Palmitoylation Regulates Regulator of G-protein Signaling (RGS) 16 Function

II. PALMITOYLATION OF A CYSTEINE RESIDUE IN THE RGS BOX IS CRITICAL FOR RGS16 GTPase ACCELERATING ACTIVITY AND REGULATION OF G\textsubscript{i}-COUPLED SIGNALING*  

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Palmitoylation is a reversible post-translational modification used by cells to regulate protein activity. The regulator of G-protein signaling (RGS) proteins RGS4 and RGS16 share conserved cysteine (Cys) residues that undergo palmitoylation. In the accompanying article (Hiol, A., Davey, P. C., Osterhout, J. L., Waheed, A. A., Fischer, E. R., Chen, C. K., Milligan, G., Druey, K. M., and Jones, T. L. Z. (2003) \textit{J. Biol. Chem.} 278, 19301–19308), we determined that mutation of NH\textsubscript{2}-terminal cysteine residues in RGS16 (Cys-2 and Cys-12) reduced GTPase accelerating (GAP) activity toward a 5-hydroxytryptamine (5-HT\textsubscript{1A})/\textsubscript{Go}\textsubscript{a1} receptor fusion protein in cell membranes. NH\textsubscript{2}-terminal acylation also permitted palmitoylation of a cysteine residue in the RGS box of RGS16 (Cys-98). Here we investigated the role of internal palmitoylation in RGS16 localization and GAP activity. Mutation of RGS16 Cys-98 or RGS4 Cys-95 to alanine reduced GAP activity on the 5-HT\textsubscript{1A}/\textsubscript{Go}\textsubscript{a1} fusion protein and regulation of adenyl cyclase inhibition. The C98A mutation had no effect on RGS16 localization or GAP activity toward purified G-protein \textsubscript{a} subunits. Enzymatic palmitoylation of RGS16 resulted in internal palmitoylation on residue Cys-98. Palmitoylated RGS16 or RGS4 WT but not C98A or C95A preincubated with membranes expressing 5-HT\textsubscript{1A}/\textsubscript{Go}\textsubscript{a1} displayed increased GAP activity over time. These results suggest that palmitoylation of a Cys residue in the RGS box is critical for GAP activity and their ability to regulate \textsubscript{G}\textsubscript{i}-coupled signaling in mammalian cells.

Activation of G-protein-coupled receptors by peptides and hormones catalyzes the exchange of GDP with GTP on the \textsubscript{a} subunit of its associated heterotrimeric G protein. The active, GTP-bound form of the \textsubscript{a} subunit interacts with effectors, initiating a signaling cascade. Deactivation of this signaling pathway is mediated by the intrinsic GTPase activity of the \textsubscript{a} subunits, which is in turn accelerated by cognate GTPase activating proteins (GAPs),\textsuperscript{1} the regulators of G-protein signaling (RGS proteins) (reviewed in Ref. 1–3).

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\textsuperscript{‡} The abbreviations used are: GAP, GTPase activating protein; RGS, the regulator of G protein signaling; pPAT, partially purified protein acyltransferase; GST, glutathione S-transferase; DRMs, detergent-resistant membranes; WT, wild-type; 5-HT, 5-hydroxytryptamine (serotonin); AMP-PNP, adenosine 5’-\textsubscript{\textgamma}-imino/triphosphate; HA, hemagglutinin; HEK, human embryonic kidney; IB, incubation buffer.
RGS16 GAP activity toward the 5-HT₁₆/Gαᵢ₁ activity in membranes but did not substantially alter membrane or lipid raft localization. The analogous mutation in RGS4 resulted in a similar reduction in GAP activity. Most importantly, direct enzymatic palmitoylation of RGS16 or RGS4 by a protein acyltransferase followed by membrane preincubation markedly increased GAP activity over time. The C98A mutation also abrogated the ability of RGS16 or RGS4 to regulate Gᵢ-mediated adenyl cyclase inhibition, confirming the significance of this internal palmitoylation site for RGS function in mammalian cells.

EXPERIMENTAL PROCEDURES

Reagents—Isoproterenol, somatostatin, EDTA, ATG, TGT, AMP-PNP, creatine phosphate, clostripain, and creatine phosphokinase were obtained from Sigma. Superfet transfection reagent was purchased from Qiagen, and pertussis toxin from Calbiochem.

Cell Culture, Proteins, and Plasmids—HEK293 and COS-7 cells were obtained from ATCC. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 5 mM glutamine, and antibiotics in a humidified 5% CO₂ incubator. HEK293 cells stably expressing a fusion protein between the human 5-HT₁₆ receptor and Gαᵢ₁ containing a C351G mutation that renders the G protein resistant to pertussis toxin were generated as previously described (34). GST-RGS16 and His₅-RGS4 were produced in Echerichia coli and purified as described (18, 35). Purified proteins were dialyzed against 50 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA, and 1% glycerol and stored at −80 °C until use. Recombinant, myristoylated rat Gαᵢ₁ and Gαᵢ₃ were purchased from Calbiochem. Plasmids directing expression of human HA-RGS4 and RGS16 WT and HA-RGS16 (C2A/C12A) have been described elsewhere (18, 34). HA-RGS16 (C98A) and HA-RGS4 (C95A) were generated using the QuikChange mutagenesis kit (Stratagene). Polyconal antiserum against mouse RGS16 (CT265), which also recognizes human RGS16, has been previously described (36).

Immunoblotting—Cell lysates were resolved on 10–20% Tris glycine gels and transferred to polyvinylidene difluoride membranes. After a 2-h blocking step in Tris-buffered saline plus 0.01% Tween 20, the blots were probed with mouse anti-HA (1:2000) or anti-HA (12CA5, Roche Diagnostics) for an additional 2 h. Signals were detected by enhanced chemiluminescence (Super Signal, Pierce) according to the manufacturer’s instructions.

Adenyl Cyclase and Single Turnover GAP Assays—These assays were performed exactly as described previously (11).

Plasma Membrane and Detergent-resistant Membrane (DRM) Fractionation, High Affinity GTPase Assay—These procedures were described in the accompanying article (33).

Purification of Protein Acyltransferase—Purification of pPAT was performed as described in detail elsewhere. Briefly, the plasma membrane from rat livers was extracted with 0.15% Triton X-100 and the PAT activity was purified over several chromatography steps, octyl-Sepharose, cerulene analogue affinity, Q-Sepharose, and palmitoyl CoA-agarose.

Enzymatic Palmitoylation of RGS Proteins—Recombinant RGS16 or RGS4 was diluted in incubation buffer (IB) (20 mM Tris-HCl, pH 7.4, 150 mM KCl, 1 mM EDTA) at a protein concentration of 0.4–1 mg/ml and incubated with the pPAT preparation (10 μg of protein), 200 μM CoA, 2 mM ATP, and 1 μM of 3,10-H-palmitoyl-CoA (5 mM/ml of 30–80 Ci/μmol, ARC Inc.). The final reaction volume was adjusted to 100 μl with IB. The reaction was stopped by the addition of sample buffer after 45 min at 30 °C. Proteins were subjected to one-dimensional PAGE and stained with MicrowaveBlue (Protiga). Acylation was determined by fluorography using the Wax technique (ISC BioExpress) following the instructions of the manufacturer.

Stoichiometry of Palmitoylation—Recombinant RGS16 (260 pmol) incubated with pPAT was diluted into IB buffer that contained 0.1% bovine serum albumin and 1% sucrose. The solutions were loaded into an Ultrafree filtration unit (Amicon) previously equilibrated with IB. After centrifugation at 2000 × g for 20 min and the filters washed once with 400 μl of 70% ethanol, then twice with IB. The insert cups were counted by liquid scintillation spectrometry, and palmitate incorporation was calculated based on observed counts and the specific activity of (3H)palmitate. Stoichiometry of labeling was determined as the ratio of picomole of palmitate to picomole of RGS16 per reaction.

Clostripain Cleavage—This procedure was carried out as in the accompanying article (33) except that recombinant RGS16 (10 μg) was used as the substrate.

Statistical Analysis—Two-tailed p values were determined by one-way analysis of variance followed by Tukey-Kramer multiple comparisons test using GraphPad InStat software. p values <0.05 were considered significant. Sigma Plot 8.0 software was used for curve fitting.

RESULTS

A Cysteine Residue in the RGS Box Is Critical for RGS16 GAP Activity—We tested the role of the conserved RGS box cysteine for the function of RGS16 and because it appeared to be a site of palmitoylation (19, 33). We transfected a cell line stably expressing a 5-HT₁₆/Gαᵢ₁ (C351G) fusion protein with HA-RGS16 WT or RGS16 (C98A) plasmids. Cells were treated with pertussis toxin to block receptor coupling to endogenous Gαᵢ₁ proteins, and membranes were prepared. Both basal and agonist-induced GTPase activity in membranes incubated with a range of 5-HT concentration were increased in the presence of WT RGS16 or compared with membranes transfected with a vector control plasmid (LacZ) as expected (37). However, GTPase activity in membranes expressing RGS16 (C98A) was decreased nearly 50% in comparison to membranes expressing WT RGS16 (Fig. 1A, upper panel). Addition of maximal 5-HT doses (10⁻⁶ to 10⁻⁴ M) resulted in a ~3.5-fold increase in activity in membranes containing WT RGS16 while the same dose resulted in only a 2.3-fold increase in activity in membranes containing RGS16 (C98A). Mutation of Cys-95 in RGS4 to alanine resulted in a similar reduction in GAP activity in this assay (Fig. 1A, lower panel). Decreased RGS16 or RGS4 levels in the membranes could not explain the loss of function of the cysteine mutants, because the amount of immunodetectable protein was comparable (Fig. 1B). A previous study showed that the GAP activity of RGS4 was unaffected by mutation of Cys-95 to Val in a single turnover assay (19). To exclude the possibility that the reduction in transfected RGS16 (C98A) activity could be a result of mutation of cysteine to alanine independent of palmitoylation, we purified WT or C98A recombinant RGS16 from E. coli, which would not undergo post-translational modification, and measured agonist-stimulated GTPase activity of the fusion protein in membranes in the presence of purified RGS16. Addition of WT RGS16 or C98A to membranes from cells expressing the 5-HT₁₆/Gαᵢ₁ protein enhanced GTPase activity equally in response to 5-HT even at subsaturating concentrations (Fig. 1C). In addition, we found no significant difference in the abilities of RGS16 WT or C98A to promote GTP hydrolysis by purified Gαᵢ in a single turnover assay (Fig. 1D). These results suggest that the loss of a cysteine thiol group was not by itself responsible for the decreased activity of RGS16 (C98A) expressed in mammalian cell membranes.

Cysteine 98 Is Essential for RGS16 and RGS4 Inhibition of Gᵢ-regulated Adenylyl Cyclase Activity—We tested the ability of the cysteine mutants to regulate Gᵢ, signaling in HEK293 cells. Somatostatin decreases adenylyl cyclase activity through Gαᵢ activation. Because decreases in basal levels of adenylyl cyclase activity are difficult to detect, we treated the cells concurrently with isoproterenol, which stimulates adenylyl cyclase through endogenous β-adrenergic receptors coupled to Gαₛ. Consistent with previous studies (18, 38), expression of WT RGS16 or WT RGS4 inhibited the negative regulation of adenylyl cyclase activity induced by somatostatin compared with vector-transfected cells (Fig. 2). In contrast, expression of RGS16 (C98A), RGS16 (C2A/C12A), or the analogous RGS4 mutants did not alter adenylyl cyclase activity compared with control cells. These results demonstrate that neither mutant was able to...
FIG. 1. Importance of a conserved cysteine residue in the RGS box for RGS16 and RGS4 GAP activity in mammalian cell membranes. A, membranes were isolated from HEK293 cells stably expressing a fusion protein between the 5-HT₁A receptor and Go_{15} (C351G) and transiently expressing LacZ (Vec), HA-RGS16, or HA-RGS4. Endogenous Go_{15} and Go_{16} activity was abrogated by pretreatment with pertussis toxin. Basal and agonist-stimulated steady-state GTPase activity of the fusion protein was determined after addition of the indicated concentrations of 5-HT after 20 min at 37 °C in the presence of co-transfected RGS16 or RGS4 WT (circles), RGS16 (C98A) (squares), RGS4 (C95A) (triangles), or vector control (Vec) (diamonds). Values represent the mean ± S.E. of four to five independent experiments. B, equivalent expression of RGS16 and RGS4 WT and mutants thereof. Equal amounts of protein (50 μg) from fusion protein-expressing HEK293 membranes were separated by SDS-PAGE and immunoblotted with anti-HA antibody. C, the C98A mutation has no effect on RGS16 GAP activity. Indicated concentrations of recombinant RGS16 WT (closed circles) or C98A (triangles) were incubated with 5-HT₁A/Go_{15} membranes in the presence of 10⁻⁶ M 5-HT as in A. GTPase activity shown is the mean ± S.E. of three independent experiments. D, GTPase activity of recombinant Go_{15} (250 nM) during a single catalytic turnover was determined in solution over the indicated time period in the presence of buffer alone (closed circles), 50 nM RGS16 WT (open circles) or C98A (triangles). Values are from a single experiment representative of five similar experiments. K_{hydrol} for Go_{15} in the absence of RGS16 was ~0.4/min, in agreement with previous studies (43).
Cysteine 98 is not required for RGS16 Plasma Membrane or Lipid Raft Localization—We delineated the membrane localization of RGS16 (C98A) by cellular fractionation and immunoblotting as previously described (33) to determine whether redistribution of the protein in membranes could account for its lack of function. The amounts of RGS16 WT or C98A found in the total membrane fraction (T) as well as the plasma membrane fraction (P) were comparable (Fig. 3A). The integrity of the fractions was verified by enrichment of the marker Na+/K+-ATPase (a plasma membrane marker, arrowheads, right panel). T, total membrane; P, plasma membrane fractions. B, cells expressing RGS16 were lysed in detergent buffer and subjected to OptiPrep gradient centrifugation. Fractions were collected (fraction 1 is from the top of the gradient, the “floating” fraction) and subjected to SDS-PAGE before immunoblotting with antibodies against RGS16 (top), caveolin (second from top) or Gαi (third from top) (markers for DRMs), and Na+/K+-ATPase (bottom), which is a marker for a plasma membrane protein not found predominantly in rafts.

Enzymatic Palmitoylation of RGS16—The aforementioned results suggest that the poor function of RGS16 (C98A) was not because of the cysteine to alanine mutation itself or a decrease in RGS16 found at the membrane. Therefore, we investigated whether palmitoylation on the RGS box cysteine could affect function of RGS16 in vitro. In the accompanying article (33), we metabolically labeled cells expressing RGS16 WT or C98A with [3H]palmitate and immunoprecipitated RGS16. We then treated immunoprecipitates with the protease clostripain, which was expected to yield a 5-kDa fragment containing Cys-98. Consistent with this prediction, we observed palmitate incorporation in a 5-kDa band only in immunoprecipitates of RGS16 WT but not C98A. To assess whether this residue underwent enzymatic palmitoylation directly, we chemically acylated recombinant RGS16 with partially purified PAM from rat liver membranes. Incubation of RGS16 WT or C98A with pPAT and [3H]palmitoyl-CoA led to incorporation of tritium in a band with the pPAT preparation (FIG. 4A, right panel), confirming that the NH2-terminal cysteine residues were the major sites of palmitoylation. RGS4 demonstrated a similar pattern of palmitoylation after incubation with the pPAT preparation (FIG. 4A, right panel). After clostripain cleavage of WT RGS16, we observed [3H]palmitate incorporation in a 5-kDa band only in samples containing RGS16 WT but not C98A. Cys-98 is not required for RGS16 localization at the plasma membrane or in lipid rafts. A, plasma membranes were isolated from cells expressing either RGS16 WT or C98A. Proteins (40 μg) were separated by SDS-PAGE and immunoblotted for RGS16 (arrows, left panel) or Na+/K+-ATPase (a plasma membrane marker, arrowheads, right panel). T, total membrane; P, plasma membrane fractions.
pPAT in a solution assay measuring single turnover GTP hydrolysis by purified Goα. We treated RGS16 or RGS4 with pPAT or boiled pPAT and found that boiling the enzyme dramatically reduced the amount of RGS16 palmitoylation, suggesting that the process was predominantly enzymatic (data not shown). We observed no significant difference in GAP activity toward Goα (RGS16) or Goα (RGS4) between RGS proteins treated with pPAT and untreated proteins (Fig. 5A) or RGS proteins preincubated with pPAT and untreated proteins (Fig. 5A) or RGS proteins preincubated with pPAT and untreated proteins (data not shown). We next measured high affinity GT-Pase activity of the 5-HT1A/Goα fusion protein in membranes incubated with recombinant RGS16 treated with pPAT. When assessed with a single time point (representing a 20-min incubation with 10⁻⁶ M 5-HT), there was little difference in GAP activity between RGS16 and pPAT-treated RGS16 (data not shown). However, because recent studies suggest that RGS proteins may bind to membranes in a time-dependent fashion and that this process may be affected by palmitoylation (22), we preincubated membranes with recombinant RGS16 or RGS4 for various times before agonist stimulation to determine whether palmitoylation affected RGS membrane binding and GAP activity. The GAP activity of RGS16 or RGS4 that had been treated with pPAT and palmitate increased significantly with longer membrane preincubation times (Fig. 5, B–C). In contrast, membranes incubated with either the pPAT preparation alone or untreated RGS proteins failed to exhibit any significant time-dependent increase in GT-Pase activity. Although the GAP activity of untreated RGS16 did not increase over time (Fig. 5B), RGS16-containing membranes displayed significantly higher absolute GT-Pase activity compared with membranes incubated with pPAT alone (2.05 ± 0.21-fold). In contrast, activity of membranes containing pPAT was equivalent to buffer-treated membranes (0.98 ± 0.19-fold), indicating that the pPAT preparation lacked intrinsic GT-Pase or GAP activity. These results suggest that palmitoylation of RGS16 and RGS4 augments GAP activity toward a membrane-bound Goα subunit and that this enhancement is facilitated by RGS-membrane interaction over time.

To confirm the sites of RGS16 palmitoylation and their relative roles in GAP activity, we first measured incorporation of [³H]palmitate into pPAT-treated, recombinant RGS16 WT or C2A/C12A and C98A mutants with or without cleavage with clostripain (Fig. 5C, right panel). Levels of tritium incorporation into full-length RGS16 (30 kDa) were compatible with the number of palmitoylated residues. No palmitoylation of the ~5-kDa band containing Cys-98 was observed with the C98A mutant after clostripain treatment, whereas the palmitoylated fragment containing Cys-98 was clearly visualized with the C2A/C12A mutant. Palmitoylation of Cys-98 in the C2A/C12A mutant was expected in this assay because recombinant RGS16 was incubated with pPAT in vitro. To determine which site of palmitoylation was responsible for increased RGS16 GAP activity induced by membrane interaction, we treated recombinant RGS16 WT, C2A/C12A, and C98A with pPAT and then preincubated proteins with 5-HT1A/Goα membranes before measuring GTPase activity in response to 5-HT. Treatment of the C2A/C12A mutant with pPAT led to a preincubation time-dependent increase in GAP activity that was mildly reduced compared with WT RGS16. After a 2-h preincubation with WT RGS16, agonist-induced GTPase activity (normalized to GTPase activity without agonist addition) of membranes was ~17 pmol/mg/min, whereas GTPase activity of membranes preincubated with the C2A/C12A mutant was reduced ~36% (11 pmol/mg/min). However, GTPase activity of membranes preincubated with pPAT-treated C98A for 2 h was reduced nearly 70% compared with membranes preincubated with pPAT-treated WT RGS16 (5 pmol/mg/min) (Fig. 5C). Similarly, the GTPase activity of membranes incubated with pPAT-treated WT RGS4 for 2 h was ~7 pmol/mg/min, whereas activity in membranes containing pPAT-treated RGS4 (C95A) was ~1.5 pmol/mg/min, an 80% decrease. Therefore, although overall palmitate incorporation was much greater for the RGS16 C98A mutant compared with the C2A/C12A mutant (30-kDa band in panel C), palmitoylation increased the GAP activity of C2A/C12A to a greater extent than C98A. Absolute levels of GAP activity were much lower for palmitoylated RGS16/RGS4 (C98A/C95A) compared with WT RGS proteins, and the activity of these mutants treated with pPAT did not increase substantially over time. From these data, we conclude that although NH₂-terminal cysteine residues are major sites of palmitoylation, internal RGS box cysteine palmitoylation is critical for full GAP activity.

**DISCUSSION**

Studies of palmitoylated residues of RGS proteins have implicated a direct effect of palmitoylation on RGS GAP activity (18, 19, 22). In the accompanying article (33), we demonstrated that NH₂-terminal palmitoylation was required for RGS16 GAP activity in cellular membranes as well as its targeting to lipid rafts. Although NH₂-terminal palmitoylation of RGS16 is not required for plasma membrane localization, it may play a role in the orientation of RGS16 within the membrane or, most intriguingly, in the facilitation of RGS16 palmitoylation on Cys-98, because we found enhanced PAT activity in DRM fractions. The question that remains is how does this internal palmitoylation affect the function of RGS16?

We investigated the mechanism and specificity of palmitoylation of cysteine 98 for RGS16 GAP activity and localization. We found that RGS16 Cys-98 is palmitoylated, similar to the
Fig. 5. RGS palmitoylation increases GAP activity. A, GTPase activity of recombinant Ga (250 nM) was assayed during a single catalytic turnover in the presence of buffer alone or RGS16 (50–100 nM), either untreated or treated with pPAT as indicated. Similar experiments were performed for RGS4 (50 nM) except that Ga (250 nM) was used. Graphs represent the mean ± S.E. of four independent experiments. B, recombinant RGS16 WT or RGS4 WT was treated with the pPAT preparation or buffer alone as described in the legend to Fig. 4 and then
II. Palmitoylation Regulates RGS16

internal palmitoylation on Cys-95 in RGS4 that has been described previously (19). Most importantly, direct enzymatic palmitoylation enhanced RGS16 and RGS4 GAP activity to a similar degree in a membrane-based assay. Mutation of Cys-98 to alanine did not affect RGS16 localization in membrane fractions or lipid rafts but resulted in a loss of incorporation of palmitate on a 5-kDa peptide containing the Cys-98 residue, significantly reducing the increase in RGS16 GAP activity induced by prolonged membrane interactions. Furthermore, mutation of Cys-98 in RGS16 or Cys-95 in RGS4 eliminated inhibition of somatostatin signaling by these RGS proteins to a similar extent as mutation of NH2-terminal palmitoylated cysteine residues (Cys-2 and Cys-12).

In agreement with these results, mutation of the analogous cysteine in RGS10, which is the sole site of palmitoylation, abolishes its ability to inhibit signaling evoked by stimulation of the gonadotropin hormone-releasing hormone receptor (GnHR) (23). In addition, auto-palmitoylation of RGS10 markedly potentiates its GAP activity when it is reconstituted in phospholipid vesicles containing both receptor and G-protein, which most closely approximates our membrane-based GTPase assay (19).

Collectively, these studies firmly establish a prominent role for NH2-terminal palmitoylation as well as palmitoylation of a cysteine residue in the RGS box for the function of RGS4, whereas we used cysteine to alanine mutants of both RGS4 and RGS16.

First, Tu et al. (19) mutated the RGS box cysteine to valine in RGS4 whereas we used cysteine to alanine mutants of both RGS4 and RGS16. Valine is a more hydrophobic residue than alanine; as a result, this substitution could affect membrane interactions independent of palmitoylation. Second, we performed enzymatic palmitoylation at physiological pH (7.4) for a short period of time (45 min), which did not appear to alter the intrinsic GAP activity in single turnover assays (Fig. 5A). In contrast, autopalmitoylation required higher pH (8) and longer incubation times to achieve significant palmitate incorporation (2–6 h). The greater efficiency of palmitoylation achieved using the pPAT preparation is reflected in the stoichiometry of labeling. WT RGS16 incorporated close to 3 mol of palmitate/mol, suggesting 3 palmitoylation sites. In contrast, the lower efficiency of autopalmitoylation (2 mol/mol) may not represent stoichiometry in mammalian cells.

Third, the composition of proteoliposomes used in the former study could affect also RGS function. Phosphatidic acid, and, to a lesser extent, phosphatidylserine, inhibits RGS4 GAP activity, which is dependent on the NH2 terminus of RGS4 (39). Tu et al. (19) utilized proteoliposomes comprised of phosphatidylserine/phosphatidylethanolamine/cholesterol hemisuccinate in a ratio of 8:5:1. It is entirely plausible that phosphatidylserine in these proportions has a more pronounced inhibitory effect on RGS4 palmitoylated at the NH2 terminus. Last, additional proteins in mammalian membrane preparations absent in lipid vesicles could synergize with palmitate to affect RGS conformation or activity. For example, RGS16 interacts with a membrane glycerophosphodiester phosphodiesterase, MIR16, through the NH2-terminal region of the RGS domain (40). Although the effect of MIR16 binding on RGS16 GAP activity has not been studied, this interaction could also contribute to RGS16 anchorage or orientation within the membrane. In addition, RGS16 undergoes phosphorylation on both serine and tyrosine residues (10, 11). RGS16 interacts with the receptor for epidermal growth factor, which mediates RGS16 tyrosine phosphorylation and enhances RGS16 GAP activity. Phosphorylation and palmitoylation may act together to enhance the GAP activity of RGS16 in membranes.

Palmitoylation at the internal cysteine may be involved in the optimal placement of RGS16 or RGS4 within the membrane after an initial docking step. Tu et al. (22) found that RGS4 binds vesicles through its NH2 terminus in a dynamic but ultimately irreversible fashion, which enhanced the GAP activity of RGS4 WT but not RGS4 (C95V) over time. Although the effect of palmitoylation was not reported in these studies, the results are consistent with our experiments demonstrating that the internal palmitoylation site in RGS16 and RGS4 is critical for the incremental increase in GAP activity induced by membrane interactions. Consistent with this notion, RGS4, which lacks the NH2-terminal a-helix and NH2-terminal palmitoylation sites that promote phospholipid vesicle binding, also displays relatively fixed GAP activity over time similar to RGS4 (C95V) (22), or RGS4/RGS16 (C95/98A) (this study).

Alternatively, palmitoylation could induce a conformational change in RGS16 or RGS4 that promotes G-protein binding and catalysis. Because enzymatic palmitoylation did not significantly affect RGS GAP activity in solution, it may fail to induce a gross structural change in the protein. However, because RGS palmitoylation could occur prior to Gα interactions, the...
structure of RGS16 or RGS4 in the membrane may be different before and after G-protein binding. Cys-95 in RGS4 lies on helix 4 in close proximity to the loop between helices 3 and 4, which forms part of the binding surface with Go switch region I. A palmitoylation group on Cys-95 in RGS4 or Cys-98 in RGS16 could re-orient the adjacent contact residues (particularly Tyr-87, Ser-88, Glu-90, and Asn-91 in human RGS16) to facilitate more efficient binding, which would be predicted to enhance GAP activity (28, 41) (Fig. 6). A recent study demonstrated that mutation of Glu-89 (mouse RGS16) to lysine did not substantiate GAP activity of both RGS16 and RGS4 in the membrane may be different before and after G-protein binding. Cys-95 in RGS4 lies on helix 4 and Go switch I that may only be evident in membranes. This binding surface could be affected by palmitoylation of the resident cysteine residue in this region.

Finally, the poor activity or RGS16 (C98A) in functional assays despite its normal localization to lipid rafts lend further support to the view that abnormal DRM localization of either RGS16 (C98A) or (C2A/C12A) cannot account for their lack of activity. Our results show that palmitoylation plays an important positive regulatory role in the GAP activity of both RGS16 and RGS4. In the future, it will be of great interest to study the regulation and activity of RGS proteins in their native cellular context, which should further illuminate specific physiological functions.

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PALMITOYLATION OF A CYSTEINE RESIDUE IN THE RGS BOX IS CRITICAL FOR RGS16 GTPase ACCELERATING ACTIVITY AND REGULATION OF Gi-COUPLED SIGNALING

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