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Palmitoylation and Plasma Membrane Targeting of RGS7 Are Promoted by α_o

Satoshi Takida, Christopher C. Fischer, and Philip B. Wedegaertner

Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania

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ABSTRACT

Regulator of G protein signaling (RGS) proteins modulate G protein signaling by acting as GTPase-activating proteins for G protein α-subunits. RGS7 belongs to a subfamily of RGS proteins that exist as dimers with the G protein β_5-subunit. In this report, we addressed the mechanisms of plasma membrane localization of β_5RGS7. When expressed in human embryonic kidney 293 cells, β_5RGS7 was found to be cytoplasmic and soluble. Expression of α_o promoted a strong redistribution of β_5RGS7 to the plasma membrane. Expression of α_q, however, failed to affect the subcellular localization of β_5RGS7. The constitutively active mutant α_oR179C, like wild-type α_o, strongly recruited β_5RGS7 to plasma membranes; however, inactive α_oG204A, RGS-insensitive α_oG184S, and lipidation-deficient α_oG2A were all defective in the ability to promote plasma membrane localization of β_5RGS7. In addition, palmitoylation of RGS7 was demonstrated, and palmitoylation required expression of α_o or α_oR179C. To examine potential palmitoylation sites of RGS7, several cysteines were substituted with serines. β_5RGS7C133S failed to localize to plasma membranes when coexpressed with α_o, suggesting cysteine 133 of RGS7 as a putative palmitoylation site. Finally, deletion of amino acids 76 to 128 of RGS7, which includes part of the disheveled, EGL-10, pleckstrin (DEP) domain, prevented α_o-mediated plasma membrane recruitment of β_5RGS7. These findings are the first to demonstrate Gα-regulated plasma membrane localization and palmitoylation of β_5RGS7 and suggest that membrane targeting of β_5RGS7 is a complex process requiring at least RGS domain-mediated interaction with α_o and RGS7 palmitoylation.

Heterotrimeric G proteins, composed of α- and $\beta\gamma$-subunits, function as a molecular switches, relaying extracellular stimuli to cytoplasmic signaling pathways. Nucleotide exchange of GTP for GDP on the α-subunit sets off a signaling cascade, whereas hydrolysis of the α-bound GTP turns off the signaling. A group of proteins called regulators of G protein signaling (RGS) accelerates this GTP hydrolysis and thus modulates the duration of the signal transduction. More than 20 subtypes of the RGS proteins have been identified and are commonly divided into six groups (Hollinger and Hepler, 2002).

RGS7 belongs to the R7 subfamily of the RGS proteins that contain a domain called the Gγ-like (GGL) domain (Sondek and Siderovski, 2001; Witherow and Slepak, 2003). Through this unique domain, RGS7 interacts with the G protein β_5-subunit, which deviates significantly from the other four β-subunits (Snow et al., 1998). Native β_5RGS7 complexes have been isolated from brain extracts (Witherow et al., 2000; Zhang and Simonds, 2000), and copurification experiments suggest that RGS7 always exists as a heterodimer with β_5 (Witherow et al., 2000). One critical role for the interaction of RGS7 and β_5 is to mutually stabilize each other. Efficient expression of RGS7 in COS-7 cells depends on coexpression of β_5 and vice versa (Snow et al., 1999; Witherow et al., 2000), and, moreover, loss of β_5 in a mouse knockout causes the complete loss of detectable RGS7 protein in retina and brain extracts (Chen et al., 2003).

Less well understood is the physiological role of β_5RGS7, in particular which G protein α-subunits interact with and are regulated by β_5RGS7. In vitro GAP assays have demonstrated that β_5RGS7 acts almost exclusively on α_o and not α_i or α_q (Posner et al., 1999; Hooks et al., 2003). In contrast, β_5RGS7 seems to show less α-subunit selectivity in various cell systems. For example, β_5RGS7 was found to regulate G$\alpha_{i/o}$ protein-coupled receptor activated K^+ channels (Keren-Raifman et al., 2001) and to attenuate Ca^{2+} mobilization mediated by α_q (Shuey et al., 1998), suggesting interaction with both α_o and α_q in cells. A recent report demonstrated

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ABBREVIATIONS: RGS, regulator of G protein signaling; GGL, Gγ-like; FRET, fluorescence resonance energy transfer; PM, plasma membrane; HEK, human embryonic kidney; HA, hemagglutinin; DEP, disheveled, EGL-10, pleckstrin; GAP, GTPase-activating protein; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; DAPI, 4,6-diamidino-2-phenylindole; PAGE, polyacrylamide gel electrophoresis.

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fluorescence resonance energy transfer (FRET) between cyan fluorescent protein-tagged α_q and yellow fluorescent protein-tagged RGS7 in transfected cells, indicating a direct protein-protein interaction between α_q and β_5RGS7 (Witherow et al., 2003). It is interesting that no FRET was observed between α_o and β_5RGS7 in those studies (Witherow et al., 2003). A difficulty in examining β_5RGS7 interactions with α-subunits has been the inability to detect stable $\alpha\beta_5$RGS7 complexes using either purified proteins or employing coimmunoprecipitation techniques. This is in striking contrast to other RGS proteins, for which it is relatively easy to demonstrate interactions with particular activated α-subunits.

To interact with G protein α-subunits, RGS proteins are likely to be targeted to plasma membranes (PM). Previous work has demonstrated that β_5RGS7 is detected in both cytosolic and membrane fractions of brain extracts and cultured cells (Rose et al., 2000; Witherow et al., 2000; Zhang et al., 2001), and a substantial amount of β_5RGS7 was detected in a nuclear fraction (Zhang et al., 2001). Immunofluorescence microscopy of endogenous β_5RGS7 in PC-12 cells or overexpressed β_5RGS7 in PC-12 or HEK293 cells indicated a predominantly cytoplasmic distribution, with little or no β_5RGS7 at the PM, along with some nuclear localization (Zhang et al., 2001; Rojkova et al., 2003). Thus, the molecular mechanisms of PM targeting β_5RGS7 are poorly defined. For some RGS proteins, interaction with a PM-localized and activated α-subunit promotes translocation of the RGS protein from the cytoplasm to PM. In addition, RGS proteins contain other membrane targeting signals, such as protein-lipid or protein-protein interaction domains or covalently bound lipids, that function to promote regulated or constitutive PM localization (Hollinger and Hepler, 2002). Palmitoylation has been demonstrated to occur on RGS7 when expressed in Sf9 insect cells (Rose et al., 2000), suggesting that this covalent modification could facilitate membrane binding of β_5RGS7.

In this report, we examined whether G protein α-subunits could promote PM localization and palmitoylation of β_5RGS7. We demonstrate that expression of α_o, but not α_q, promotes a redistribution of β_5RGS7 from the cytoplasm to PM. An analysis of α_o mutants suggests that the GTP-bound active form, rather than the GDP-bound form, of α_o preferentially induces PM targeting of β_5RGS7. In addition, we demonstrate that α_o promotes palmitoylation of RGS7, and our results suggest that Cys 133 of RGS7 is a site of palmitoylation.

Materials and Methods

Cell Culture. HEK293 and COS-7 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and maintained at 37°C in a 95% air, 5% CO_2-humidified atmosphere.

Expression Vectors. pcDNA3.1 encoding α_o or triple hemagglutinin epitope-tagged human RGS7 (S1 or S2) was purchased from the Guthrie cDNA Resource Center (Sayre, PA). RGS7(S2) is full-length cDNA, and RGS7(S1) lacks amino acids from 76 to 128. A plasmid for myc-His-tagged β_5 was provided by David P. Siderovski (University of North Carolina, Chapel Hill, NC). α_o mutants α_oG2A, (α_oC3S), α_oR179C, α_oG184S, and α_oG204A were created using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), as were triple hemagglutinin epitope-tagged RGS7C120S, RGS7C133S, and RGS7C206S. The deletion mutants RGS7Δ 17–75 and RGS7Δ 17–112 were generated by sequential polymerase chain reaction amplification using pcDNA3.1-RGS7(S2) as a template, and then subcloned into pcDNA3.1 as a KpnI-XhoI fragment.

Transfection. Unless otherwise noted, cells were seeded 1 day before transfection. An indicated amount of DNA constructs was transfected into cells using FuGene 6 (Roche, Indianapolis, IN).

Immunofluorescence Microscopy. Cells were fixed with 3.7% formaldehyde in phosphate-buffered saline for 15 min and permeabilized by incubation in blocking buffer (2.5% nonfat milk and 1% Triton X-100 in Tris-buffered saline) for 20 min. Cells were then incubated with primary antibodies indicated in blocking buffer for 1 h. The cells were washed with blocking buffer and incubated in a 1:250 dilution of a goat anti-mouse or a goat anti-rabbit antibody conjugated with either Alexa 488 or Alexa 594 for 30 min. The coverslips were washed with 1% Triton X-100 in Tris-buffered saline, rinsed in distilled water, and mounted on glass slides with Prolong or Prolong Gold antifade reagent (Molecular Probes, Eugene, OR). Only cells displaying low to moderate levels of fluorescence were examined. Images were recorded with a Olympus BX60 microscope and Sony DKC-5000 digital camera or using an Olympus BX61 microscope and Hamamatsu ORCA-ER digital camera controlled by Slidebook v4.0 (Intelligent Imaging Innovations, Denver, CO). For deconvolved images, image stacks were deconvolved using a constrained iterative algorithm in Slidebook v4.0, and images of "*x-y*" planes through the middle of cells are presented. Images were transferred to Adobe Photoshop for digital processing.

Cell Fractionation Assay. Soluble and particulate fractions were isolated as described previously (Evanko et al., 2000; Takida and Wedegaertner, 2003). Densitometric quantitation of relative amounts in soluble versus particulate fractions was performed using a Kodak DC40 imaging system.

Palmitoylation Assay. Wild-type and mutants of RGS7 were transfected into COS-7 cells in conjunction with β_5 in the absence or presence of α_o or α_oR179C. 36 h after transfection, the cells were metabolically labeled with [^{3}H]palmitate for 3 h then lysed. RGS7 was immunoprecipitated using an anti-HA polyclonal antibody. The samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The membrane was sprayed with EnHance (PerkinElmer Life and Analytical Sciences) and exposed to Hyperfilm MP (Amersham Biosciences) at −80°C for 24 to 60 days. After fluorography, the RGS7 protein was detected by immunoblotting using an anti-HA monoclonal antibody. COS-7 cells were used for palmitoylation rather than HEK293 cells because we routinely observe better palmitate labeling of proteins using COS-7 cells (Evanko et al., 2000).

Materials. The anti-α_o polyclonal antibody was provided by David R. Manning (University of Pennsylvania). Anti-HA polyclonal and anti-α_q polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). 12CA5 and 9E10 monoclonal antibodies were from Roche and Covance (Berkeley, CA), respectively.

Results

α_o Specifically Promoted PM Targeting of β_5RGS7. We examined effects of α-subunit expression on localization of the β_5RGS7 complex in HEK293 cells. Both β_5 and RGS7 are enriched in brain with little or no expression in other tissues (Rose et al., 2000; Witherow and Slepak, 2003; Zhang et al., 2000), and α_o is lacking from HEK293 cells (Wang et al., 1999). Thus, this cell line facilitates studies independently of endogenous counterparts. β_5 and RGS7 were expressed in HEK293 cells, and then localization of the complex was visualized using an anti-HA monoclonal antibody to detect HA-tagged RGS7. β_5RGS7 displayed a diffuse distribution throughout the cytoplasm (Fig. 1A, a). Efficient expression of RGS7 requires coexpression of β_5 (Snow et al.,

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1999; Witherow et al., 2000; Chen et al., 2003), and β_5 and RGS7 have been shown to form tight complexes and to colocalize (Witherow et al., 2000; Zhang et al., 2001); thus, it is likely that localization of RGS7 is representative of the β_5RGS7 complex, and we refer to RGS7 detection as β_5RGS7 herein. Coexpression of α_o (Fig. 1A, d) led to strong PM localization of β_5RGS7 (Fig. 1A, b). In contrast, α_q (Fig. 1A, e) did not promote β_5RGS7's PM targeting (Fig. 1A, c), suggesting that α_o selectively induces PM localization β_5RGS7. Cells were also stained with DAPI to define the location of nuclei (Fig. 1A, f–h).

To further examine subcellular localization of RGS7, we carried out a cell fractionation assay. Transfected cells were lysed in a hypotonic buffer, and the soluble and particulate fractions were separated by ultracentrifugation. Proteins in each fraction were analyzed by immunoblotting using 12CA5 anti-HA monoclonal antibody for RGS7. When expressed alone, β_5RGS7 was mostly found in the soluble fraction (Fig. 1B, lanes 1 and 2), whereas coexpression of α_o resulted in a significant shift of β_5RGS7 from the soluble to particulate fraction (Fig. 1B, lanes 3 and 4). In contrast, expression of α_q did not change the predominantly soluble distribution of β_5RGS7 (Fig. 1B, lanes 5 and 6). The results were consistent with the observations of immunofluorescence microscopy, suggesting that α_o specifically promotes PM localization of β_5RGS7 in HEK293 cells.

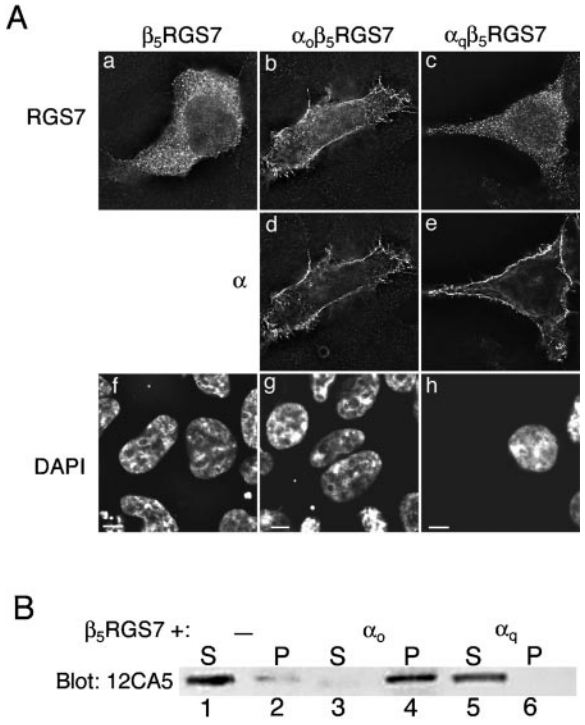

Fig. 1. α_o promotes membrane targeting of β_5RGS7. A, expression vectors for β_5 (450 ng) and RGS7 (550 ng) were transfected into HEK293 cells in conjunction with pcDNA3 (500 ng) (a and f), α_o (500 ng) (b, d, and g), or α_q (500 ng) (c, e, and h). Thirty-six hours after transfection, immunofluorescence staining and deconvolution microscopy were performed to visualize transiently expressed proteins. Primary antibodies used were monoclonal anti-HA 12CA5 antibody for RGS7 (a–c), rabbit polyclonal anti-α_o (d), or rabbit polyclonal anti-α_q antibody (e). Cells were also stained with DAPI to visualize nuclei (f–h). Images shown are representative of more than 100 cells examined in more than three experiments. Bar, 5 μm. B, HEK293 cells expressing β_5 and RGS7 (lanes 1 and 2), β_5, RGS7, and α_o (lanes 3 and 4), or β_5, RGS7, and α_q (lanes 5 and 6) were lysed, and cell lysates were separated by high-speed centrifugation into soluble (S) and particulate (P) fraction as described under *Materials and Methods*. RGS7 was detected by Western blotting with an anti-HA monoclonal antibody. The blot shown is representative of at least three independent experiments. Quantitation of immunoblots from multiple fractionation experiments provides the following ratios ± S.E. of RGS7 in soluble:particulate fractions when coexpressed with β_5 and the following α-subunit: no α, S:P = 96:4 ± 1 (n = 3); α_o, S:P = 7:93 ± 1 (n = 3); α_q, S:P = >95:<5 (no detectable RGS7 band in P fraction) (n = 3).

Effects of α_o Mutants on Subcellular Localization of β_5RGS7. To investigate whether PM localization of β_5RGS7 is preferentially mediated by inactive or active forms of α_o, we next tested the effects of two α_o mutants, α_oG204A and α_oR179C. α_oG204A is unable to undergo activating conformational changes and is thus considered to be locked in the inactive GDP-bound form. On the other hand, α_oR179C is constitutively active because of a greatly reduced ability to hydrolyze GTP. RGS7 and β_5 were expressed with α_oR179C or α_oG204A and localization of the complex was examined as described above. Coexpression of α_oR179C promoted pronounced PM localization of β_5RGS7 (Fig. 2A, a–d), whereas, in the presence of α_oG204A, the β_5RGS7 dimer predominantly exhibited cytoplasmic, dispersed distribution (Fig. 2A, e–h). In cell fractionation, β_5RGS7 was, as described above, mostly found in the soluble fraction (Fig. 2B, lanes 1 and 2) and coexpression of α_oR179C led to an almost complete shift of the RGS7 band from the soluble to the particulate fraction (Fig. 2B, lanes 3 and 4). On the other hand, α_oG204A promoted an increase in the amount of RGS7 in the particulate fraction, but the shift was much less compared with that induced by α_oR179C (Fig. 2B lanes 5 and 6). We observed that cells expressing transfected α_oG204A and β_5RGS7 at high levels displayed some PM localization of β_5RGS7 (data not shown), which probably accounts for the β_5RGS7 band in the particulate fraction (Fig. 2B, lanes 5 and 6). Figure 2C shows that the expression level of RGS7 was not markedly changed upon coexpression of various α_o mutants. Taken together, our findings imply that activated, GTP-bound α_o preferentially mediates PM localization of β_5RGS7.

The α_o-subunit is modified with fatty acids, and we examined whether this lipidation is required for α_o to promote PM recruitment of β_5RGS7. A 14-carbon saturated myristate attaches to glycine at position 2, and a 16-carbon palmitate modifies cysteine at position 3 of α_o. α_oG2A is devoid of both myristate and palmitate because myristoylation is a prerequisite for palmitoylation. When the β_5RGS7 dimer was expressed in conjunction with α_oG2A, β_5RGS7 displayed virtually no PM localization (Fig. 2A, i–l) and remained in the soluble fraction (Fig. 2B, lane 7 and 8), suggesting that lipid modification of α_o is required for it to mediate PM localization of β_5RGS7.

Moreover, we examined β_5RGS7's subcellular localization in the presence of an α_oG184S mutant. This mutant, termed RGS-insensitive, has been shown to have a reduced ability to interact with the RGS domain of a RGS protein (Lan et al., 1998). When α_oG184S was expressed together with β_5RGS7, a majority of transfected cells displayed predominantly cytoplasmic distribution of β_5RGS7 (Fig. 2A, m–p). In the fractionation assay, substantially less shift of β_5RGS7 to the particulate fraction was induced by α_oG184S compared with wild-type α_o and α_oR179C (Fig. 2B, lanes 9 and 10). The presence of some increased β_5RGS7 in the particulate fraction is probably due to some membrane localization when proteins are expressed at very high levels, because fraction-

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ation experiments use a population of cells and thus do not distinguish between cells expressing different levels of the proteins. As was the case when α_oG204A was expressed, when cells expressed α_oG184S proteins at high levels, some PM localization of β_5RGS7 was observed (data not shown). The observations with α_oG184S suggest that interaction of α_o with the RGS domain is important in α_o-mediated PM localization of β_5RGS7.

Next, we considered the possibility that the failure of α_q to promote PM localization of β_5RGS7 (Fig. 1) was due to a requirement for the α-subunit to be sufficiently activated. Thus, we tested the ability of the constitutively active mutant α_qR183C to affect localization of β_5RGS7. As was the case with α_q, α_qR183C failed to promote PM localization of β_5RGS7 (Fig. 2). When coexpressed with α_qR183C, β_5RGS7 remained in the cytoplasm of cells (Fig. 2A, q–t) and was found predominantly in the soluble fraction (Fig. 2B, lanes 11 and 12). These results confirm that α_q, in contrast to α_o, is not effective at promoting PM localization of β_5RGS7.

α_o Induced Palmitoylation of β_5RGS7. It has been shown that some RGS proteins are modified with palmitate, a fatty acid known to serve as a membrane targeting signal, and a previous report demonstrated that RGS7 incorporated palmitate when expressed with β_5 in Sf9 insect cells (Rose et al., 2000). We looked at whether RGS7 is palmitoylated in mammalian cells. RGS7 and β_5 were transfected into COS-7 cells in the absence or presence of α_o or α_oR179C. Cells were metabolically labeled with [^{3}H]palmitate and incorporation of radioactivity into RGS7 was analyzed as described under *Materials and Methods*. Control transfection of empty vector showed no nonspecific binding of the radioactivity (Fig. 3, top, lane 1). No incorporation of radiolabeled palmitate into RGS7 was seen without α_o expression (Fig. 3, top, lane 2). On the other hand, RGS7 incorporated radioactive palmitate in the presence of α_o or α_oR179C (Fig. 3, top, lanes 3 and 4). Expression of RGS7 was confirmed by Western blotting (Fig. 3, bottom).

The site(s) of palmitoylation on RGS7 have not been identified, but three cysteine residues that could serve as potential palmitoylation sites exist in the region between the DEP and GGL domains (Rose et al., 2000). We thus replaced each of those cysteines with serine to create the RGS7 mutants RGS7C120S, RGS7C133S, and RGS7C206S, and tested for α_o-promoted PM localization. The mutants were expressed in conjunction with β_5 and α_o, and their localization was detected by using the 12CA5 antibody. RGS7C120S was not detected at the PM; however, its expression was extremely low (data not shown); thus, we could not conclusively demonstrate a defect in α_o-promoted PM localization. β_5RGS7C133S expression was somewhat variable, but the typical expression level was approximately 25 to 50% of wild-type β_5RGS7 (Fig. 4B, lanes 5–7). Although expressed at a reduced level, RGS7C133S retained binding to β_5 as determined by pull-down experiments using the hexahistidine tag

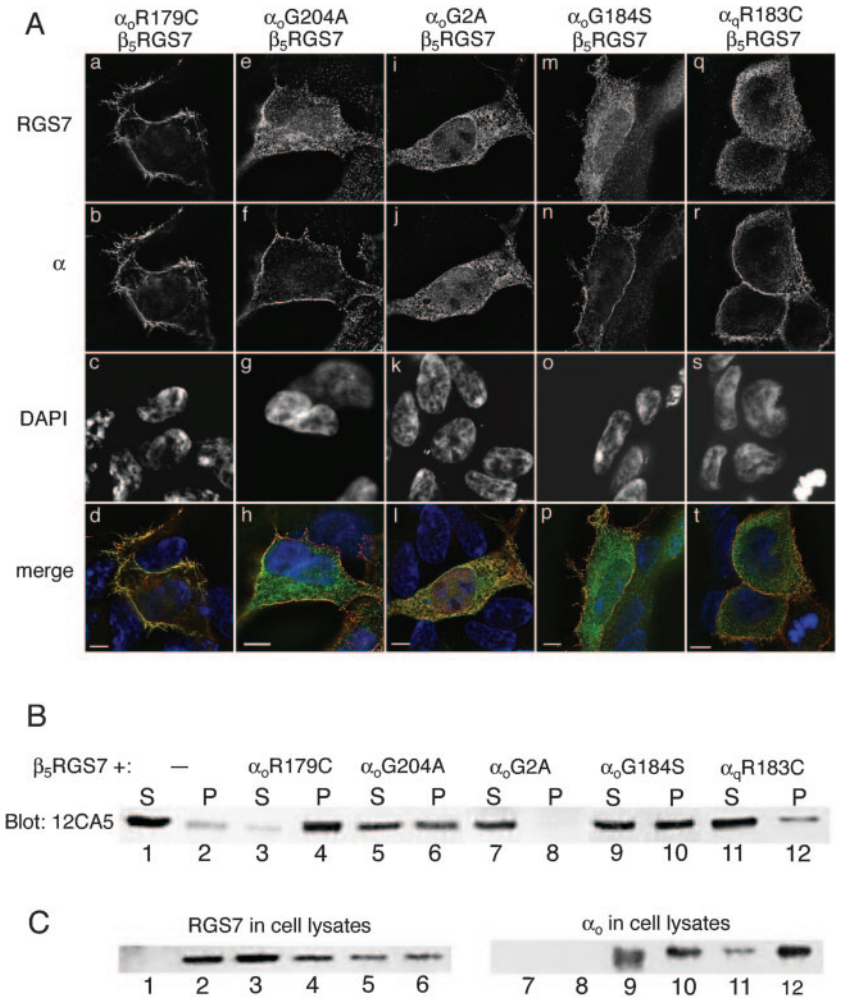

Fig. 2. Effect of α_o mutants on membrane targeting of β_5RGS7. A, HEK293 cells were transfected with plasmids containing β_5 (450 ng) and RGS7 (550 ng) along with α_oR179C (500 ng) (a–d), α_oG204A (500 ng) (e–h), α_oG2A (500 ng) (i–l), α_oG184S (500 ng) (m–p), or α_qR183C (500 ng) (q–t). Transfected proteins were visualized by immunofluorescence staining and deconvolution microscopy as described under *Materials and Methods* using a mouse monoclonal anti-HA 12CA5 antibody for β_5RGS7 (a, e, i, m, and q) and a rabbit polyclonal anti-α_o antibody for α_o (b, f, j, and n) or a rabbit polyclonal anti-α_q antibody (r). Cells were also stained with DAPI to visualize nuclei (c, g, k, o, and s). Merged images with RGS7 (green), α_o or α_q (red), and nuclei (blue) are also shown (d, h, l, p, and t). Images shown are representative of more than 100 cells examined in more than three experiments. Bar, 5 μm. B, transfected cells expressing indicated proteins were lysed, and soluble and particulate fractions were prepared as described, resolved by SDS-PAGE, and immunoblotted with 12CA5 anti-HA monoclonal antibody to visualize RGS7. The blot shown is representative of at least three independent experiments. Quantitation of immunoblots from multiple fractionation experiments provides the following ratios ± S.E. of RGS7 in soluble/particulate fractions when coexpressed with β_5 and the following α-subunit: α_oR179C, S:P = 17:83 ± 12 (n = 5); α_oG204A, S:P = 52:48 ± 4 (n = 3); α_oG2A, S:P = 88:12 ± 9 (n = 3); α_oG184S, S:P = 65:35 ± 16 (n = 4); α_qR183C, S:P = 92:8 ± 0.1 (n = 2). C, total cell lysates from HEK293 cells transfected with pcDNA3 alone (lanes 1 and 7), or vectors for β_5 and RGS7 (lanes 2 and 8), or vectors for β_5 and RGS7 along with α_oR179C (lanes 3 and 9), α_oG204A (lanes 4 and 10), α_oG2A (lanes 5 and 11), or α_oG184S (lanes 6 and 12) were immunoblotted with 12CA5 anti-HA monoclonal antibody (lanes 1–6) or anti-α_o polyclonal antibody (lanes 7–12).

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on β_5 (data not shown). We thus compared localization of β_5RGS7C133S with wild-type β_5RGS7. In immunofluorescence microscopy, β_5RGS7C133S displayed a diffuse cytoplasmic distribution even when expressed with PM-localized α_o (Fig. 4A, a and c). When assayed by subcellular fractionation, β_5RGS7C133S was partially recruited to the particulate fraction when coexpressed with α_o (Fig. 4B, lanes 1 and 2), although the portion of RGS7C133S in the particulate was significantly reduced compared with wild-type RGS7 when coexpressed with α_o (Fig. 1B, lanes 3 and 4). On the other hand, β_5RGS7C206S was strongly recruited to PM (Fig. 4A, b and d) or the particulate fraction (Fig. 4B, lanes 3 and 4) by expression of α_o. Together, the data with the cysteine mutants indicate that Cys133 of RGS7 is critical for PM localization of β_5RGS7 and suggest that Cys133 is a putative palmitoylation site.

Amino Acids 76 to 128 Are Crucial for PM Targeting. It has been shown that deletion of the DEP domain in RGS9 resulted in its mislocalization (Martemyanov et al., 2003b). To test a role of the DEP domain and its flanking region in RGS7 subcellular localization, we examined several RGS7 mutants that have deletions of all or portions of the DEP domain. The RGS7 DEP domain comprises amino acids 17 to 112 (Wong et al., 2000). RGS7Δ 17–75, a deletion of the N-terminal portion of the DEP domain, and RGS7Δ 17–112, a deletion of the entire DEP domain, were expressed together with β_5 and α_o, but RGS7Δ 17–75 and RGS7Δ 17–112 protein was almost undetectable, as assessed by Western blotting (Fig. 5A, lanes 2 and 3). RGS7Δ 76–128, which lacks the C-terminal portion of the DEP domain and additional flanking residues, displayed a substantially greater level of expression, although slightly reduced compared with wild-type RGS7 (Fig. 5A, lanes 1 and 4). It is noteworthy that β_5RGS7Δ 76–128 was refractory to α_o-promoted PM localization (Fig. 5B, a); it remained in the cytoplasm, implying that the deleted region is important in α_o-mediated PM localization of the complex.

Discussion

We demonstrated herein that β_5RGS7 is mostly cytoplasmic and soluble when expressed in HEK293 cells, but coexpression of α_o promoted a strong redistribution of β_5RGS7 to the PM. Expression of α_q, however, did not elicit a similar PM recruitment of β_5RGS7. PM localization of β_5RGS7 was promoted by constitutively active α_oR179C, but not the inactive mutant α_oG204A. Moreover, our results suggest that PM recruitment of β_5RGS7 is mediated, at least in part, through interaction of its RGS domain with α_o, because the RGS-insensitive α_oG184S mutant showed a decreased ability to recruit β_5RGS7 to the PM. In addition to PM recruitment of β_5RGS7, we demonstrate that expression of α_o is required for detectable palmitoylation of RGS7 in COS-7 cells. Finally, mutational analysis of RGS7 indicates that Cys133 is a potential site of palmitoylation. These studies are thus the first to demonstrate regulated PM localization and palmitoylation of RGS7.

The preferred G protein α-subunit target of β_5RGS7 remains controversial. In GAP assays using purified proteins, β_5RGS7 is highly selective for α_o and exhibits no GAP activity on α_q (Posner et al., 1999; Hooks et al., 2003). However, when expressed in cells, β_5RGS7 can regulate α_o-, α_i-, and

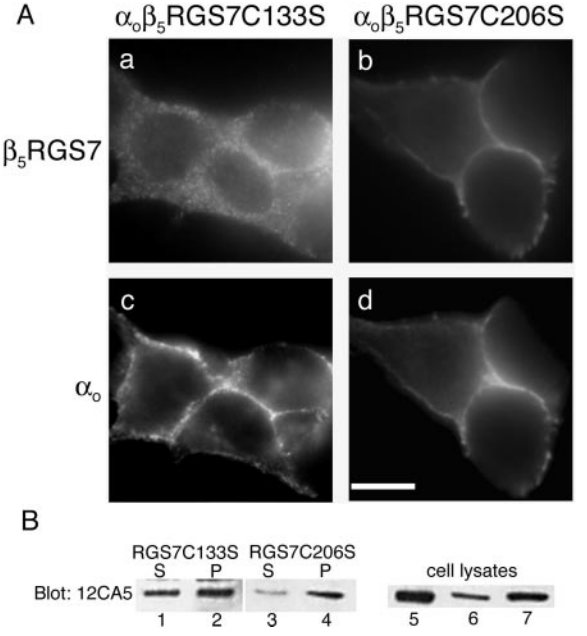

Fig. 4. Subcellular localization of RGS7 cysteine mutants. A, HEK293 cells were transfected with expression plasmids for α_o (500 ng), β_5 (450 ng), and RGS7C133S (550 ng) (a and c) or α_o (500 ng), β_5 (450 ng), and RGS7C206S (550 ng) (b and d). Thirty-six hours after transfection, cells were fixed and subjected to immunofluorescence staining as described under *Materials and Methods*. Primary antibodies used were a rabbit polyclonal anti-α_o antibody for the α_o-subunit and a mouse monoclonal anti-HA 12CA5 antibody for β_5RGS7. Images shown are representative of greater than 100 cells examined in more than three experiments. Bar, 5 μm. B, HEK293 cells expressing α_o, β_5, and RGS7C133S (lanes 1 and 2) or α_o, β_5, and RGS7C206S (lanes 3 and 4) were lysed, and cell lysates were separated by high-speed centrifugation into soluble (S) and particulate (P) fractions as described under *Materials and Methods*. RGS7 cysteine mutants were detected by Western blotting with an anti-HA monoclonal antibody. Note that RGS7C133S (lanes 1 and 2) required a longer exposure to visualize because of lower expression levels. Expression levels of RGS7 (lane 5), RGS7C133S (lane 6), and RGS7C206S (lane 7) when coexpressed with α_o and β_5 are compared in 12CA5 anti-HA immunoblot of total cell lysates (lanes 5–7). The blots shown are representative of at least three independent experiments. Quantitation of immunoblots from multiple fractionation experiments provides the following ratios ± S.E. of each RGS7 cysteine mutant in soluble:particulate fractions when coexpressed with β_5 and α_o: RGS7C133S, S:P = 47:53 ± 4 (n = 3); RGS7C206S, S:P = 12:88 ± 8 (n = 5).

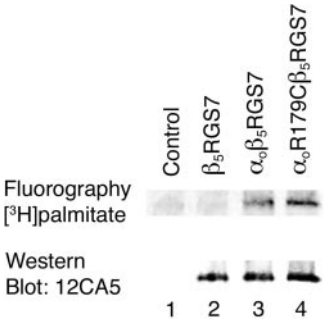

Fig. 3. Palmitoylation of RGS7. COS-7 cells were transfected with pcDNA3 (4.5 μg) (lane 1), or expression constructs for β_5 (1.35 μg) and RGS7 (1.65 μg) along with pcDNA3 (1.5 μg) (lane 2), α_o (1.5 μg) (lane 3), or α_oR179C (1.5 μg) (lane 4). Cells were labeled for 3 h with 1 mCi/ml of [^{3}H]palmitate. β_5RGS7 was immunoprecipitated using an anti-HA polyclonal antibody. The samples were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was exposed to a film at −80°C. Thereafter, the membrane was subjected to Western blotting for RGS7. The blots shown are representative of two experiments.

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α_q-dependent signaling pathways (Shuey et al., 1998; Kovoor et al., 2000; Witherow et al., 2000; Keren-Raifman et al., 2001; Zhang et al., 2002; Witherow et al., 2003; Ghavami et al., 2004). In our studies, α_o but not α_q was able to promote PM localization of β_5RGS7 in cells. Thus, our assays of PM recruitment are consistent with selectivity of β_5RGS7 for α_o. We were surprised to find our studies in contrast to a recent report showing a FRET signal between wild-type α_q and β_5RGS7 in cells but no FRET signal between α_o and β_5RGS7 (Witherow et al., 2003). We observed no PM recruitment of β_5RGS7 when wild-type α_q was expressed. An explanation for these seemingly contradictory results is not clear. It is interesting that stable complexes between purified β_5RGS7 and α_o, either GDP- or GDP-AlF_4^--bound, cannot be detected (Posner et al., 1999), and coimmunoprecipitation and pull-down approaches from cell lysates have likewise failed to isolate α_o or α_q bound to β_5RGS7 (data not shown) (Witherow et al., 2000). It seems as if the binding of β_5RGS7 to α_o is relatively weak compared with other RGS/Gα pairs (Posner et al., 1999), and thus the PM recruitment of β_5RGS7 by α_o may provide a surrogate method for monitoring association of β_5RGS7 and α_o in cells. In addition, unidentified proteins probably influence the affinity and selectivity of β_5RGS7 binding to α-subunits. For example, the related complex, β_{5L}RGS9–1, fails to form a stable complex with α_t or α_o using purified proteins unless the α_t effector cGMP phosphodiesterase-subunit γ is also included (Martemyanov and Arshavsky, 2002; Martemyanov et al., 2003a). The identification of similar affinity adaptors, possibly α_o or α_q effectors, will shed light on β_5RGS7/Gα specificity in vivo.

How does α_o mediate RGS7's PM localization? Our results suggest a model in which the RGS domain of RGS7 interacts preferentially with active α_o. The inactive α_oG204A, a mutant that can serve a dominant-negative function because of an inability to undergo activating conformational changes, failed to induce strong PM localization of β_5RGS7, whereas constitutively active mutant α_oR179C promoted strong PM recruitment of β_5RGS7. It is interesting that, in our hands, wild-type α_o was just as effective as α_oR179C in recruiting β_5RGS7 to the PM in transient transfection experiments. We suspect that wild-type α_o did so most probably because some fraction of the overexpressed protein was in fact active; it is not uncommon for overexpressed α-subunits to show some ability to activate signaling pathways, even in the absence of receptor stimulation or an activating mutation. On the other hand, high amounts of overexpressed wild type α_o may simply overcome its lower affinity compared with activated α_o for RGS7. Active α_o probably interacts directly with β_5RGS7's RGS domain to induce PM recruitment, and this proposal is supported by the failure of RGS-insensitive α_oG184S to promote strong PM localization of β_5RGS7; however, the degree to which the G184S disrupts interaction with RGS7 has not been demonstrated (Lan et al., 1998) Our results are consistent with a number of other studies showing that certain activated α-subunits can selectively recruit RGS domain-containing proteins to the PM (Druey et al., 1998; Heximer et al., 2001; Bhattacharyya and Wedegaertner, 2003; Day et al., 2003; Masuho et al., 2004). There have been suggestions, though no direct demonstrations, that complexes of β_5 and R7 RGS family members, such as RGS7, can interact with inactive α-subunits via β_5 and thus form a novel G protein heterotrimer. Although we cannot rule out the possibility that PM recruitment of β_5RGS7 is mediated by β_5 binding to α_o, our results with α_o mutants are more consistent with the model that PM localization of β_5RGS7 is mediated, at least in part, through RGS domain binding to active α_o.

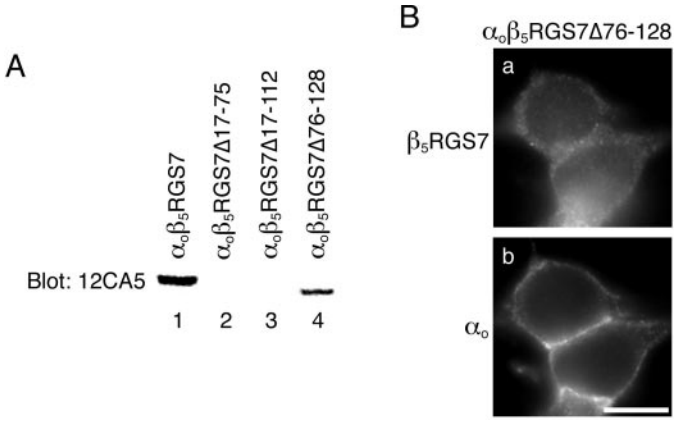

Fig. 5. RGS7 DEP domain deletions. A, cells transiently expressing α_o (500 ng) and β_5 (450 ng) in conjunction with wild-type RGS7 (550 ng) (lane 1), RGS7(Δ 17–75) (550 ng) (lane 2), RGS7(Δ 17–112) (550 ng) (lane 3), or RGS7(Δ 76–128) (550 ng) (lane 4) were solublized and analyzed by SDS-PAGE and Western blotting using the 12CA5 monoclonal antibody. B, expression vectors for α_o (500 ng), β_5 (450 ng), and RGS7(Δ 76–128) (550 ng) were transfected into HEK293 cells. Thirty-six hours after transfection, immunofluorescence staining was carried out to visualize transiently expressed proteins as described under *Materials and Methods*. Images shown are representative of more than 100 cells examined in more than three experiments. Bar, 5 μm.

However, the presence of the RGS domain of RGS7 seems not to be sufficient for α_o-induced PM localization of β_5RGS7. For example, mutants of RGS7 containing an intact RGS domain but with partial deletion of the DEP domain, RGS7Δ 76–128, or the mutation C133S were deficient in α_o-mediated PM recruitment. We identified palmitoylation as a modification of RGS7, and our results suggest that palmitoylation serves as a membrane targeting signaling. A previous report demonstrated that RGS7 was palmitoylated when expressed in Sf9 cells, but we could not detect palmitoylation of RGS7 when expressed together with β_5 in COS-7 cells. Coexpression of α_o or α_oR179C with β_5RGS7, however, promoted RGS7 palmitoylation. Thus, our palmitoylation assays were consistent with observations of PM localization; both palmitoylation and PM localization of β_5RGS7 were promoted by α_o. To address potential sites of RGS7 palmitoylation, several cysteines were mutated individually to serines and α_o-promoted PM localization was determined. β_5RGS7C206S was recruited to the PM just like wild-type β_5RGS7. In contrast, β_5RGS7C133S displayed a clear defect in PM recruitment. These results are consistent with the idea that Cys133 is a site for palmitoylation of RGS7, although requirements for very long exposure times for palmitate labeling and variable expression of RGS7C133S precluded us from definitively demonstrating that it fails to incorporate palmitate. In addition, we cannot rule out that additional sites of palmitoylation, such as potentially Cys120, exist in RGS7.

Palmitoylation has been identified in several RGS proteins and in some it has been demonstrated to influence their GAP activity. Palmitoylation of RGS4 was shown to inhibit its GAP activity toward α_z (Tu et al., 1999), whereas palmitoylation of RGS16 increased GAP function (Osterhout et al., 2003). Rose et al. (2000) showed that, when purified from Sf9 cells, membrane-bound RGS7, which is palmitoylated, and

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cytosolic, nonpalmitoylated RGS7 equipotently stimulated the GTPase activity of α_o, suggesting that palmitoylation has no effect on RGS7's GAP activity. Thus, for RGS7, a primary function of palmitoylation is probably to facilitate PM targeting of β_5RGS7.

Despite the recent breakthrough in identification of *bona fide* palmitoyltransferases in yeast (Lobo et al., 2002; Linder and Deschenes, 2003, 2004), molecular mechanisms underlying palmitoylation in mammalian cells are still unclear. We have found that mutants of the small GTPase Sar1, which are known to inhibit vesicle transport between ER and Golgi along the exocytic pathway, had no effect on α_o-mediated membrane localization of β_5RGS7 (data not shown). Thus, our findings suggest that RGS7's PM targeting and, presumably, palmitoylation are independent of the conventional exocytic pathway. The α_o-subunit itself is modified with palmitate, and its palmitoylation has been shown to be Brefeldin A-insensitive (Gonzalo and Linder, 1998), thus suggesting that α_o does not require a functional Golgi, and by extension does not use the classic exocytic pathway, for palmitoylation and PM targeting. Furthermore, we showed that a lipidation defective α_oG2A mutant was unable to induce β_5RGS7's PM localization (Fig. 2A, c), and α_oC3S, which is myristoylated but not palmitoylated, was also unable to recruit β_5RGS7 to the PM (data not shown), suggesting that palmitoylation of α_o is required for PM targeting of the β_5RGS7 complex. Whether the same palmitoyltransferase can catalyze attachment of palmitate to α_o and RGS7 remains to be seen.

DEP domains may play roles in subcellular targeting of proteins (Martemyanov et al., 2003b), although the mechanisms are not clear. RGS7Δ 76–128 was defective in α_o-promoted PM localization. This may indicate that the DEP domain plays a unique role in facilitating membrane targeting. On the other hand, the 76–128 deletion may affect the ability of Cys133 to undergo palmitoylation. Our attempts to resolve this question by additional DEP domain deletions were thwarted by very poor expression of RGS7Δ 17–75 and RGS7Δ 17–112. These results suggest that deletion of the N-terminal portion of RGS7's DEP domain creates an unstable protein; moreover, these deletion experiments raise the possibility that amino acids 112–128 could be a critical region for both stability and PM localization of RGS7. A recent report proposed that DEP domains influence subcellular targeting by interacting with SNARE or SNARE-like proteins, and R9AP, which interacts with the DEP domain of RGS9, seems to have a SNARE-like domain (Martemyanov et al., 2003b). In light of this proposal, it is particularly interesting that another recent report used two-hybrid studies to identify snapin, a SNARE complex protein, as a protein that interacts with the N terminus of RGS7 in a region that partially includes the DEP domain (Hunt et al., 2003).

In conclusion, this study demonstrated that α_o can specifically induce palmitoylation and PM localization of RGS7. To our knowledge, it is the first report to show regulated membrane targeting of RGS7 and to begin to characterize the molecular mechanisms involved.

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Address correspondence to: Philip B. Wedegaertner, Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, 233 South 10th Street, 839 BLSB, Philadelphia, PA 19107. E-mail: p_wedegaertner@mail.jci.tju.edu

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