Receptors, Gα subunits, and RGS proteins all undergo palmitoylation. One type of microdomain has been characterized by its detergent insolubility, low density on centrifugation, and enrichment of cholesterol and sphingolipids (49). These cholesterol/sphingolipid rafts have a high affinity for saturated, long chain fatty acids such as palmitate (50), and many proteins found in rafts are palmitoylated (51). Cysteine residues that undergo palmitoylation on endothelial nitric-oxide synthase, $p56^{lck}$, and GAP-43 are critical for targeting those proteins to these membrane domains (52–54). Concentration of these low abundance proteins to small areas within the membrane may be necessary for their action within cells. These domains may differ between mammalian cells and yeast cells to explain the results of Srinivasa and colleagues (20), who found relatively preserved activity and plasma membrane localization of palmitoylation-defective RGS4 in yeast.

The regulation of RGS proteins in cells is poorly understood. Their powerful catalytic activities suggest that they must be carefully regulated. Transcriptional control is likely to represent one means of modulating RGS expression (3), but the reversible nature of palmitoylation would also allow it to control the function of the protein. Palmitate on Gα turns over upon activation (14–16) and inhibits RGS activity (44). Likewise, palmitoylation, with its inherent reversibility, may prove to harness the action of RGS proteins on Gα subunits.

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