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

I. MUTATION OF AMINO-TERMINAL CYSTEINE RESIDUES ON RGS16 PREVENTS ITS TARGETING TO LIPID RAFTS AND PALMITOYLATION OF AN INTERNAL CYSTEINE RESIDUE

Abel Hioi‡§, Penelope C. Davey‡§, James L. Osterhout¶, Abdul A. Waheed‡, Elizabeth R. Fischer†, Ching-Kang Chen‡‡, Graeme Milligan‡‡, Kirk M. Druey‡‡, and Teresa L. Z. Jones‡‡‡

From the ‡Metabolic Diseases Branch, NIDDK, National Institutes of Health, Bethesda, Maryland 20892, the §Molecular Signal Transduction Section, Laboratory of Allergic Diseases, NIAID, National Institutes of Health, Rockville, Maryland 20892, the ¶Microscopy Branch, NIAID, National Institutes of Health, Hamilton, Montana 59840, the †Department of Ophthalmology, University of Utah, Salt Lake, Utah 84112, and the ‡‡Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, United Kingdom

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§§ To whom correspondence should be addressed: Bldg. 10, Rm. 9C101, National Institutes of Health, Bethesda, MD 20892-1802. Tel.: 301-496-8711; Fax: 301-496-0200; E-mail: tlzj@helix.nih.gov.

† Both authors contributed equally to this work.

‡ Both authors contributed equally to this work.

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Heterotrimeric G proteins transmit extracellular signals by coupling cell surface receptors to intracellular effectors (1, 2). The heterotrimer consists of Gα subunits that bind GDP/GTP and Gβγ subunits that form a hydrophobic complex. Upon activation by a receptor, the Gα subunit exchanges GDP for GTP and in this active state modulates effectors. Regulators of G-protein signaling (RGS) proteins bind to Gα subunits during GTP hydrolysis and accelerate GTP hydrolysis. RGS proteins are a family of over 20 proteins that share significant homology in a domain called the RGS box that binds the Gα subunit (3–5). This family of proteins can be subdivided on the basis of other domains outside the RGS box. RGS16 shares significant homology with RGS4 and RGS5 and consists of short amino- and carboxyl-terminal ends on either side of the RGS box.

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I. MUTATION OF AMINO-TERMINAL CYSTEINE RESIDUES ON RGS16 PREVENTS ITS TARGETING TO LIPID RAFTS AND PALMITOYLATION OF AN INTERNAL CYSTEINE RESIDUE

Regulators of G-protein signaling (RGS) proteins down-regulate signaling by heterotrimeric G-proteins by accelerating GTP hydrolysis on the Gα subunits. Palmitoylation, the reversible addition of palmitate to cysteine residues, occurs on several RGS proteins and is critical for their activity. For RGS16, mutation of Cys-2 and Cys-12 blocks its incorporation of [3H]palmitate and ability to turn-off Gα and Gq signaling and significantly inhibited its GTPase activating protein activity toward a Gα subunit fused to the 5-hydroxytryptamine receptor 1A, but did not reduce its plasma membrane localization based on cell fractionation studies and immunoelectron microscopy. Palmitoylation can target proteins, including many signaling proteins, to membrane microdomains, called lipid rafts. RGS16 in rat liver membranes and overexpressed RGS16 in COS cells, but not the nonpalmitoylated cysteine mutant of RGS16, localized to lipid rafts. However, disruption of lipid rafts by treatment with methyl-β-cyclodextrin did not decrease the GTPase activating protein activity of RGS16. The lipid raft fractions were enriched in protein acyltransferase activity, and RGS16 incorporated [3H]palmitate into a peptide fragment containing Cys-98, a highly conserved cysteine within the RGS box. These results suggest that the amino-terminal palmitoylation of an RGS protein promotes its lipid raft targeting and allows palmitoylation of a poorly accessible cysteine residue that we show in the accompanying article (Osterhout, J. L., Waheed, A. A., Hioi, A., Ward, R. J., Davey, P. C., Nini, L., Wang, J., Milligan, G., Jones, T. L. Z., and Druey, K. M. (2003) J. Biol. Chem. 278, 19309–19316) was critical for RGS16 and RGS4 GAP activity.

The abbreviations used are: RGS, regulator of G protein signaling; 5-HT, 5-hydroxytryptamine; 5-HT1A/Gα1, fusion protein of the 5-HT receptor subtype 1A with the mutant (C361G) Gα1 protein; PBS, phosphate-buffered saline; CD, methyl-β-cyclodextrin; GAP, GTPase activating protein; WT, wild type; DRM, detergent-resistant membranes; HA, hemagglutinin; PAT, protein acyltransferase; HEK, human embryonic kidney; Tricine, N-(2-hydroxyethyl)glycine; AMP-PNP, adenosine 5’-(β,γ-imino)triphosphate; HB, homogenization buffer.
Purification—The cDNA for mouse RGS16 was inserted into the pcDNA3 vector (Invitrogen). Cysteine to alanine mutations for residues 2 and 12 in RGS16 were described previously (9). The cDNA for human RGS16 was inserted into the pcDNA3 vector with the DNA sequence for the hemagglutinin epitope in-frame at the 5’ end of the cDNA for RGS16. Mutation of Cys-98 in HA-RGS16 was performed using the QuikChange mutagenesis kit (Stratagene). Construction of the pcDNA3 vector containing the cDNA for the 5-hydroxytryptamine (serotonin) receptor subtype 1A fused in-frame at the 5’ end of the CT-265 antiserum or a polyclonal antibody to the HA epitope (anti-HA.11, Covance Inc.), and antibodies to cavin (BD Transduction Laboratories) and Na+/K+-ATPase (Biomol Research Laboratories). Antibody binding was detected by enhanced chemiluminescence (Amer sham Biosciences). For immunoprecipitation, 500 µg of protein was incubated with 5 µl of the CT-265 antiserum or a polyclonal antibody to the HA epitope (Santa Cruz Biotechnologies) and prepared as described (26) except that after separation by SDS-PAGE, the tritium-labeled proteins were transferred to nitrocellulose membranes. Fluorography was performed by coating the membranes with EA wax (EA Biotech Ltd.) and placing in film cassettes with MS film (Kodak) at −70°C. The exposure times ranged from 3 to 7 days.

Preparation of Membranes for GTPase Assay—48 h after transfection with plasmids encoding HA-RGS16 (WT or mutants), HEK-293 cells stably expressing the 5-HT4A/Ga11 activity. 48 h after transfection, the cells were scraped and harvested in PBS and centrifuged at 2,000 × g for 10 min at 4°C. The cell pellet was resuspended in binding buffer (10 mM HEPES (pH 7.4), 25 mM KCl, 0.5 mM MgCl2, 1 mM dithiothreitol, 200 µM EDTA, pH 7.5, 80 mM Tris, pH 7.5, 1 µM GTP) spiked with 50,000 cpm of [γ-32P]GTP (Amersham Biosciences, 3000 Ci/mmol) and incubated for 20 min at 30°C. The reactions were stopped by addition of ice-cold 10% (w/v) activated charcoal in 50 mM phosphoric acid followed by centrifugation at 10,000 × g for 20 min at 4°C. Supernatants containing free [γ-32P]phosphate were analyzed using liquid scintillation spectrometry. Nonspecific GTPase activity was determined in simultaneous reactions containing 100 mM GTP.

Plasma Membrane Isolation—Livers (6–9 g) from male rats, CD strain (Charles River Laboratories), were rapidly excised, washed in ice-cold buffer (IB) consisting of 0.25M sucrose, 5 mM Tris-HCl, pH 7.4, and 1 mM MgCl2 and cut into about 2–3-mm pieces. The rat liver pieces or COS cell pellets were homogenized in 5 volumes of HB with 10 strokes in a Dounce homogenizer, and the liver samples were filtered through four layers of moistened gauze. The COS cell homogenate or the filtered homogenate from liver underwent centrifugation at 3,000 × g for 5 min. The supernatant was saved, and homogenization and centrifugation of the pellet was repeated in half the original volume of HB. The supernatants were combined and centrifuged at 3,000 × g for 10 min. The pellet (total membrane fraction) was suspended in HB to obtain the final buffer concentration to 1.42M sucrose in 5 mM MgCl2, pH 7.4, and 1 mM MgCl2 and cut into about 2–3-mm pieces. The rat liver pieces or COS cell pellets were homogenized in 5 volumes of HB in 10 strokes at a Dounce homogenizer, and the liver samples were filtered through four layers of moistened gauze. The COS cell homogenate or the filtered homogenate from liver underwent centrifugation at 3,000 × g for 5 min. The supernatant was saved, and homogenization and centrifugation of the pellet was repeated in half the original volume of HB. The supernatants were combined and centrifuged at 3,000 × g for 10 min. The pellet (total membrane fraction) was suspended to bring the final buffer concentration to 1.42M sucrose in 5 mM MgCl2, pH 7.4, and 1 mM MgCl2 and used as the source of plasma membrane proteins. The suspension was adjusted to 1.42M sucrose in 5 mM MgCl2, pH 7.4, and 1 mM MgCl2 and used as the source of plasma membrane proteins.

Cell Culture, Transfection, and Metabolic Labeling—Stable transfections of HEK293 cells with the plasmid containing the 5-HT4A/Ga11 fusion protein was performed and the cells maintained as described (25). COS-7 and HEK293 cells were transfected using 10 µg of DNA in 50 µl of the CT-265 antiserum or a polyclonal antibody to the HA epitope (anti-HA.11, Covance Inc.), and antibodies to cavin (BD Transduction Laboratories) and Na+/K+-ATPase (Biomol Research Laboratories). Antibody binding was detected by enhanced chemiluminescence (Amer sham Biosciences). For immunoprecipitation, 500 µg of protein was incubated with 5 µl of the CT-265 antiserum or a polyclonal antibody to the HA epitope (Santa Cruz Biotechnologies) and prepared as described (26) except that after separation by SDS-PAGE, the tritium-labeled proteins were transferred to nitrocellulose membranes. Fluorography was performed by coating the membranes with EA wax (EA Biotech Ltd.) and placing in film cassettes with MS film (Kodak) at −70°C. The exposure times ranged from 3 to 7 days.

Preparation of Membranes for GTPase Assay—48 h after transfection with plasmids encoding HA-RGS16 (WT or mutants), HEK-293 cells stably expressing the 5-HT4A/Ga11 fusion protein were treated overnight with pertussis toxin (50 mg/ml) to eliminate endogenous Ga11 activity. 48 h after transfection, the cells were scraped and harvested in PBS and centrifuged at 2,000 × g for 10 min at 4°C. The cell pellet was resuspended in binding buffer (10 mM HEPES (pH 7.4), 25 mM KCl, 0.5 mM MgCl2, 1 mM dithiothreitol, 200 µM EDTA, pH 7.5, 80 mM Tris, pH 7.5, 1 µM GTP) spiked with 50,000 cpm of [γ-32P]GTP (Amersham Biosciences, 3000 Ci/mmol) and incubated for 20 min at 30°C. The reactions were stopped by addition of ice-cold 10% (w/v) activated charcoal in 50 mM phosphoric acid followed by centrifugation at 10,000 × g for 20 min at 4°C. Supernatants containing free [γ-32P]phosphate were analyzed using liquid scintillation spectrometry. Nonspecific GTPase activity was determined in simultaneous reactions containing 100 mM GTP.

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Thin sections were cut with an RMC MT-7000 ultramicrotome (Boeckeler, Tucson, AZ) stained with 1% uranyl acetate and observed at 80 kV on a Philips CM-10 transmission electron microscope (FEI, Hillsboro, OR). Images were acquired with an AMT digital camera (Advanced Microscopy Techniques) and processed using Adobe Photoshop version 7 (Adobe Systems).

**Detergent-resistant Membrane (DRM) Partitioning and Cellular Cholesterol Depletion and Determination**—Lipid raft isolation was carried out as previously described (29, 30). Briefly, cell pellets from transfected COS or HEK293 cells, or the plasma membrane fraction from rat livers were resuspended and incubated for 20 min at 4 °C in cold buffer containing 0.5% Triton X-100 to give a detergent to protein ratio of 5:1. The samples were adjusted to 35% (v/v) OptiPrep and the DRM fraction separated by centrifugation on a 50%/35% OptiPrep gradient. For depletion of cellular cholesterol, cells were incubated in serum-free Dulbecco’s modified Eagle medium containing 10 mM methyl-β-cyclodextrin (Sigma) for 30 min and then harvested. The concentration of cholesterol in the cell lysates was determined as described (21).

**Protein Acyltransferase Activity**—HEK293 cells stably expressing the 5-HT1A/Galpha1 fusion protein were treated with detergent and separated on an OptiPrep gradient as described above except six fractions of 0.78 ml were taken. Three μg of protein from each fraction was incubated for 45 min at 30 °C with 10 μg of purified RGS16, 200 μM coenzyme A, 2 mM ATP, and 1 μl of [9,10-^3^H]palmitate (American Radiolabeled Chemicals, 30–60 Ci/mmol) in a final volume of 100 μl as described (31). The reaction was stopped by the addition of sample buffer and the proteins separated by SDS-PAGE. The gels were either stained with Microwave-Blue (Protiga, Frederick, MD), or the proteins were transferred to nitrocellulose paper and prepared for fluorography.

**Clostridin Treatment**—After the final washing step, immunoprecipitates bound to protein A-Sepharose (Amersham Biosciences) were resuspended in 30 μl of a buffer containing 75 mM NaPO4 and 2.5 mM dithiothreitol with and without 1.5 units of clostripain (Sigma) and incubated for 2 h at room temperature. The reaction was stopped by the addition of sample buffer (Novex) and boiling. The peptides were separated by SDS-PAGE on 10–20% Tricine gels (Invitrogen), and prepared for fluorography. MS films (Eastman Kodak) were exposed to the nitrocellulose membranes for 3 days at -70 °C.

**RESULTS**

**GAP Activity of the C2A/C12A Mutant after Transfection**—In a previous report (9), we found that two amino-terminal cysteine residues (Cys-2 and Cys-12) on RGS16 were critical for palmitoylation and in vitro function, but were not necessary for membrane attachment and in vitro GAP activity. We tested whether the failure of the C2A/C12A mutant to turn-off G_i and G_q signaling was because of the inability of the mutant to accelerate the GTP hydrolysis of the Go subunit in cellular membranes. To test GAP activity, we used a fusion protein in which the carboxyl terminus of the 5-HT1A receptor is fused to the amino terminus of a mutant form of Go alpha1 (C351G) that is resistant to pertussis toxin treatment (25). In HEK293 cells stably expressing the fusion protein, GTPase activity of the fused Go subunit in response to 5-HT is determined after inactivating endogenous Go and Go proteins by pertussis toxin. This method reduces GTP turnover of the endogenous Go and Go proteins, as well as ensuring a 1:1 stoichiometry of receptor to Go subunit (32). For these experiments, the stably transfected cells expressing the fusion protein were transiently transfected to co-express either the WT RGS16 or the C2A/C12A mutant with a hemagglutinin (HA) epitope tag at the amino terminus. The level of expression of these proteins in the membrane was similar (Fig. 1A). As expected, 5-HT increased GTPase activity in membranes expressing the fusion protein alone (Fig. 1B, open diamonds). Membranes from cells co-expressing WT RGS16 showed a marked additional increase in GTPase activity in response to 5-HT (Fig. 1B, filled circles). The 5-HT-induced GTPase activity in membranes from cells expressing the C2A/C12A mutant was only slightly better than the membranes from the vector-transfected cells (Fig. 1B, filled squares). We tested whether the C2A/C12A mutation itself could affect RGS16 GAP activity by adding purified RGS16 proteins expressed in bacteria to the membranes of the 5-HT1A/Go alpha1 fusion-protein-expressing cells. Addition of WT RGS16 or RGS16 (C2A/C12A) to the membranes resulted in a nearly identical increase in 5-HT-evoked GTPase activity at each RGS16 concentration (Fig. 1C). These results indicate that the poor function of the nonpalmitoylated C2A/C12A mutant of RGS16 within cells (Fig. 1B) (9) was likely because of altered intracellular processing or targeting.

**Plasma Membrane Localization of the WT and Mutant RGS16**—We investigated whether palmitoylation was critical for the plasma membrane localization of RGS16 because G protein signaling occurs on the cytoplasmic face of the plasma membrane and other signaling proteins such as Go alpha1 (33), Go alpha (34), H-Ras (35), and p59(126) (36) require palmitoylation for plasma membrane localization. COS cells transfected with the WT RGS16 or the double cysteine mutant, C2A/C12A, were homogenized and subcellular fractions were obtained by gradi-

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**FIG. 1.** GAP activity of an amino-terminal cysteine mutant of RGS16 after transfection or reconstitution. A and B, HER293 cells stably expressing a fusion protein between the 5-HT1A receptor and Go alpha1 (C351G) were transiently transfected with plasmids containing LocZ, as a control, HA-RGS16 or HA-RGS16 with Cys-2 and Cys-12 mutated to alanine (C2A/C12A). The cells were pretreated with pertussis toxin to block the activity of the endogenous Go and Go proteins. 48 h after transfection, membranes were prepared and 50 μg of protein was separated by SDS-PAGE and prepared for immunoblotting with an antibody to the HA epitope (A) or for agonist-stimulated steady-state GTPase activity as determined after addition of the indicated concentrations of 5-HT and incubation for 20 min at 37 °C (B). C, the WT and C2A/C12A mutant of RGS16 were expressed in bacteria, purified, and incubated with membranes of HEK293 cells expressing the 5-HT1A/ Go alpha1 fusion protein. High-affinity GTPase activity was determined in the presence of 1 μM 5-HT. For panels B and C, values represent the mean ± S.E. of four independent experiments each. For panel A, molecular mass markers in kDa are shown to the left.
centrifugation. Both the WT RGS16 and the nonpalmitoylated C2A/C12A mutant were found to a similar degree in the fraction that was enriched in the plasma membrane protein, Na+/K+ -ATPase (Fig. 2). In these experiments, we expressed RGS16 without any epitope tags and used the CT-265 antiserum raised against the whole protein. As in previous experiments (9), we found two bands for RGS16 after separation by SDS-PAGE and immunoblotting with the CT-265 antiserum. These two bands are found in rat liver membranes (Fig. 4A) and after expression of the HA-tagged RGS16 when the CT-265 antiserum is used for detection. The lower band could represent a degradation product missing part of the amino terminus because it is not detected with the HA antibody (Fig. 1A).

We also used immunoelectron microscopy to determine the intracellular localization of the WT RGS16 and nonpalmitoylated C2A/C12A mutant in HEK293 cells transfected with HA-tagged RGS16. Immunoblotting of cell lysates from HEK293 cells expressing RGS16 proteins with an anti-HA antibody revealed only one band at about 30 kDa (Fig. 1A). Plasma membrane staining was seen for cells expressing the WT RGS16 and the C2A/C12A mutant (Fig. 3, A and B). RGS16-expressing cells incubated without the HA antibody or vector-transfected cells showed only a trivial amount of staining (Fig. 3, C and D). These results indicate that the nonpalmitoylated C2A/C12A mutant was localized to the plasma membrane in mammalian cells as well as WT RGS16. Therefore, the poor function of nonpalmitoylated C2A/C12A mutant of RGS16 cannot be explained simply by mislocalization from the plasma membrane.

**Lipid Raft Targeting**—Another function of palmitoylation is targeting proteins to membrane microdomains, called lipid rafts (16, 17). Gq subunits, including Goq and Gqα15, which are regulated by RGS16, are located in lipid rafts (37, 38). We isolated lipid rafts by treating cells or membranes with cold, Triton X-100 and separating the DRM fraction by OptiPrep gradient centrifugation. Endogenous RGS16 in rat liver plasma membrane partitioned to DRM fractions 1 and 2 (Fig. 4A). Goq was also found in DRM fraction 1. Na+/K+ -ATPase, a plasma membrane protein not found in lipid rafts (30), was in higher density fractions 7–9. RGS16 after transfection in COS cells was also found in the DRM fractions (Fig. 4B). Caveolin, a lipid raft protein, and Goq were in DRM fractions 1 and 2, and Na+/K+ -ATPase was in higher density fractions 6–9 in these cells. The RGS16 in the detergent-soluble fractions would contain not only any of the membrane-bound RGS16 not in lipid rafts, but also a significant amount (about 50% of the total) of both the WT and C2A/C12A mutant found in the cytosolic fraction after transfection (data not shown) (9). The nonpalmitoylated, C2A/C12A mutant of RGS16 showed a shift away from the DRM fraction to intermediate fractions 2–5. This result suggests that palmitoylation was important in targeting...
RGS16 to lipid rafts. The plasma membrane may contain multiple microdomains with different densities after detergent treatment and gradient centrifugation (23) and the nonpalmitoylated C2A/C12A mutant may localize to such a domain.

We then tested the role of lipid rafts for RGS16 function because the decrease in GAP activity of the nonpalmitoylated C2A/C12A mutant could be due to a decrease of this mutant in the lipid rafts. We disrupted lipid rafts by treating cells with methyl-β-cyclodextrin (CD), a cholesterol-binding agent that depletes cellular cholesterol. In HEK293 cells, treatment with 10 mM CD for 30 min depleted the cellular cholesterol by 42% (mean ± S.E. of three experiments). WT RGS16 and Gβi were in DRM fraction 1 (Fig. 6A). The WT RGS16 was distributed to DRM fraction 1 and the detergent-soluble fractions. Thus, the inability of the nonpalmitoylated, C2A/C12A mutant of RGS16 to increase the GTPase activity of the 5-HT1A/Gαi1 fusion protein compared with the WT RGS16 was unlikely to be solely because of mistargeting away from lipid rafts, because the 5-HT1A/Gαi1 fusion protein was not in the lipid raft fraction. Taken together, the results with cyclodextrin treatment and DRM fractionation suggest that RGS16 did not require co-localization with its cognate Go subunit in a lipid raft to act as a GAP in cell membranes.

Lipid rafts are the home to many acylated proteins (16, 17). A recent report showed an enrichment of PAT activity in a low density membrane fraction (23). We tested the PAT activity of the DRM and detergent-soluble fractions after OptiPrep gradient centrifugation of the HEK293 cells stably expressing the 5-HT1A/Gαi1 fusion protein. As in other experiments, caveolin and Gβi were in the lower density fractions 1–2 and Na+/K+-ATPase in the higher density fractions 4–6. The fractions were incubated with [3H]palmitate and purified RGS16 as the substrate, and the level of tritium incorporation into RGS16 was determined by fluorography (Fig. 6B). Staining of the gels with Coomassie Blue showed equivalent amounts of RGS16 in each lane (data not shown). An increase in tritium incorporation was seen in the 30-kDa band for samples incubated with fractions 1 and 2. In agreement with a previous study using different fractionation methods (23), these results show that PAT activity was enriched in membrane fractions containing proteins found in lipid rafts.

**Palmitoylation of an Internal Cysteine Residue**—We investigated whether another defect was present in the C2A/C12A
were obtained from Refs. 4, 5, and 46. The underlined residues are in contact with the switch region of Gαi, in the crystal structure of the RGS4-Gαi complex (44). The RGS sequences were obtained from Refs. 4, 5, and 46–48.

### Table I. Mammalian RGS proteins with a cysteine residue in the α4 helix of the RGS box

| RGS proteins with Cys-98 in RGS16 | Cysteine residues in other positions in the α4 helix<sup>a</sup> | No cysteine residues in the α4 helix<sup>b</sup> |
|----------------------------------|---------------------------------------------------------------|--------------------------------------------------|
| RGS1                            | RGS13 PDZRhoGEF (−1)                                         | RGS6 (Val)                                       |
| RGS2                            | RGS14 p115RhoGEF (−3, −4)                                    | RGS7 (Val)                                       |
| RGS3                            | RGS16 LARG (−4)                                              | RGS-PX1 (Val)                                   |
| RGS4                            | RGS18 D-ACKAP2 (Ala)                                         |                                                  |
| RGS5                            | RGS-GAP GRK2 (Ile)                                           |                                                  |
| RGS8                            | RGSZ1 GRK3 (Ile)                                             |                                                  |
| RGS9                            | RET-RGS1                                                    |                                                  |
| RGS10                           | Conductin                                                   |                                                  |
| RGS11                           | Axin                                                        |                                                  |
| RGS12                           |                                                              |                                                  |

<sup>a</sup> Position of cysteine relative to Cys-98 in RGS16 is shown in parentheses.

<sup>b</sup> The residue in the Cys-98 position is shown in parentheses.

#### FIG. 7. [3H]Palmitate incorporation into RGS16. A, COS cells were transfected with vector alone or with the cDNAs for the WT or C2A/C12A mutant of RGS16. Two days after transfection, the cells were incubated with [3H]palmitate, homogenized, and separated into particulate and soluble fractions. The RGS16 proteins in the particulate fractions underwent immunoprecipitation with the CT-265 antiserum to RGS16 separation by SDS-PAGE and fluorography with exposure to MS film for 7 days. B and C, COS cells were transfected with vector alone or with the cDNAs for the HA-tagged WT or C98A mutants of RGS16. Two days after transfection, the cells were incubated with [35S]methionine (B) or [3H]palmitate (C), homogenized, and separated into particulate and soluble fractions. The RGS16 proteins in the particulate fractions underwent immunoprecipitation with the HA antiserum to RGS16 and the immunoprecipitates were treated with or without the protease, clostripain (clostr.). The reaction was stopped by the addition of sample buffer and the proteins separated by SDS-PAGE using 10–20% Tricine gels and prepared for fluorography and exposure to film for 2 (B) or 7 (C) days. The arrow indicates the full-length RGS16 and the arrowhead indicates the 5-kDa band. The molecular mass markers in kDa are shown to the left.

mutant of RGS16 to explain its diminished GAP activity. Another role for palmitoylation on Cys-2 and Cys-12 could be palmitoylation of an internal cysteine residue that is conserved within the RGS box of many RGS proteins (Table I). Palmitoylation on Cys-95 in RGS4 and Cys-66 in RGS10 modifies the GAP activity of these proteins (13). RGS4 requires Cys-2 and Cys-12 for autopalmitoylation of Cys-95, and [3H]palmitate incorporation on Cys-2 and Cys-12 precedes that on Cys-95 (13). COS cells expressing the WT RGS16 or C2A/C12A mutant after transient transfection were metabolically labeled with [3H]palmitate, and the RGS16 proteins in the particulate fractions were immunoprecipitated with the CT-265 antiserum. Both RGS16 WT and C2A/C12A were expressed to a similar degree in the membrane fractions (data not shown). Tritium incorporation was seen in a 30-kDa band for the cells expressing RGS16 WT, but not for the RGS16 (C2A/C12A)-expressing cells (Fig. 7A).

We tested for an additional site in RGS16 that was critical for palmitoylation by transfecting COS cells with vector alone or with the cDNAs for the WT RGS16 or a mutant in which Cys-98 is changed to alanine, and metabolically labeling with [3H]palmitate or [35S]methionine. The C2A/C12A mutant was not used in these experiments because it did not incorporate [3H]palmitate after metabolic labeling in cells (Fig. 7A). RGS16 was immunoprecipitated and treated with the protease, clostripain, which cleaves peptides after arginine residues. Complete cleavage of RGS16 with clostripain results in a 4926-dalton fragment, the peptide from residues 64 to 105 that contains Cys-98 as the only cysteine residue, and in smaller fragments (3390 to 409 daltons). Immunoprecipitation of the [35S]methionine-labeled fraction showed that both of the proteins were expressed and proteolyzed to the same degree (Fig. 7B). The 4926-dalton fragment does not contain any methionine residues and would not be detected by this method. Separation by SDS-PAGE of the [3H]palmitate-labeled peptide after clostripain treatment, followed by fluorography, showed a band at about 5 kDa for the WT RGS16-transfected cells that was not present in the vector or C98A-transfected cells (Fig. 7C). We often saw a tritium-labeled band at about 16 kDa that most likely represents a degradation product of RGS16 because it was not seen in the vector-transfected cells. This result suggests that WT RGS16 undergoes palmitoylation on the Cys-98 residue and that this modification requires Cys-2 and Cys-12. The functional effects of palmitoylation on Cys-98 are reported in the accompanying article (24).

### DISCUSSION

Regulation of G protein signaling occurs at many steps during the GTPase activation cycle. RGS proteins accelerate the...
GTP hydrolysis of Go subunits to return them to their basal state. For RGS proteins to work, they need to be at the cytoplasmic face of the plasma membrane where they can bind to the Go subunit in its transition state of GTP hydrolysis. We found that cysteine residues on the amino terminus of RGS16 were important for accelerating the GTPase activity of the Go subunit at the membrane, for targeting RGS16 to lipid rafts, and for palmitoylation of an internal cysteine residue. Based on these results, we propose a new function of protein palmitoylation: the targeting of proteins to lipid rafts that are enriched in palmitoyltransferase activity to allow palmitoylation of poorly accessible cysteine residues.

Plasma Membrane Targeting—Previous studies with RGS16 and a closely related protein, RGS4, showed that an amphipathic α helix near the amino terminus is a key determinant in plasma membrane targeting for these proteins in yeast (14, 39). Mutation of hydrophobic and basic residues in this amphipathic helix encompassing the first 33 amino acids, decreases the plasma membrane localization and biologic activity of these proteins (14, 39), whereas mutation of palmitoylation sites had a minor effect on localization (12, 14). In addition to the amphipathic α helix, the amino-terminal end of the RGS domain of RGS16 interacts with an integral membrane protein, MIR16, found at the plasma membrane in liver cells (40). Sites for palmitoylation are often found near membrane targeting signals (41). A two-signal model of membrane targeting predicts that proteins first find a docking partner at the membrane and then undergo palmitoylation that locks the protein in place (42, 43). The utility of palmitoylation for membrane targeting is then dependent on the affinity of the targeting signal for the binding partner. For RGS16, the loss of palmitoylation did not significantly change the plasma membrane localization as detected by fractionation studies and electron microscopy in mammalian cells. Therefore, the amphipathic α helix and possibly other targeting signals are adequate to keep RGS16 at the plasma membrane without palmitoylation stabilizing the binding. The significant amount of RGS16 found in the cytosolic fraction after overexpression may be because of a saturation of RGS16 membrane binding sites.

Lipid Raft Targeting and RGS16 Function—Lipid rafts contain signaling proteins and are critical for a number of signaling pathways (18–20). We found RGS16 in lipid rafts and the amino-terminal cysteine residues were necessary for this targeting. A simple explanation for the poor signaling function of the C2A/C12A mutant would be mistargeting from the lipid rafts. Yet, the GAP activity of RGS16 was intact after disrupting lipid rafts, which are enriched in PAT activity. Within the environment of increased PAT activity, rotation of the α helix or the whole protein that exposes Cys-98 to the membrane could lead to its palmitoylation. In the following article (24), we show that palmitoylation on Cys-98 greatly enhanced the GAP activity of RGS16. RGS16 does not quickly take a seat at the membrane, but instead nestles in until it finds a comfortable position to optimally interact with the Go subunit.

Acylation has many roles for protein function including membrane attachment, plasma membrane and lipid raft targeting, and conformational changes. The function of palmitoylation, like phosphorylation, another reversible protein modification, is likely to be specific for each protein. For RGS16, palmitoylation may have two roles: 1) amino-terminal palmitoylation directs the proteins to lipid rafts to allow palmitoylation of an internal cysteine residue; and 2) palmitoylation of this internal cysteine residue increases the GAP activity of the protein through changes in the conformation or orientation at the membrane. In the future, we may find even more functions of palmitoylation in protein regulation.

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MUTATION OF AMINO-TERMINAL CYSTEINE RESIDUES ON RGS16 PREVENTS ITS TARGETING TO LIPID RAFTS AND PALMITOYLATION OF AN INTERNAL CYSTEINE RESIDUE

Abel Hiol, Penelope C. Davey, James L. Osterhout, Abdul A. Waheed, Elizabeth R. Fischer, Ching-Kang Chen, Graeme Milligan, Kirk M. Druey and Teresa L. Z. Jones

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