Palmitoylation and Plasma Membrane Targeting of RGS7 Are Promoted by δ "

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Abbreviations used: G protein, guanine nucleotide-binding protein; RGS, regulator of G protein signaling; PM, plasma membrane; DEP, disheveled, EGL-10, pleckstrin; HEK293 cells, human embryonic kidney cells; COS7, African green monkey kidney cells; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride
Abstract

Regulator of G protein signaling (RGS) proteins modulate G protein signaling by acting as GTPase-activating proteins for G protein $\alpha$ subunits. RGS7 belongs to a subfamily of RGS proteins that exist as dimers with the G protein $\beta_5$ subunit. In this report, we addressed the mechanisms of plasma membrane localization of $\beta_5$RGS7. When expressed in HEK293 cells, $\beta_5$RGS7 was found to be cytoplasmic and soluble. Expression of $\alpha_o$ promoted a strong redistribution of $\beta_5$RGS7 to the plasma membrane. Expression of $\alpha_q$, however, failed to affect the subcellular localization of $\beta_5$RGS7. The constitutively active mutant $\alpha_oR179C$, like wild type $\alpha_o$, strongly recruited $\beta_5$RGS7 to plasma membranes; however, inactive $\alpha_oG204A$, RGS-insensitive $\alpha_oG184S$, and lipidation deficient $\alpha_oG2A$ were all defective in the ability to promote plasma membrane localization of $\beta_5$RGS7. In addition, palmitoylation of RGS7 was demonstrated, and palmitoylation required expression of $\alpha_o$ or $\alpha_oR179C$. To examine potential palmitoylation sites of RGS7 several cysteines were substituted with serines. $\beta_5$RGS7C133S failed to localize to plasma membranes when co-expressed with $\alpha_o$, suggesting cysteine 133 of RGS7 as a putative palmitoylation site. Lastly, deletion of amino acids 76-128 of RGS7, which includes part of the DEP domain, prevented $\alpha_o$-mediated plasma membrane recruitment of $\beta_5$RGS7. These findings are the first to demonstrate G$\alpha_o$ regulated plasma membrane localization and palmitoylation of $\beta_5$RGS7 and suggest that membrane targeting of $\beta_5$RGS7 is a complex process requiring at least RGS domain-mediated interaction with $\alpha_o$ and RGS7 palmitoylation.
Introduction

Heterotrimeric G proteins, composed of $\alpha$ and $\beta\gamma$ subunits, function as a molecular switches, relaying extracellular stimuli to cytoplasmic signaling pathways. Nucleotide exchange of GTP for GDP on the $\alpha$ subunit sets off a signaling cascade, whereas hydrolysis of the $\alpha$-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. Over 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 G gamma-like (GGL) domain (Sondek and Siderovski, 2001; Witherow and Slepak, 2003). Through this unique domain RGS7 interacts with the G protein $\beta_5$ subunit which deviates significantly from the other four $\beta$ subunits (Snow et al., 1998). Native $\beta_5$RGS7 complexes have been isolated from brain extracts (Witherow et al., 2000; Zhang and Simonds, 2000), and co-purification experiments suggest that RGS7 always exists as a heterodimer with $\beta_5$ (Witherow et al., 2000). One critical role for the interaction of RGS7 and $\beta_5$ is to mutually stabilize each other. Efficient expression of RGS7 in COS-7 cells depends on co-expression of $\beta_5$ and vice versa (Snow et al., 1999; Witherow et al., 2000), and, moreover, loss of $\beta_5$ 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 for $\beta_5$RGS7, and specifically which G protein $\alpha$ subunits interact with and are regulated by $\beta_5$RGS7. In vitro GAP assays have demonstrated that $\beta_5$RGS7 acts almost exclusively on $\alpha_o$, and not $\alpha_i$ or $\alpha_q$ (Hooks et al., 2003; Posner et al., 1999). In contrast, $\beta_5$RGS7 appears to show less $\alpha$ subunit selectivity in various cell systems. For example,
b5RGS7 was found to regulate Gαi/o protein-coupled receptor activated K+ channels (Keren-Raifman et al., 2001) and to attenuate Ca2+ mobilization mediated by Gq (Shuey et al., 1998), suggesting interaction with both Gα0 and Gαq in cells. A recent report demonstrated fluorescence resonance energy transfer (FRET) between CFP-tagged Gαq and YFP-tagged RGS7 in transfected cells, indicating a direct protein-protein interaction between Gαq and b5RGS7 (Witherow et al., 2003). Interestingly, no FRET was observed between Gα0 and b5RGS7 in those studies (Witherow et al., 2003). A difficulty in examining b5RGS7 interactions with Gα subunits has been the inability to detect stable Gα5RGS7 complexes using either purified proteins or employing co-immunoprecipitation techniques. This is in striking contrast to other RGS proteins for which it is relatively easy to demonstrate interactions with particular activated Gα subunits.

To interact with G protein Gα subunits, RGS proteins are likely to be targeted to plasma membranes (PM). Previous work has demonstrated that b5RGS7 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 b5RGS7 was detected in a nuclear fraction (Zhang et al., 2001). Immunofluorescence microscopy of endogenous b5RGS7 in PC12 cells or overexpressed b5RGS7 in PC12 or HEK293 cells indicated a predominantly cytoplasmic distribution, with little or no b5RGS7 at the PM, along with some nuclear localization (Rojkova et al., 2003; Zhang et al., 2001). Thus, the molecular mechanisms of PM targeting of b5RGS7 are poorly defined. For some RGS proteins, interaction with a PM-localized and activated Gα 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 $\delta_5$RGS7.

In this report, we examined whether G protein $\alpha$ subunits could promote PM localization and palmitoylation of $\delta_5$RGS7. We demonstrate that expression of $\alpha_\omega$, but not $\alpha_q$, promotes a redistribution of $\delta_5$RGS7 from the cytoplasm to PM. An analysis of $\alpha_\omega$ mutants suggests that the GTP-bound active form, rather than the GDP-bound form, of $\alpha_\omega$ preferentially induces PM targeting of $\delta_5$RGS7. In addition, we demonstrate that $\alpha_\omega$ 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 COS7 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% CO₂-humidified atmosphere.

Expression Vectors  pcDNA3.1 encoding α or 3xHA-tagged human RGS7 (S1 or S2) was purchased from the Guthrie cDNA Resource Center (Sayre, PA, http://www.cdna.org). RGS7(S2) is full length cDNA, and RGS7(S1) lacks amino acids from 76 to 128. A plasmid for myc-His tagged β5 was provided by David P. Siderovski (University of North Carolina) α mutants, αG2A, (αC3S), αR179C, αG184S, and αG204A were created using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). So were 3xHA-tagged RGS7C120S, RGS7C133S, and RGS7C206S. The deletion mutants RGS7D17-75 and RGS7D17-112 were generated by sequential PCR 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 (PBS) for 15 min and permeabilized by incubation in blocking buffer (2.5% nonfat milk and 1% Triton X-100 in TBS) 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 TBS, 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 COS7 cells in conjunction with \( \beta_5 \) in the absence or presence of \( \beta_\alpha \) or \( \beta_\alpha R179C \). 36 hr after transfection the cells were metabolically labeled with \([3H]\)palmitate for 3 hr then lysed. RGS7 was immunoprecipitated using an anti-HA polyclonal antibody. The samples were separated by SDS-PAGE and transferred onto polyvinidene difluoride membrane. The membrane was sprayed with EnHance (PerkinElmer Life Science) and exposed to Hyperfilm MP (Amersham Biosciences) at –80 °C for 24 – 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 polyclonal antibody was provided by David R. Manning (University of Pennsylvania). Anti-HA polyclonal and anti-α polyclonal antibodies were from Santa Cruz Biotechnology. 12CA5 and 9E10 monoclonal antibodies were from Roche and Covance (Berkeley, CA), respectively.
Results

\[ \text{\textit{\textbf{\( o \) specifically promoted PM targeting of \( \beta_5\)RGS7.}} \]

We examined effects of \( o \) subunit expression on localization of the \( \beta_5\)RGS7 complex in HEK293 cells. Both \( \beta_5 \) 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 \( o \) is lacking from HEK293 cells (Wang et al., 1999). Thus, this cell line facilitates studies independently of endogenous counterparts. \( \beta_5 \) 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. \( \beta_5\)RGS7 displayed a diffuse distribution throughout the cytoplasm (Figure 1A a). Efficient expression of RGS7 requires co-expression of \( \beta_5 \) (Chen et al., 2003; Snow et al., 1999; Witherow et al., 2000), and \( \beta_5 \) 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 \( \beta_5\)RGS7 complex, and we refer to RGS7 detection as \( \beta_5\)RGS7 herein. Co-expression of \( o \) (Figure 1A d) led to strong PM localization of \( \beta_5\)RGS7 (Figure 1A b). In contrast, \( q \) (Figure 1A e) did not promote \( \beta_5\)RGS7’s PM targeting (Figure 1A c), suggesting that \( o \) selectively induces PM localization \( \beta_5\)RGS7. Cells were also stained with DAPI to define the location of nuclei (Figure 1A f-h).

To further look at 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, \( \beta_5\)RGS7 was mostly found in the soluble fraction (Figure 1B lanes 1 and 2) whereas coexpression of \( o \) resulted in a significant shift of \( \beta_5\)RGS7 from the soluble to particulate fraction.
(Figure 1B lanes 3 and 4). In contrast, expression of \( \alpha_q \) did not change the predominantly soluble distribution of \( \beta_5 \)RGS7 (Figure 1B lanes 5 and 6). The results were consistent with the observations of immunofluorescence microscopy, suggesting that \( \alpha_o \) specifically promotes PM localization of \( \beta_5 \)RGS7 in HEK293 cells.

**Effects of \( \alpha_o \) mutants on subcellular localization of \( \beta_5 \)RGS7.** To investigate whether PM localization of \( \beta_5 \)RGS7 is preferentially mediated by inactive or active forms of \( \alpha_o \), we next tested the effects of two \( \alpha_o \) mutants, \( \alpha_o G204A \) and \( \alpha_o R179C \). \( \alpha_o G204A \) is unable to undergo activating conformational changes and is thus considered to be locked in the inactive GDP-bound form. On the other hand, \( \alpha_o R179C \) is constitutively active due to a greatly reduced ability to hydrolyze GTP. RGS7 and \( \beta_5 \) were expressed with \( \alpha_o R179C \) or \( \alpha_o G204A \) and localization of the complex was examined as described above. Coexpression of \( \alpha_o R179C \) promoted pronounced PM localization of \( \beta_5 \)RGS7 (Figure 2A a-d) whereas, in the presence of \( \alpha_o G204A \), the \( \beta_5 \)RGS7 dimer predominantly exhibited cytoplasmic, dispersed distribution (Figure 2A e-h). In cell fractionation, \( \beta_5 \)RGS7 was, as described above, mostly found in the soluble fraction (Figure 2B lanes 1 and 2) and coexpression of \( \alpha_o R179C \) led to an almost complete shift of the RGS7 band from the soluble to particulate fraction (Figure 2B lanes 3 and 4). On the other hand, \( \alpha_o G204A \) promoted an increase in the amount of RGS7 in the particulate fraction, but the shift was much less compared to that induced by \( \alpha_o R179C \) (Figure 2B lanes 5 and 6). We observed that cells expressing transfected \( \alpha_o G204A \) and \( \beta_5 \)RGS7 at high levels displayed some PM localization of \( \beta_5 \)RGS7 (not shown), which probably accounts for the \( \beta_5 \)RGS7 band in the particulate fraction (Figure 2B lanes 5 and 6). Figure 2C shows that the expression level of RGS7 was not markedly changed upon co-expression of
various $\alpha$ mutants. Taken together, our findings imply that activated, GTP-bound $\alpha$ preferentially mediates PM localization of $\beta_5$RGS7.

The $\alpha$ subunit is modified with fatty acids, and we examined whether this lipidation is required for $\alpha$ to promote PM recruitment of $\beta_5$RGS7. A 14 carbon saturated myristate attaches to glycine at position 2, and a 16 carbon palmitate modifies cysteine at position 3 of $\alpha$. $\alpha$G2A is devoid of both myristate and palmitate because myristoylation is a prerequisite for palmitoylation. When the $\beta_5$RGS7 dimer was expressed in conjunction with $\alpha$G2A, $\beta_5$RGS7 displayed virtually no PM localization (Figure 2A i-l) and remained in the soluble fraction (Figure 2B lane 7 and 8), suggesting that lipid modification of $\alpha$ is required for it to mediate PM localization of $\beta_5$RGS7.

Moreover, we examined $\beta_5$RGS7’s subcellular localization in the presence of an $\alpha$G184S 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 $\alpha$G184S was expressed together with $\beta_5$RGS7, a majority of transfected cells displayed predominantly cytosplasmic distribution of $\beta_5$RGS7 (Figure 2A m-p). In the fractionation assay, substantially less shift of $\beta_5$RGS7 to the particulate fraction was induced by $\alpha$G184S compared to wild type $\alpha$ and $\alpha$R179C (Figure 2B lanes 9 and 10). The presence of some increased $\beta_5$RGS7 in the particulate fraction is probably due to some membrane localization when proteins are expressed at very high levels, since fractionation experiments utilize a population of cells and thus do not distinguish between cells expressing different levels of the proteins. As was the case when $\alpha$G204A was expressed, when cells expressed $\alpha$G184S proteins at high levels some PM localization of $\beta_5$RGS7 was observed (data not shown). The observations with $\alpha$G184S suggest that interaction of $\alpha$ with the RGS domain is important in $\alpha$-mediated PM localization of $\beta_5$RGS7.
Next, we considered the possibility that the failure of \( \alpha_q \) to promote PM localization of \( \beta_5 \)RGS7 (Figure 1) was due to a requirement for the \( \alpha \) subunit to be sufficiently activated. Thus, we tested the ability of the constitutively active mutant \( \alpha_q R183C \) to affect localization of \( \beta_5 \)RGS7. As was the case with \( \alpha_q \), \( \alpha_q R183C \) failed to promote PM localization of \( \beta_5 \)RGS7 (Figure 2). When co-expressed with \( \alpha_q R183C \), \( \beta_5 \)RGS7 remained in the cytoplasm of cells (Figure 2A q-t) and was found predominantly in the soluble fraction (Figure 2B lanes 11 and 12). These results confirm that \( \alpha_q \), in contrast to \( \alpha_o \), is not effective at promoting PM localization of \( \beta_5 \)RGS7.

\( \alpha_o \) induced palmitoylation of \( \beta_5 \)RGS7. 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 \( \beta_5 \) in Sf9 insect cells (Rose et al., 2000). We looked at whether RGS7 is palmitoylated in mammalian cells. RGS7 and \( \beta_5 \) were transfected into COS7 cells in the absence or presence of \( \alpha_o \) or \( \alpha_o R179C \). Cells were metabolically labeled with \([3H]\)palmitate and incorporation of radioactivity into RGS7 was analyzed as described in Materials and Methods. Control transfection of empty vector showed no nonspecific binding of the radioactivity (Figure 3 upper panel lane 1). No incorporation of radiolabeled palmitate into RGS7 was seen without \( \alpha_o \) expression (Figure 3 upper panel lane 2). On the other hand, RGS7 incorporated radioactive palmitate in the presence of \( \alpha_o \) or \( \alpha_o R179C \) (Figure 3 upper panel lanes 3 and 4). Expression of RGS7 was confirmed by Western blotting (Figure 3 lower panel).

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 αo-promoted PM localization. The mutants were expressed in conjunction with β5 and αo, and their localization was detected by using the 12CA5 antibody. RGS7C120S was not detected at the PM; however, it’s expression was extremely low (not shown) and thus we could not conclusively demonstrate a defect in αo-promoted PM localization. β5RGS7C133S expression was somewhat variable, but the typical expression level was approximately 25-50% of wild type β5RGS7 (Figure 4B lanes 5-7). Although expressed at a reduced level, RGS7C133S retained binding to β5 as determined by pull down experiments utilizing the hexahistidine tag on β5 (data not shown). We thus compared localization of β5RGS7C133S with wild type β5RGS7. In immunofluorescence microscopy, β5RGS7C133S displayed a diffuse cytoplasmic distribution even when expressed with PM-localized αo (Figure 4A a and c). When assayed by subcellular fractionation, β5RGS7C133S was partially recruited to the particulate fraction when co-expressed with αo (Figure 4B lanes 1 and 2) although the portion of RGS7C133S in the particulate was significantly reduced compared to wild type RGS7 when co-expressed with αo (Figure 1B lanes 3 and 4). On the other hand, β5RGS7C206S was strongly recruited to PM (Figure 4A b and d) or the particulate fraction (Figure 4B lanes 3 and 4) by expression of αo. Collectively, the data with the cysteine mutants indicate that Cys133 of RGS7 is critical for PM localization of β5RGS7, and suggest that Cys133 is a putative palmitoylation site.

**Amino acids 76-128 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). RGS7\[17-75, a deletion of the N-terminal portion of the DEP domain, and RGS7\[17-112, a deletion of the entire DEP domain, were expressed together with \( \beta_5 \) and \( \alpha_o \), but RGS7\[17-75 and RGS7\[17-112 protein was almost undetectable, as assessed by Western blotting (Figure 5A lanes 2 and 3). RGS7\[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 to wild type RGS7 (Figure 5A lanes 1 and 4). Importantly, \( \beta_5 \)RGS7\[76-128 was refractory to \( \alpha_o \)-promoted PM localization (Figure 5B a); it remained in the cytoplasm, implying that the deleted region is important in \( \alpha_o \)-mediated PM localization of the complex.
Discussion

We demonstrated herein that $\beta_5$RGS7 is mostly cytoplasmic and soluble when expressed in HEK293 cells, but co-expression of $\alpha_o$ promoted a strong redistribution of $\beta_5$RGS7 to the PM. Expression of $\alpha_q$, however, did not elicit a similar PM recruitment of $\beta_5$RGS7. PM localization of $\beta_5$RGS7 was promoted by constitutively active $\alpha_o$R179C, but not the inactive mutant $\alpha_o$G204A. Moreover, our results suggest that PM recruitment of $\beta_5$RGS7 is mediated, at least partly, through interaction of its RGS domain with $\alpha_o$, since the RGS-insensitive $\alpha_o$G184S mutant showed a decreased ability to recruit $\beta_5$RGS7 to the PM. In addition to PM recruitment of $\beta_5$RGS7, we demonstrate that expression of $\alpha_o$ is required for detectable palmitoylation of RGS7 in COS-7 cells. Lastly, 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 $\alpha$ subunit target of $\beta_5$RGS7 remains controversial. In GAP assays using purified proteins, $\beta_5$RGS7 is highly selective for $\alpha_o$ and exhibits no GAP activity on $\alpha_q$ (Hooks et al., 2003; Posner et al., 1999). However, when expressed in cells, $\beta_5$RGS7 can regulate $\alpha_o$, $\alpha_i$, and $\alpha_q$ dependent signaling pathways (Ghavami et al., 2004; Keren-Raifman et al., 2001; Kovoor et al., 2000; Shuey et al., 1998; Witherow et al., 2003; Witherow et al., 2000; Zhang et al., 2002). In our studies, $\alpha_o$ but not $\alpha_q$ was able to promote PM localization of $\beta_5$RGS7 in cells. Thus, our assays of PM recruitment are consistent with selectivity of $\beta_5$RGS7 for $\alpha_o$. Surprisingly, our studies are in contrast to a recent report showing a FRET signal between wild type $\alpha_q$ and $\beta_5$RGS7 in cells but no FRET signal between $\alpha_o$ and $\beta_5$RGS7 (Witherow et al.,...
2003). We observed no PM recruitment of \( b_5 \)RGS7 when wild type \( a_q \) was expressed. An explanation for these seemingly contradictory results is not clear. Interestingly, stable complexes between purified \( b_5 \)RGS7 and \( a_o \), either GDP- or GDP-\( \text{AlF}_4^- \)-bound, cannot be detected (Posner et al., 1999), and co-immunoprecipitation and pull down approaches from cell lysates have likewise failed to isolate \( a_o \) or \( a_q \) bound to \( b_5 \)RGS7 (data not shown) (Witherow et al., 2000). It appears as if the binding of \( b_5 \)RGS7 to \( a_o \) is relatively weak compared to other RGS/G\( a \) pairs (Posner et al., 1999), and thus the PM recruitment of \( b_5 \)RGS7 by \( a_o \) may provide a surrogate method for monitoring association of \( b_5 \)RGS7 and \( a_o \) in cells. In addition, unidentified proteins likely influence the affinity and selectivity of \( b_5 \)RGS7 binding to \( a \) subunits. For example, the related complex, \( b_5 \)RGS9-1, fails to form a stable complex with \( a_t \) or \( a_o \) using purified proteins unless the \( a_t \) effector cGMP phosphodiesterase subunit \( \text{PDE} \) is also included (Martemyanov and Arshavsky, 2002; Martemyanov et al., 2003a). The identification of similar affinity adaptors, possibly \( a_o \) or \( a_q \) effectors, will shed light on \( b_5 \)RGS7/G\( a \) specificity in vivo.

How does \( a_o \) mediate RGS7’s PM localization? Our results suggest a model in which the RGS domain of RGS7 interacts preferentially with active \( a_o \). The inactive \( a_o \)G204A, a mutant that can serve a dominant negative function due to an inability to undergo activating conformational changes, failed to induce strong PM localization of \( b_5 \)RGS7, while constitutively active mutant \( a_o \)R179C promoted strong PM recruitment of \( b_5 \)RGS7. Interestingly, in our hands wild type \( a_o \) was just as effective as \( a_o \)R179C in recruiting \( b_5 \)RGS7 to the PM in transient transfection experiments. We suspect that wild type \( a_o \) did so most likely because some fraction of the overexpressed protein was in fact active; it is not uncommon for overexpressed \( a \) subunits to show some ability to activate signaling pathways even in the absence of receptor stimulation or an activating
mutation. Alternatively, high amounts of overexpressed wild type $\alpha_o$ may simply overcome its lower affinity compared to activated $\alpha_o$ for RGS7. Active $\alpha_o$ likely interacts directly with $\beta_5$RGS7’s RGS domain to induce PM recruitment, and this proposal is supported by the failure of RGS-insensitive $\alpha_o$G184S to promote strong PM localization of $\beta_5$RGS7; 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 $\alpha$ subunits can selectively recruit RGS domain-containing proteins to the PM (Bhattacharyya and Wedegaertner, 2003; Day et al., 2003; Druey et al., 1998; Heximer et al., 2001; Masuho et al., 2004). There have been suggestions, though no direct demonstrations, that complexes of $\beta_5$ and R7 RGS family members, such as RGS7, can interact with inactive $\alpha$ subunits via $\beta_5$ and thus form a novel G protein heterotrimer. Although we cannot rule out that PM recruitment of $\beta_5$RGS7 is mediated by $\beta_5$ binding to $\alpha_o$, our results with $\alpha_o$ mutants are more consistent with the model that PM localization of $\beta_5$RGS7 is mediated, at least partly, through RGS domain binding to active $\alpha_o$.

However, the presence of the RGS domain of RGS7 appears not to be sufficient for $\alpha_o$-induced PM localization of $\beta_5$RGS7. For example, mutants of RGS7 containing an intact RGS domain but with partial deletion of the DEP domain, RGS7D76-128, or the mutation C133S were deficient in $\alpha_o$-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 $\beta_5$ in COS-7 cells. Co-expression of $\alpha_o$ or $\alpha_o$R179C with $\beta_5$RGS7, however, promoted RGS7 palmitoylation. Thus, our palmitoylation assays were consistent with observations of PM localization; both palmitoylation and PM
localization of $\alpha_5$RGS7 were promoted by $\alpha_0$. To address potential sites of RGS7 palmitoylation several cysteines were mutated individually to serines and $\alpha_0$-promoted PM localization was determined. $\alpha_5$RGS7C206S was recruited to the PM just like wild type $\alpha_5$RGS7. In contrast, $\alpha_5$RGS7C133S displayed a clear defect in PM recruitment. These results are consistent with Cys133 being a site for palmitoylation of RGS7, although requirements for very long exposures 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 $\alpha_z$ (Tu et al., 1999) while palmitoylation of RGS16 increased GAP function (Osterhout et al., 2003). Hepler and colleagues showed that, when purified from Sf9 cells, membrane-bound RGS7, which is palmitoylated, and cytosolic, non-palmitoylated RGS7 equipotently stimulated the GTPase activity of $\alpha_0$, suggesting that palmitoylation has no effect on RGS7’s GAP activity (Rose et al., 2000). Thus, for RGS7, a primary function of palmitoylation is probably to facilitate PM targeting of $\alpha_5$RGS7.

Despite the recent breakthrough in identification of bona fide palmitoyltransferases in yeast (Linder and Deschenes, 2003; Linder and Deschenes, 2004; Lobo et al., 2002), 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 $\alpha_0$-mediated membrane localization of $\alpha_5$RGS7 (data not shown). Thus, our findings suggest
that RGS7’s PM targeting and, presumably, palmitoylation are independent of the conventional exocytic pathway. The αo subunit itself is modified with palmitate, and its palmitoylation has been shown to be Brefeldin A insensitive (Gonzalo and Linder, 1998), thus suggesting that αo does not require a functional Golgi, and by extension does not utilize the classical exocytic pathway, for palmitoylation and PM targeting. Furthermore, we showed that a lipidation defective αoG2A mutant was unable to induce β5RGS7’s PM localization (Figure 2A c), and αoC3S, which is myristoylated but not palmitoylated, was also unable to recruit β5RGS7 to the PM (not shown) suggesting that palmitoylation of αo is required for PM targeting of the β5RGS7 complex. Whether the same palmitoyltransferase can catalyze attachment of palmitate to αo and RGS7 remains to be seen.

DEP domains may play roles in subcellular targeting of proteins (Martemyanov et al., 2003b), though the mechanisms are not clear. RGS7Δ76-128 was defective in αo-promoted PM localization. This may indicate that the DEP domain plays a unique role in facilitating membrane targeting. Alternatively, 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 RGS7Δ17-75 and RGS7Δ17-112. These results suggest that deletion of the N-terminal portion of RGS7’s DEP domain creates an unstable protein, and, 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, appears 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 manuscript demonstrated that \( \text{a} \) 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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References


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Footnotes

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Figure Legends

Figure 1. αo promotes membrane targeting of β5RGS7. A, expression vectors for β5 (450 ng) and RGS7 (550 ng) were tranfected into HEK293 cells in conjunction with pcDNA3 (500 ng) (a and f), αo (500 ng) (b, d, and g), or αq (500 ng) (c, e, and h). 36 h after transfection, immunofluorescence staining and deconvolution microscopy were performed to visualize transiently expressed proteins. Primary antibodies utilized were monoclonal anti-HA 12CA5 antibody for RGS7 (a-c), rabbit polyclonal anti-αo (d) or rabbit polyclonal anti-αq antibody (e). Cells were also stained with DAPI to visualize nuclei (f-h). Images shown are representative of greater than 100 cells examined in more than three experiments. Bar, 5 μm. B, HEK293 cells expressing β5 and RGS7 (lanes 1 and 2), β5, RGS7, and αo (lanes 3 and 4), or β5, RGS7, and αq (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 co-expressed with β5 and the following α subunit: no α, S:P=96:4 ± 1 (n=3); αo, S:P=7:93 ± 1 (n=3); αq, S:P=>95:<5 (no detectable RGS7 band in P fraction) (n=3).

Figure 2. Effect of αo mutants on membrane targeting of β5RGS7. A, HEK293 cells were transfected with plasmids containing β5 (450 ng) and RGS7 (550 ng) along with αoR179C (500 ng) (a-d), αoG204A (500 ng) (e-h), αoG2A (500 ng) (i-l), αoG184S (500 ng) (m-p), or αqR183C (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 \( \beta_5 \)RGS7 (a, e, i, m, and q) and a rabbit polyclonal anti-\( \alpha_o \) antibody for \( \alpha_o \) (b, f, j, and n) or a rabbit polyclonal anti-\( \alpha_q \) antibody (r). Cells were also stained with DAPI to visualize nuclei (c, g, k, o, and s). Merged images with RGS7 (green), \( \alpha_o \) or \( \alpha_q \) (red), and nuclei (blue) are also shown (d, h, l, p, and t). Images shown are representative of greater than 100 cells examined in more than three experiments. Bar, 5 \( \mu \)m. 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 co-expressed with \( \beta_5 \) and the following \( \alpha \) subunit: \( \alpha_o \)R179C, S:P=17:83 ± 12 (n=5); \( \alpha_o \)G204A, S:P=52:48 ± 4 (n=3); \( \alpha_o \)G2A, S:P=88:12 ± 9 (n=3); \( \alpha_o \)G184S, S:P=65:35 ± 16 (n=4); \( \alpha_q \)R183C, 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 \( \beta_5 \) and RGS7 (lanes 2 and 8), or vectors for \( \beta_5 \) and RGS7 along with \( \alpha_o \)R179C (lanes 3 and 9), \( \alpha_o \)G204A (lanes 4 and 10), \( \alpha_o \)G2A (lanes 5 and 11), or \( \alpha_o \)G184S (lanes 6 and 12) were immunoblotted with 12CA5 anti-HA monoclonal antibody (lanes 1-6) or anti-\( \alpha_o \) polyclonal antibody (lanes 7-12).

**Figure 3. Palmitoylation of RGS7.** COS7 cells were transfected with pcDNA3 (4.5 \( \mu \)g) (lane 1), or expression constructs for \( \beta_5 \) (1.35 \( \mu \)g) and RGS7 (1.65 \( \mu \)g) along with pcDNA3 (1.5 \( \mu \)g) (lane 2), \( \alpha_o \) (1.5 \( \mu \)g) (lane 3), or \( \alpha_o \)R179C (1.5 \( \mu \)g) (lane 4). Cells were labeled for 3 h with 1 mCi/ml of \( ^{3} \)H]palmitate. \( \beta_5 \)RGS7 was
immunoprecipitated using an anti-HA polyclonal antibody. The samples were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The PVDF membrane was exposed to a film at –80°C. Subsequently, the membrane was subjected to Western blotting for RGS7. The blots shown are representative of two experiments.

**Figure 4. Subcellular localization of RGS7 cysteine mutants.** A, HEK293 cells were transfected with expression plasmids for a (500 ng), b5 (450 ng) and RGS7C133S (550 ng) (a and c) or a (500 ng), b5 (450 ng) and RGS7C206S (550 ng) (b and d). 36 h after transfection, cells were fixed and subjected to immunofluorescence staining as described under Materials and Methods. Primary antibodies used were a rabbit polyclonal anti-a antibody for the a subunit and a mouse monoclonal anti-HA 12CA5 antibody for b5RGS7. Images shown are representative of greater than 100 cells examined in more than three experiments. Bar, 5 μm. B, HEK293 cells expressing a, b5 and RGS7C133S (lanes 1 and 2), or a, b5 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 due to lower expression levels. Expression levels of RGS7 (lane 5), RGS7C133S (lane 6), and RGS7C206S (lane 7) when co-expressed with a and b5 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 co-expressed with $b_5$ and $\alpha_0$: RGS7C133S, S:P=47:53 ± 4 (n=3); RGS7C206S, S:P=12:88 ± 8 (n=5).

**Figure 5. RGS7 DEP domain deletions.** A, Cells transiently expressing $\alpha_0$ (500 ng) and $b_5$ (450 ng) in conjunction with wild type RGS7 (550 ng) (lane 1), RGS7(D17-75) (550 ng) (lane 2), RGS7(D17-112) (550 ng) (lane 3), or RGS7(D76-128) (550 ng) (lane 4) were solublized and analyzed by SDS-PAGE and Western blotting using the 12CA5 monoclonal antibody. B, Expression vectors for $\alpha_0$ (500 ng), $b_5$ (450 ng) and RGS7(D76-128) (550 ng) were transfected into HEK293 cells. 36 h after transfection, immunofluorescence staining was carried out to visualize transiently expressed proteins as described under Materials and Methods. Images shown are representative of greater than 100 cells examined in more than three experiments. Bar, 5 μm.
Figure 1
Figure 2
Figure 3

Fluorography

$[^3H] $palmitate

Western Blot: 12CA5

Control  $\beta_5$RGS7  $\alpha_o \beta_5$RGS7  $\alpha_o R179C \beta_5$RGS7

1  2  3  4
**Figure 4**

(A) Immunofluorescence images showing the distribution of RGS7 in cells expressing different combinations of α<sub>o</sub>β<sub>5</sub> and RGS7 variants. 
- **a**: β<sub>5</sub>RGS7C133S
- **b**: α<sub>o</sub>β<sub>5</sub>RGS7C206S

(B) Blot analysis using the 12CA5 antibody to detect RGS7C133S and RGS7C206S in cell lysates. 
- **S**: unphosphorylated samples
- **P**: phosphorylated samples

Samples 1 and 2 show phosphorylation of RGS7C133S, while samples 3 and 4 show phosphorylation of RGS7C206S. Samples 5 to 7 represent cell lysates with different phosphorylation states.
A

Blot: 12CA5

1  2  3  4

B

\(\alpha_0\beta_5\text{RGS7}\Delta 76-128\)

\(\beta_5\text{RGS7}\)

Figure 5