Palmitoylation of a Conserved Cysteine in the Regulator of G Protein Signaling (RGS) Domain Modulates the GTPase-activating Activity of RGS4 and RGS10*

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RGS4 and RGS10 expressed in Sf9 cells are palmitoylated at a conserved Cys residue (Cys95 in RGS4, Cys66 in RGS10) in the regulator of G protein signaling (RGS) domain that is also autopalmitoylated when the purified proteins are incubated with palmitoyl-CoA. RGS4 also autopalmitoylates at a previously identified cellular palmitoylation site, either Cys2 or Cys12. The C2A/C12A mutation essentially eliminates both autopalmitoylation and cellular [3H]palmitate labeling of Cys95. Membrane-bound RGS4 is palmitoylated both at Cys95 and Cys212, but cytosolic RGS4 is not palmitoylated. RGS4 and RGS10 are GTPase-activating proteins (GAPs) for the G,Gi, and Gq families of G proteins. Palmitoylation of Cys95 on RGS4 or Cys66 on RGS10 inhibits GAP activity 80–100% toward either Goα or Goα in a single-turnover, solution-based assay. In contrast, when GAP activity was assayed as acceleration of steady-state GTPase in receptor-G protein proteoliposomes, palmitoylation of RGS10 potentiated GAP activity ≥20-fold. Palmitoylation near the N terminus of C95V RGS4 did not alter GAP activity toward soluble Goα and increased Goα GAP activity about 2-fold in the vesicle-based assay. Dual palmitoylation of wild-type RGS4 remained inhibitory. RGS protein palmitoylation is thus multi-site, complex in its control, and either inhibitory or stimulatory depending on the RGS protein and its sites of palmitoylation.

Palmitoylation is increasingly recognized as a frequent and important modification of eukaryotic signaling proteins. Palmitoylated proteins include G protein α subunits and monomeric GTP-binding proteins (e.g., p21ras), G protein-coupled receptors, RGS1 proteins, and protein kinases. Unlike prenylation and myristoylation, palmitoylation is reversible, allowing for its regulation. Protein-bound palmitate turns over continuously in cells (see Ref. 1 for review).

Knowledge of the mechanism and control of protein palmitoylation remain rudimentary. Depalmitoylation may be the rate-limiting event in palmitate turnover. Duncan and Gilman (2) recently identified a palmitoyl-protein thioesterase that is probably the enzyme that depalmitoylates Go subunits and which may also depalmitoylate other proteins. The enzyme that transfers palmitate to proteins has not been clearly identified. Dunphy et al. (3) partially purified an activity from hepatic membranes that accelerates palmitoylation of Go using Pal-CoA as donor, potentially a protein-palmitoyl transferase. However, Go subunits can autopalmitoylate in vitro at the physiologically correct Cys residues (4), and G protein-coupled receptors may also autopalmitoylate (5, 6). Autopalmitoylation is somewhat slower than palmitoylation in vivo, but the correlation between Cys residues that autopalmitoylate in vitro and those that are naturally palmitoylated suggests that autopalmitoylation is at least involved in the physiological process. The Cys residues that selectively autopalmitoylate may do so because their thiol groups have an unusually low pl, because they are at relatively hydrophobic surfaces and are thus exposed to Pal-CoA, or because these proteins contain ancillary residues that catalyze the reaction. The ability of a specific residue to autopalmitoylate would provide selectivity to the palmitoylation process, and the protein studied by Dunphy et al. (3) might act non-selectively either as a Pal-CoA carrier protein or as a non-selective transferase.

The functions of protein palmitoylation are an active area of study. In the case of p21ras and Go subunits, palmitoylation helps anchor the proteins to the plasma membrane (1, 7, 8). Palmitoylation of Go subunits also enhances their binding to Gβγ and desensitizes them to the GAP activity of RGS proteins (9, 10). Palmitoylation has been reported to enhance the activity of a G protein-coupled receptor kinase (11), and mutations of the palmitoylated Cys residue in the C-terminal region of G protein-coupled receptors themselves have resulted in a variety of effects (8). In none of these cases is it completely clear whether palmitoylation alters the structure of the palmitoylated protein itself, whether the palmitoyl group forms or blocks part of a protein-protein interface, or whether it only increases binding to hydrophobic surfaces.

It has recently been reported that several RGS proteins are also palmitoylated in cells (12–14). RGS proteins are a family of variably selective GAPs for members of the G,Gi, and Gq families of heterotrimeric G proteins (15). RGS proteins inhibit G protein signaling in fungi and roundworms (16–18) and their overexpression can inhibit signaling in mammalian cells (see Ref. 15 for review). RGS proteins are also important in modulating the decay kinetics of G protein signaling (19–23). The closely related proteins RGS4 and RGS16 are palmitoylated at Cys2 and/or Cys12 (13, 14), and mutation of these residues diminished the ability of RGS16 to inhibit cellular signaling (14). Membrane-bound GAIP is also palmitoylated near its N terminus, but in a cysteine string that characterizes a separate
RGS protein subfamily (12). We report here that RGS4 can autopalmitoylate stoichiometrically in vitro at two sites, one near the N terminus and the other in the conserved RGS box domain. RGS10, which lacks the N-terminal site, also autopalmitoylates at the conserved Cys residue. Autopalmitoylation of both proteins correlates well with their palmitoylation in cells. We report further that dual palmitoylation can either inhibit or potentiate GAP activity depending on the site of the RGS protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wild-type and mutant RGS4 and RGS10 were purified from *Escherichia coli* as described (24). RGS4 was purified from Sf9 cells by essentially the same procedure. Gaα5, (25, 26), GB72 (25), and m2AChR (27) were produced from Sf9 cells. Myristoylated Gaα1 was purified from *E. coli* (28). [3H,32P]GTP and [3H]Pal-CoA were synthesized and purified as described (4, 29). [9,10-3H]Palmitic acid was purchased from NEN Life Science Products.

**Mutagenesis of RGS4 cDNA**—The Cys mutants C2A/C12A/C33A and C2A/C12A RGS4 were prepared by sequential polymerase chain reaction reactions. C33A RGS4 cDNA was first prepared by substituting codon 33 by GCG. The product and the wild-type cDNA were cut with NcoI and BamHI and ligated in-frame into a modified pQE60 (Qiagen) that encodes the sequence MGH6MG before the cloning site (30). In subsequent reactions, codons 2 and 12 were replaced by GCC. The final cDNA products, C2A/C12A and C2A/C12A/C33A RGS4, were cloned into the modified pQE60 vector as described above. The ΔN57 RGS4 deletion mutation was generated by polymerase chain reaction with the primer 5′-GATCCATGGCGAATCTGGCGTGAATCGCGGAAA and was inserted into pVL1392 modified to include a NcoI site at the 5′ end of the multiple cloning site. Recombinant baculoviruses were produced as described previously (27). The plasmid that encodes RGS10 box was prepared as described (30).

**Expression and Palmitoylation of RGS Proteins in Sf9 Cells**—For labeling with [3H]palmitate and small scale preparation, Sf9 cells (4 × 10⁶) were infected with RGS4 or RGS10 virus for 32 h prior to metabolic labeling. Sodium [3H]palmitate (1 mCi) was suspended in 2 ml of IPL-41 that contained 1% ethanol and 2% heat-inactivated fetal calf serum. Cells were incubated in this medium for 1 h. The cells were harvested by centrifugation, washed with 2 ml of phosphate-buffered saline, and suspended in 0.5 ml of lysis buffer (20 mM NaHepes (pH 8.0), 2 mM MgCl₂, 1 mM 2-mercaptoethanol, 1 mM phenylmethylsulfon fluoride, 10 μg/ml leupeptin). Cells were lysed by 5 freeze/thaw cycles, followed 10 passes through a 25-gauge needle. The lysates were centrifuged at 100,000 × g at 4 °C for 20 min in a Beckman TL100.3 rotor. The supernatant (cytosolic fraction) was removed, and the pellet (crude membrane) was resuspended in lysis buffer. Both fractions could then be analyzed by SDS-PAGE. For purification of active RGS protein, the pellet was solubilized by resuspending and stirring for 30 min at 4 °C in 100 mM NaCl, 50 mM NaHepes (pH 8.0), 0.5% deoxycholate, 0.5% Triton X-100, 0.1% SDS, 1 mM 2-mercaptoethanol, and the protease inhibitors listed above. After centrifugation for 30 min as described above, the supernatant was diluted 5-fold with 50 mM NaHepes (pH 8.0) and applied to nitrocellulose acid-Ni²⁺-agarose and purified as described for RGS proteins expressed in *E. coli* (24). RGS4 from the cytosolic fraction was purified similarly.

**Autopalmitoylation of RGS Proteins**—Autopalmitoylation of RGS proteins was performed as described previously for Ga subunits with slight modification (4, 10). Routinely, 5 μM RGS protein was incubated with 100 μM radioative or non-radioactive Pal-CoA for up to 6 h at 30 °C in 50 mM NaHepes (pH 8.0), 0.005% Lubrol PX, and 100 mM 2-mercaptoethanol. Residual free Pal-CoA was removed by adsorbing the palmitoylated RGS protein to nitrocellulose acid-Ni²⁺-agarose followed by elution as described (24). To assess the degree of palmitoylation, the preparation was either precipitated with 10% trichloroacetic acid and isolated either on a glass fiber filter (10) or by SDS-PAGE before liquid scintillation counting. Gels were stained with Coomassie Blue to detect proteins and appropriate slices were solubilized with 30% H₂O₂ for 16 h at 60 °C. GSG concentrations were determined by Amido Black staining (31) using bovine serum albumin as standard.

**Electrophoresis and Immunoblotting**—SDS-PAGE was performed as described (10). Gradient gels (15–22%) were used for CNBr-cleaved samples. Samples for PAGE were prepared as described (10) to maintain palmitoylation during denaturation. Proteins were transferred to nitrocellulose as described (10) and immunoblots were developed according to instructions in the ECL kit (Amersham Pharmacia Biotech). CNBr Cleavage of RGS4—RGS4 (10 μg/ml, 50–100 pmol) in 70% (v/v) formic acid was mixed with 100 μg/ml CNBr and 1 mg/ml tryptophan and was incubated under argon in the dark at room temperature for 20 h. Products were diluted in water and dried under vacuum.

**Mass Spectrometry**—Palmitylated or non-palmitylated RGS4 was alkylated by incubation with 15 mM N-ethylmaleimide to block free thiol groups and digested with CNBr. The mixture was resolved with SDS-PAGE, the gels were stained by Copper Stain (Bio-Rad), and appropriate bands were cut and extracted with 17% formic acid, 33% 2-propanol (32). The molecular masses of peptides were determined by matrix-assisted laser desorption ionization/time of flight mass spectrometry using a matrix of 3,5-dimethoxy-4-hydroxycinnamic acid on a Voyager-DE spectrometer (PE Biosystems).

**GAP Assays**—GAP activity was assayed in two formats. In the simpler assay, purified Go is first bound to [γ-32P]GTP, and the rate of hydrolysis of the [γ-32P]GTP-Go is measured in detergent solution in the presence and absence of the GAP. In this assay, GAP activity is defined as the increase in the first-order hydrolysis rate constant or is approximated as an increase in the initial rate of hydrolysis (26, 33). Such single-turnover GAP assays, using either 2 nm Goα2345[P]GTP at 15 °C or ~10 nm Gaα1 [γ-32P]GTP at 8 °C, were performed as described (10, 26). A more sensitive and presumably more physiological assay for GAP activity monitors the enhancement of agonist-stimulated steady-state GTase activity in proteoliposomes reconstituted with receptor and heterotrimeric G protein. Reconstitution of purified m2AChR with either Gα or Gβ and was performed as described (24). RGS proteins were usually incubated with the vesicles for 1 h at 30 °C prior to assay conditions for measuring carbachol-stimulated GTase activity in this system have been described (24).

**RESULTS**

RGS4 and RGS10 Autopalmitoylate in Vitro and Are Naturally Palmitoylated in Sf9 Cells at a Highly Conserved Residue in the RGS Box—When RGS10 or RGS4 were incubated with [3H]Pal-CoA under the conditions originally described by Duncan and Gilman (4) for autopalmitoylation of Ga subunits, they incorporated 3H through a bond that was sensitive to both NH₂OH and DTT (Fig. 1A), presumably a palmitoyl thioester. Both RGS4 (13) and RGS10 are also naturally palmitoylated in Sf9 cells, as indicated by labeling with [3H]palmitic acid (Fig. 1B).
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Fig. 2. Localization of [3H]palmitoylation sites on RGS4. A, CNBr peptide mapping. [3H]Palmitoylated RGS4 was either prepared by autopalmitoylation in vitro or purified from Sf9 cells grown with [3H]palmitate as described in the legend to Fig. 1. Samples (20–40 pmol) were analyzed by SDS-PAGE either without further treatment or after cleavage by CNBr as described under “Experimental Procedures,” as shown. The gel was stained with Coomassie Blue (left panel) and then subjected to fluorography (right panel) for 72 h. The fourth lane contained 10^7 cpm of free [3H]Pal-CoA to indicate its migration in the gel. B, labeling of C95V RGS4. RGS4, wild-type or C95V, was either metabolically labeled in Sf9 cells and then purified (left panels) or was labeled with [3H]palmitate by autopalmitoylation in vitro (right panels). Samples, either untreated (25 pmol) or CNBr-treated (50 pmol), were analyzed by SDS-PAGE. The gel was stained with Coomassie Blue (CB) and then subjected to fluorography (H) for 40 h. Radioactive bands were then cut out, hydrolyzed with H_2O_2, and counted. [3H]Palmitate incorporation is expressed either as cpm/pmol (left) or as mol of palmitate/mol of RGS4 (right).

1B). In vivo [3H]palmitoylation was essentially limited to the RGS protein found in the particulate fraction. Little label was found on soluble RGS protein despite the fact that about 30% of RGS4 and almost all of the RGS10 were found in the cytoplasmic fraction.

The extent of in vitro autopalmitoylation of RGS10 was approximately 1 mol of palmitate/mol (Fig. 1A). A truncated RGS10 consisting only of the conserved RGS box was also labeled to about 1 mol/mol with approximately similar kinetics. Because Cys_66 is the only Cys residue in the RGS10 box, its palmitoylation accounts for that observed in full-length RGS10. Intact RGS4 incorporated 2 mol of palmitate/mol, but an N-terminal truncation mutant of RGS4 was labeled to only 1 mol/mol. This finding suggests that one palmitoylation site lies in the RGS4 box and that the other lies in the N-terminal region. Because Cys_66 in RGS10 is the only highly conserved Cys residue in the RGS protein family, its palmitoylation suggests that the corresponding Cys residue in RGS4, Cys_95, might be the site of palmitoylation in the RGS4 box. Both Cys_9 and Cys_12 are candidate sites for the N-terminal palmitoylation site (13, 14), and we did not try to distinguish between them in this study.

We analyzed the palmitoylation sites of RGS4 by a combination of CNBr peptide mapping and mutagenesis. As shown in Fig. 2A, autopalmitoylated RGS4 contained [3H]palmitate in two CNBr-generated peptides, one of about 15,000 Da and the other of about 2000 Da. Both peptides were also labeled in RGS4 that had been palmitoylated in Sf9 cells. The palmitoylated peptides were identified both by Edman sequencing and mass spectrometry as Lys_{20}–Met_{141}, which includes most of the RGS4 box, and Cys_{9}–Met_{19}. Comparison of the masses of these peptides in samples prepared from palmitoylated and non-palmitoylated samples indicated that each incorporated 1 palmitoyl group. Mutation of Cys_{95} decreased palmitoylation of intact RGS4 that was labeled either in vitro or in vivo, and essentially eliminated incorporation of palmitate into the ~15-kDa Lys_{20}–Met_{141} peptide (Fig. 2B). This finding, coupled with the unique palmitoylation of the homologous Cys_{66} residue in RGS10, indicates that Cys_{95} is the site of palmitoylation in the RGS4 box. In autopalmitoylated RGS4, Cys_{95} and the more N-terminal site were each labeled to approximately 1 mol/mol. In Sf9 cells, however, more [3H]palmitate was incorporated into the N-terminal site than into Cys_{95} under our standard conditions for Sf9 cell growth. This difference might reflect either relatively more incorporation of palmitate into the N-terminal site or more complete turnover at that site, although net incorporation of label into RGS4 was essentially complete within 1 h.

To study the interdependence of palmitoylation of the N-terminal region and the RGS box, we mutated Cys_9 and Cys_12, which are probable sites of cellular palmitoylation in both RGS4 and RGS16 (13, 14). Surprisingly, mutation of these sites eliminated all RGS4 autopalmitoylation, including autopalmitoylation of Cys_{95} (Fig. 3B). To determine whether N-terminal palmitoylation precedes Cys_{95} autopalmitoylation in wild-type RGS4, we monitored autopalmitoylation of both sites in vitro. As shown in Fig. 3C, autopalmitoylation at Cys_{95} lagged somewhat behind autopalmitoylation at the N terminus. While the lag was not great, it was reproducible in three similar experiments. These findings indicate that palmitoylation of the N-terminal site is kinetically favored over Cys_{95} palmitoylation. Consistent with this idea, the ΔN57 mutant of RGS4 and RGS10 (which is not N-terminally palmitoylated) both autopalmitoylate very slowly (t_{1/2} ~ 2 h, compared with a t_{1/2} of 30 min for wild-type RGS4). These results suggest that initial N-terminal palmitoylation promotes subsequent palmitoylation in the RGS4 box. N-terminal palmitoylation is not absolutely required for autopalmitoylation within the RGS box because both Cys_{95} in the RGS4 box construct and Cys_{95} in RGS10 do slowly autopalmitoylate. Coupling of palmitoylation in the RGS box with that in the N-terminal region also appears to hold true in cells, where the C2A/C12A mutation eliminates all RGS4 palmitoylation (13).

Palmitoylation in the RGS Box Inhibits G Protein GAP Activity in Solution-based Assays—Autopalmitoylation of RGS4 inhibited its GAP activity essentially completely when activity was measured in a single-turnover assay in detergent solution (Fig. 3A). Upon incubation with Pal-CoA, GAP activity declined with a time course similar to that of incorporation of palmitate (data not shown; see Fig. 4 for similar data for RGS10). Incubation of autopalmitoylated RGS4 with DTT largely reversed inhibition (Fig. 3A), and incubation of RGS4 without Pal-CoA was without effect. The GAP assays shown in Fig. 3A used G_{α_q}-GTP as substrate, but similar results were obtained in similar experiments that used G_{α_q}-GTP as substrate and Triton X-100 instead of Lubrol PX.

Mutation of Cys_{95} in RGS4 blocked inhibition of GAP activity by incubation with Pal-CoA, suggesting that inhibition is me-
Proteins (25 pmol) were incubated with [3H]Pal-CoA, and autopalmitoylation was blocked by mutation of either Cys 95 itself or of the N-terminal Cys residue. DTT-treated and untreated samples were then assayed for GAP activity. 

Regulation of RGS Proteins by Palmitoylation

Palmitoylation of RGS4 at Cys95 blocks its GAP activity. RGS4 (5 μM) was incubated at 30 °C either alone (Δ) or with (○) 100 μM Pal-CoA. At the times shown, 5-μl aliquots (25 pmol) were diluted and assayed for GAP activity toward Gαi-GTP. At 120 min, half of each of the remaining two samples were treated with 50 mM DTT for 60 min at 30 °C (○). DTT-treated and untreated samples were then assayed for GAP activity. B, palmitoylation of Cys95 accounts for RGS4 inhibition and can be blocked by mutation of either Cys95 itself or of the N-terminal Cys residues. Samples of mutant or wild-type RGS4 (5 μM) were incubated at 30 °C with 100 μM Pal-CoA for 2 h. Gz GAP activity was assayed and measured for GAP activity using Gz, GTP as substrate. In a parallel incubation, [3H]Pal-CoA (also 100 μM) was used to monitor the progress of palmitoylation as described in the legend to Fig. 1 (○).

Palmitoylation of RGS10 Potentiates GAP Activity in a Vesicle-Based Assay System—In contrast to the inhibition described above, palmitoylation of RGS10 markedly stimulated its GAP activity as measured during receptor-stimulated, steady-state GTP hydrolysis. The agonist-stimulated GTPase activity of unilamellar phospholipid vesicles that contained heterotrimeric Gαi and m2AChR was measured in the presence of increasing concentrations of RGS10 (Fig. 5). When agonist-bound receptor drives GDP/GTP exchange in these vesicles, hydrolysis of Gαi-bound GTP becomes rate-limiting and a GAP increases steady-state hydrolysis until the overall reaction again approaches the rate of receptor-catalyzed GDP/GTP exchange (29, 34, 35). In m2AChR-Gi vesicles, RGS10 increases agonist-stimulated GTPase activity about 10-fold at 5 μM, the highest concentration tested (Fig. 5). When agonist-bound receptor drives GDP/GTP exchange in these vesicles, hydrolysis of Gαi-bound GTP becomes rate-limiting and a GAP increases steady-state hydrolysis until the overall reaction again approaches the rate of receptor-catalyzed GDP/GTP exchange (29, 34, 35). In m2AChR-Gi vesicles, RGS10 increases agonist-stimulated GTPase activity about 10-fold at 5 μM, the highest concentration tested (Fig. 5).

Palmitoylation at Cys95, Both C2A/C12A and C2A/C12A/C33A RGS4 were also inhibited by incubation with Pal-CoA (Fig. 3B). As would be predicted by its inability to incorporate palmitate at Cys95, both C2A/C12A and C2A/C12A/C33A RGS4 were also inhibited by incubation with Pal-CoA (Fig. 3B).

Palmitoylation also inhibited the GAP activity of RGS10 when activity was measured in the solution-based assay (Fig. 4). Again, fractional inhibition of GAP activity paralleled fractional palmitoylation and suggested that stoichiometrically palmitoylated RGS10 is inhibited 80–90% at this substrate concentration. Control incubations without Pal-CoA did not cause inhibition (Fig. 4), and inhibition was reversed by incubation with DTT (not shown). Because RGS10 is palmitoylated only on Cys66, these data combine with those of Fig. 3 to indicate that palmitoylation of the conserved Cys residue in the RGS box blocks the GAP activity of these RGS proteins in the single-turnover assay.

Palmitoylation of RGS10 inhibits Gαi GAP activity in detergent solution. RGS10 (5 μM) was incubated at 30 °C either alone (●) or with 100 μM Pal-CoA (○). At the times shown, 5-μl aliquots were withdrawn and measured for GAP activity using Gz, GTP as substrate. In a parallel incubation, [3H]Pal-CoA (also 100 μM) was used to monitor the progress of palmitoylation as described in the legend to Fig. 1 (○).
somes that contained m2AChR (0.27 nM m2AChR) and trimeric Gz (0.95 nM). Steady-state GTPase activity was then assayed in the presence of 1 μM carbachol. Final concentrations of RGS proteins were 10 nM.

We then tested the GAP activities of samples of RGS4 purified from Sf9 cell membranes could only incorporate about 1.0–1.2 mol of palmitate/mol, suggesting that labeling is blocked because one or both sites are naturally palmitoylated in cells to a total of 0.8–1.0 mol of palmitate/mol of RGS4. Based on these data and on the ratio of [3H]palmitate labeling at the two sites (about 2; Fig. 2), we estimate that RGS4 bound to Sf9 cell membranes is about 60% palmitoylated at the N-terminal site and 30% palmitoylated at Cys95 to produce an average total palmitoylation of about 1 mol of palmitate/mol of RGS4.

We then tested the GAP activities of samples of RGS4 purified from either the membrane or cytoplasmic fraction of Sf9 cells, both without treatment and after complete in vitro autopalmitoylation. In the solution-based single-turnover assay, non-palmitoylated RGS4 purified from Sf9 cytosol can auto-palmitoylate to approximately 2 mol of palmitate/mol, as was the case for RGS4 purified from E. coli (Fig. 1A). In contrast, RGS4 purified from Sf9 cell membranes could only incorporate about 1.0–1.2 mol of palmitate/mol, suggesting that labeling is blocked because one or both sites are naturally palmitoylated in cells to a total of 0.8–1.0 mol of palmitate/mol of RGS4. Based on these data and on the ratio of [3H]palmitate labeling at the two sites (about 2; Fig. 2), we estimate that RGS4 bound to Sf9 cell membranes is about 60% palmitoylated at the N-terminal site and 30% palmitoylated at Cys95 to produce an average total palmitoylation of about 1 mol of palmitate/mol of RGS4.

We then tested the GAP activities of samples of RGS4 purified from either the membrane or cytoplasmic fraction of Sf9 cells, both without treatment and after complete in vitro autopalmitoylation. In the solution-based single-turnover assay, non-palmitoylated RGS4 purified from Sf9 cytosol was about 50% more active as a GAP than was the partially palmitoylated RGS4 purified from the membrane fraction (Fig. 5B). Partial palmitoylation at Cys95 is thus inhibitory in solution as expected, and partial palmitoylation near the N terminus had little if any effect. Autopalmitoylation at both sites completely inhibited GAP activity, consistent with the experiment shown in Figs. 3 and 7A. When GAP activity was measured during steady-state GTP hydrolysis by m2AChR-Gz vesicles, the opposite order was observed. Partially palmitoylated RGS4 from the membrane fraction was nearly twice as active as RGS4 from cytoplasm, consistent with significant stimulation by palmitoylation in the N-terminal region (Fig. 8C). Again, complete palmitoylation of both RGS4 preparations inhibited their activities to the same low level. These data, taken together, suggest that RGS4 palmitoylated near its N terminus is more active as a GAP when assayed at a membrane surface and that a second palmitoyl group added in the RGS box inhibits activity regardless of the palmitoylation state of the N-terminal site.

DISCUSSION

These data establish that multi-site palmitoylation of an RGS protein, within the RGS box and near the N terminus, can either potentiate or inhibit its G protein GAP activity. We were able to determine that these distinct sites in RGS4 are both

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**TABLE I**

| Cys mutations on RGS4 GAP activity | k_{obs} (min⁻¹) |
|-----------------------------------|----------------|
| Control                           | 0.17           |
| WT                                | 0.87           |
| C2A/C12A                          | 0.087          |
| C2A/C12A/C33A                     | 0.028          |
| C95V                              | 0.14           |

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**DISCUSSION**

These data establish that multi-site palmitoylation of an RGS protein, within the RGS box and near the N terminus, can either potentiate or inhibit its G protein GAP activity. We were able to determine that these distinct sites in RGS4 are both...
palmitoylated in cells because both sites in RGS4 are efficiently autopalmitoylated in vitro, apparently in the same order. RGS10 also autopalmitoylated essentially quantitatively at a single site in the RGS box. Near quantitative in vitro labeling allowed identification by peptide mapping and mass spectrometry of a conserved Cys residue in the RGS box, Cys95 in RGS4 and Cys96 in RGS10, as the major site of both autopalmitoylation and cellular palmitoylation within the RGS box. This is the only Cys residue in the RGS10 box, and palmitoylation within the RGS box was eliminated by C95V mutation. This Cys residue is conserved in all mammalian RGS proteins (including the RGS4 box was eliminated by C95V mutation. This Cys only Cys residue in the RGS10 box, and palmitoylation within the RGS box. This is the correct Cys residues out of 13) is multiply palmitoylated (12).

RGS proteins are the most recently recognized group of cellularly palmitoylated proteins that also autopalmitoylate at the correct Cys residues in vitro. Ga subunits were the first (4), GAP-43 palmitoylates at a regulatory site (36), and G protein-coupled receptors probably autopalmitoylate as well (5, 37, 38). RGS4 autopalmitoylated fairly quickly, such that palmitoylation was complete in about 30 min. The other RGS constructs autopalmitoylated with t1/2 = 2 h. The specificity of autopalmitoylation for specific Cys residues and the general correlation between the rates for in vitro autopalmitoylation and cellular palmitoylation resurrect the question of whether autopalmitoylation is a significant physiological mechanism for protein palmitoylation (1, 4). Although Dunphy et al. (3) have described a cellular activity that enhances the rate of Ga palmitoylation (see also Ref. 38), no protein palmitoyltransferase has been identified at either the protein or DNA level. Druey et al. (14, 39) also required a fraction from liver to palmitoylate RGS16 in vitro, but that activity might be the acyl-CoA synthetase needed to form Pal-CoA from labeled palmitate. Although it may seem intuitively unlikely that autopalmitoylation accounts totally for protein palmitoylation in cells, it remains plausible that autopalmitoylation is adequate in a local environment rich in Pal-CoA. 

Peptide mapping of RGS4 and RGS10 that was labeled by [3H]palmitate in Sf9 cells allowed us to demonstrate that both sites, Cys2 or Cys12 in RGS4 and Cys96 in the RGS box, are palmitoylated naturally. Palmitoylation in the RGS box was detected previously because for intact RGS4 and, presumably, for RGS16 (14), it essentially depends on the prior palmitoylation of the more N-terminal site. Thus, mutation of Cys2 and Cys12 to Ala eliminated both their own palmitoylation and that of Cys95 (Refs. 13 and 14 and Fig. 3B). Removal of the N-terminal domain of RGS4 also slowed palmitoylation, and the absence of an N-terminal palmitoylation site in RGS10 probably accounts in part for its slow palmitoylation in vitro and modest palmitoylation in cells. The same pattern is apparent true for RGS16, a close homolog of RGS4, when it is expressed in COS cells (14). Sequential palmitoylation may also occur in the RGSZ family (24), in which an N-terminal cysteine string (9 Cys residues out of 13) is multiply palmitoylated (12).
There are at least three plausible explanations for why both autopalmitoylation and cellular palmitoylation in the RGS box are accelerated by initial palmitoylation near the N terminus. First, initial N-terminal palmitoylation may help secure the RGS protein or the RGS box domain to a membrane or micelle that contains Pal-CoA (3). More likely, N-terminal palmitoylation actively facilitates palmitoylation in the box, probably by altering the conformation of the RGS protein such that the Cys<sup>95</sup> thiol group is more exposed, more reactive or both. This thiol group is relatively inaccessible according to the structure of the RGS4-Go<sub>o</sub> complex described by Tesmer et al. (40). Finally, it is possible that the N-terminal palmitoyl-cysteine thioester acts catalytically and directly transfers its palmitoyl group to the Cys residue in the box, after which it can be subsequently repalmitoylated. Interaction of the cysteine residues at the N terminus with Cys<sup>95</sup> is at least consistent with the fact that AN57 RGS4 palmitoylates faster than does the C2A/C12A mutant, in which the N-terminal domain may unproductively occlude Cys<sup>95</sup>. Unfortunately, the N terminus of RGS4 is not visible in the only available crystal structure of RGS4 (40), and the spatial relationship between the two Cys residues is unknown.

The cellular palmitoylation state of the RGS proteins is regulated both by palmitoylation and depalmitoylation. The combined data of Figs. 1B, 2, and 4A suggest that the palmitate groups on RGS proteins are in constant turnover in S9 cells, and the same appears true in COS cells (14). At steady state, RGS4 was 50–60% palmitoylated in S9 cell membranes according the capacity of purified RGS4 to add more [³H]palmitate when exposed to [³H]Pal-CoA in vitro (Fig. 8). CNBr peptide mapping of steady-state palmitoylated RGS4 (Fig. 2) suggests further that palmitoyl groups are found at both the N-terminal position and at Cys<sup>95</sup>, indicating that palmitate turns over at both sites. Labeling at both sites reached steady state in about 1 h, suggesting that removal of palmitate is essentially continuous and is required for addition of new [³H]palmitate label. We do not know how depalmitoylation of each site may be regulated, however, and rapid hydrolysis of palmitate at the N-terminal site could lead to the creation of significant RGS4 that is only palmitoylated at Cys<sup>95</sup>.

Palmitoylation of RGS4 and RGS10 either inhibited or stimulated GAP activity depending on which assay system was used and, for RGS4, which of the two sites was palmitoylated. Palmitoylation of Cys<sup>95/96</sup> in RGS4 and RGS10 uniformly inhibited their GAP activities in the single-turnover assay in detergent solution. Inhibition was sufficiently complete that we could not determine whether it was caused by a decrease in k<sub>cat</sub>, an increase in k<sub>off</sub>, or both. The extent of inhibition was similar for both G<sub>i</sub> and G<sub>q</sub> substrates and was observed with either Triton X-100 or Lubrol PX. Because Cys<sup>95/96</sup> lies in the middle of helix 4 and is oriented inward toward helix 5 (40), it seems likely that palmitoylation inhibits GAP activity by shifting the packing of the central four-helix bundle to alter the structure of the site that binds the Go-GTP substrate. The cysteine thiol group is itself not required for GAP activity because RGS6, RGS7, and the active C95A mutant all have a Val residue at this site. Inhibition also does not appear to result simply from a net increase in hydrophobicity because palmitoylation at the N terminus had no effect on intrinsic GAP activity (Fig. 7A).

In contrast to the inhibition observed in the single turnover assay, palmitoylation at the N terminus of RGS4 or at Cys<sup>95</sup> of RGS10 potentiated GAP activity measured in the vesicle-based, steady-state assay. GTPase activity in this assay depends on the coordinated stimulation of GDP/GTP exchange by receptor and of GTP hydrolysis by the GAP (29, 33). Potentiation in this assay probably reflects either increased hydrophobicity that attaches the RGS protein more firmly to the vesicles or enhanced interaction with some other component of the system (receptor or Gβγ). Interaction of RGS proteins with receptors has been proposed based on receptor-selective action of RGS4 in pancreatic acinar cells (41, 42) and interaction with Gβγ has been supported by the effects of RGS proteins on the regulation of Gβγ-gated K<sup>+</sup> channels (20, 21, 43).

It is likely that palmitoylation enhances GAP activity at least in part by increasing affinity for membranes. For RGS10, which is quite hydrophilic and does not bind appreciably to membrane (Fig. 1B) or to phospholipid bilayers (data not shown), palmitoylation would provide localization at the membrane surface. Increased local concentration at the site where G protein and receptor interact could account for the enhanced potency shown in Fig. 5. RGS4 is intrinsically more hydrophobic because of its N-terminal domain (Ref. 44, and data not shown). The differential hydrophobic effect of RGS4 palmitoylation would therefore not be as great as with RGS10 and would potentiate GAP activity less. Thus, only palmitoylation at the N terminus could cause net stimulation, and this effect would not overcome the inhibition by palmitoylation at Cys<sup>95</sup> (Fig. 7). In this context it is interesting that Druey et al. (14) found that mutation of Cys<sup>92</sup> and, to a lesser extent, Cys<sup>95</sup> in RGS16 diminished its ability to inhibit signaling by G<sub>i</sub> or G<sub>q</sub>, suggesting that N-terminal palmitoylation may contribute to GAP activity in cells. It will be important to determine whether Cys<sup>97</sup> in RGS16, homologous to Cys<sup>95</sup> in RGS4, is palmitoylated under these conditions. Alternatively, cellular effects of mutating N-terminal Cys residues may not reflect only the absence of palmitoylation. For example, a synthetic peptide that corresponds to residues 1–33 of RGS4 inhibited signaling through an apparently GAP-independent mechanism, and substitution of Cys residues in this peptide eliminated this inhibition (41). Thus, while it is clear that palmitoylation of RGS proteins occurs and can alter their activities in vitro, it will be important to find which Cys residues are palmitoylated in cells, how palmitoylation is controlled and what effects palmitoylation of specific Cys residues has on RGS protein function.

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