Gβγ and Palmitate Target Newly Synthesized Gαz to the Plasma Membrane

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The subcellular location of a signaling protein determines its ability to transmit messages accurately and efficiently. Three different lipid modifications tether heterotrimeric G proteins to membranes: α subunits are myristoylated and/or palmitoylated, and γ subunits are prenylated. In a previous study, we examined the role of lipid modifications in maintaining the membrane attachment of a G protein α subunit, αz, which is myristoylated and palmitoylated (Morales, J., Fishburn, C. S., Wilson, P. T., and Bourne, H. R. (1998) Mol. Biol. Cell 9, 1–14). Now we extend this analysis by characterizing the mechanisms that target newly synthesized αz to the plasma membrane (PM) and analyze the role of lipid modifications in this process. In comparison with newly synthesized αz, which is palmitoylated but not myristoylated, αz moves more rapidly to the membrane fraction following synthesis in the cytosol. Newly synthesized αz associates randomly with cellular membranes, but with time accumulates at the PM. Palmitoylated αz is present only in PM-enriched fractions, whereas a nonpalmitoylated mutant of αz (αzC3A) associates less stably with the PM than does wild-type αz. Expression of a C-terminal fragment of the β-adrenoreceptor kinase, which sequesters free βγ, impairs association of both αz and αzC3A with the PM, suggesting that the α subunit must bind βγ in order to localize at the PM. Based on these findings, we propose a model in which, following synthesis on soluble ribosomes, myristoylated αz associates randomly and reversibly with membranes; upon association with the PM, αz binds βγ, which promotes its palmitoylation, thus securing it in the proper place for transmitting the hormonal signal.

Accurate signal transmission from cell surface receptors to intracellular effectors requires that the appropriate signaling proteins interact to form highly co-ordinated pathways. This coordination depends critically on the correct cellular locations of the participating proteins. Heterotrimeric G proteins are peripheral membrane proteins, localized at the plasma membrane (PM),2 which convey signals from activated receptors to downstream effectors (1, 2). Covalent lipid attachments on the α subunit and on the heterodimeric βγ subunit tether G proteins to the cytoplasmic face of membranes: isoprenylation of the γ-polypeptide at its C terminus provides a strong hydrophobic anchor for βγ, whereas α subunits are modified at their N termini by myristoylation and/or palmitoylation (3–5). G proteins must localize at the PM in order to interact with receptors and many of their effectors. How they reach the PM following their synthesis on ribosomes is not well understood. In a previous study, we examined the role of lipid modifications in maintaining the membrane attachment of G protein α subunits (6). We now extend this analysis by characterizing the mechanisms that target newly synthesized α subunits to the PM and analyze the role of lipid modifications in this process.

Posttranslational lipid modifications can control the association of otherwise cytosolic proteins with membranes. Studies of Ras, Src kinases, and the myristoylated alanine-rich protein kinase C substrate show that a combination of two membrane-binding signals enables each of these proteins to associate specifically and tightly with its target membrane (7–9). For G protein α subunits in the α family, these two signals are the fatty acyl chains myristate and palmitate (4, 6, 10).

Myristate and palmitate are 14- and 16-carbon saturated fatty acids that together provide high membrane avidity for the α subunit (reviewed in Ref. 11). The different properties of these lipids suggest, however, that their functional roles differ also. Whereas myristoylation occurs co-translationally in the cytosol, palmitoylation takes place postranslationally in the membrane fraction. Myristate is attached via a stable amide linkage to an N-terminal glycine residue, and by itself provides only weak attachment to membranes. Palmitate is bound by a reversible thioester linkage to cysteine residues, and its greater hydrophobicity provides approximately 15-fold stronger membrane affinity, as compared with myristate (11). In addition to providing hydrophobicity, palmitate helps determine the subcellular location of a protein. Mutation of the palmitoylated cysteines in α subunits, Ras and Src kinases causes these proteins to mislocalize and redistribute from the PM to intracellular membranes (6, 12–15). Conversely, cytosolic proteins translocate specifically to the PM when fused to the N-terminal palmitoylated sequence of a Src kinase (16, 17). In addition to these roles, palmitate and myristate may mediate or modulate direct protein-protein interactions; for example, βγ shows greater affinity for αz or αi subunits when they are palmitoylated or myristoylated, respectively (10, 18, 19).

In an earlier study, we presented immunocytochemical evidence that palmitate enables G protein α subunits to associate specifically with the PM, whereas myristate and βγ stabilize both membrane attachment and the presence of palmitate (6). Here, we have sought to determine how newly synthesized G proteins are targeted to membranes in a manner that reflects their presence at the PM, where they can contribute to signal transduction.

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1 The abbreviations used are: PM, plasma membrane; βARK, β-adrenoreceptor kinase; ct, C terminus; BFA, Brefeldin A; CHO, Chinese hamster ovary; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; SNAP-25, synaptosomal-associated protein of 25 kDa; IM, intracellular membrane; mAb, monoclonal antibody.

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protein α subunits are targeted specifically to the PM, their functional location in the cell. Using metabolic labeling and biochemical fractionation on sucrose density gradients, we have followed the subcellular location of newly synthesized recombinant αi in CHO cells. The results of these experiments confirm and extend our previous inferences from immunocytochemistry. Moreover, they suggest a model in which, following synthesis, myristoylated αi associates randomly and reversibly with cellular membranes until it reaches the PM, where association with β and attachment of palmitate trap it at the appropriate location for transmitting the hormonal signal.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glutathione S-transferase (GST) recombinant proteins, MBP recombinant proteins, p58 antibody, and antibodies against the EE, HA, and HR epitopes were obtained from Berkeley Antibody Co. (Berkeley, CA). Mouse IgG fractions were obtained from C-terminus (Denver, CO). RAP was obtained from Chiron (Emeryville, CA). [3H]Leucine, [35S]Methionine, and [35S]Sulfate were obtained from NENLife Science Products. [3H]Palmitic acid was obtained from NEN Life Science Products.

**Cell Culture and Transfection**—CHO-K1 cells were propagated in minimal essential medium α with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) (complete medium) at 37 °C in 5% CO2 in a humidified incubator. Cells were transfected and stable cell lines established as described previously (6). Transient transfection with the βARK-ct-adenovirus and LacZ-adenovirus vectors was performed by incubating 4 × 106 cells/100-mm plate in 2 ml of serum-free minimal medium α containing 50 μl of concentrated virus stock for 2 h, followed by addition of 8 ml of complete medium and incubation overnight. Cells were replated the following day and used in experiments 48 h after transfection.

**Metabolic Labeling**—CHO-KI cells (2 × 107/150 mm plate), stably expressing the epitope-tagged αi (αi, EE), αC3A (αC3A, EE), αi,HA, or FLAG αi opioid receptor, were starved for 1 h in methionine- and cysteine-free Dulbecco’s modified Eagle’s medium without 1% FBS for 2 h, followed by addition of 8 ml of complete medium and incubation overnight. Cells were replated the following day and used in experiments 48 h after transfection.

**Subcellular Fractionation**—For each condition, one confluent 150-mm plate was metabolically labeled and then washed in ice-cold Ca2+/Mg2+-free phosphate-buffered saline and incubated for 4 h in serum-free minimal essential medium α containing 5 mM sodium pyruvate and 1.0 mM Ca2+/Mg2+.

**Enzyme Linked Immunosorbent Assay (ELISA)**—CHO-K1 cells were propagated in minimal essential medium α with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) (complete medium) at 37 °C in 5% CO2 in a humidified incubator. Cells were transfected and stable cell lines established as described previously (6). Transient transfection with the βARK-ct-adenovirus and LacZ-adenovirus vectors was performed by incubating 4 × 106 cells/100-mm plate in 2 ml of serum-free minimal medium α containing 50 μl of concentrated virus stock for 2 h, followed by addition of 8 ml of complete medium and incubation overnight. Cells were replated the following day and used in experiments 48 h after transfection.

**Membranes—**CHO-KI cells were labeled with [3H]palmitic acid as described previously (21, 22). FLAG-tagged αi (αi, EE), αC3A (αC3A, EE), αi,HA, or FLAG αi opioid receptor, were starved for 1 h in methionine- and cysteine-free Dulbecco’s modified Eagle’s medium without 1% FBS for 2 h, followed by addition of 8 ml of complete medium and incubation overnight. Cells were replated the following day and used in experiments 48 h after transfection.

**Subcellular Fractionation**—For each condition, one confluent 150-mm plate was metabolically labeled and then washed in ice-cold Ca2+/Mg2+-free phosphate-buffered saline and incubated for 4 h in serum-free minimal essential medium α containing 5 mM sodium pyruvate and 1.0 mM Ca2+/Mg2+.

**Immunoprecipitation of αi and recombinant αi, wild-type or αiC3A by immunoblotting cell extracts with an αi antibody (αi, Santa Cruz Biotechnology, Santa Cruz, CA) or EE mAb, Santa Cruz Biotechnology, Santa Cruz, CA) or EE mAb, respectively. Varying amounts of membranes (P100 fraction) from cells expressing either αi, wild-type or αiC3A, solubilized in sample buffer, were subjected to SDS-PAGE and blotted with the appropriate antibody. Bands were visualized by ECL and signal intensity was quantitated as described above. Signal intensity per cell equivalent was calculated from samples in which signal intensity varied directly with amount of cell extract (that is, linear portions of the extract-intensity curve), and normalized by comparison with a standard curve of signal intensity derived from blots, with the same antibodies, of known amounts of αiEE purified from SF9 cells as described (23).

**RESULTS**

Experiments were performed on stably transfected CHO cell lines expressing the epitope-tagged α subunits αi, EE, αC3A-EE (palmitoylation-defective mutant) or αi, HA. Quantitative Western analysis revealed cellular contents of αi and αC3A-EE in the stable cell lines comparable to that of endogenous αi (2). Subcellular localization of αi (2 × 106 molecules per cell data not shown); cellular content of β was not significantly altered (data not shown). We have previously shown that the addition of the EE or HA epitope tags does not alter signaling or membrane attachment properties of these α subunits (21, 22) and that the αC3A-EE palmitoylation-defective mutant incorporates myristate but not palmitate (22).

**Myristoylation Allows Rapid Association of αi with Cellular Membranes**—Because mutational removal of the myristoyla-
tion site of \( \alpha_s \) (\( \alpha_s \)G2A) produces a cytosolic protein that is neither myristoylated nor palmitoylated (6, 22), we sought a different approach to gain insight into the potential role of myristate on targeting of newly synthesized \( \alpha_s \). To do so, we compared the rate of membrane association of \( \alpha_s \) with that of a second \( \alpha \) subunit, the \( \alpha \) subunit (\( \alpha_1 \)) of G\(_r\), because \( \alpha_1 \) is naturally subject only to palmitoylation but not myristoylation (4).

Separation of \(^{35}\)S-methionine labeled cells expressing \( \alpha_s \)EE or \( \alpha_s \)HA into cytosolic S100 and particulate P100 fractions shows that newly synthesized \( \alpha_s \)HA associates much more slowly with membranes than does \( \alpha_s \)EE (Fig. 1). Following a 10-min pulse, 54% of \( \alpha_s \)EE was membrane-bound, compared with only 10% for \( \alpha_s \)HA (Fig. 1A), suggesting that myristate allows an initial rapid association of \( \alpha_s \)EE with cellular membranes. In keeping with the idea that \( \alpha_s \)EE and \( \alpha_s \)HA are synthesized on soluble ribosomes, both proteins began in the cytosol fraction and moved to particulate fractions; as expected, both were found almost exclusively in the P100 fraction when chased for longer periods (Fig. 1B).

Subcellular Fractionation of Newly Synthesized \( \alpha_s \)—To obtain a more detailed picture of the distribution of newly synthesized \( \alpha_s \) among the membranes of subcellular organelles, we fractionated metabolically labeled cells expressing \( \alpha_s \)EE on sucrose gradients and immunoprecipitated radiolabeled \( \alpha_s \)EE from each fraction. PM-enriched lighter membrane fractions separated well from heavier intracellular membranes. Fig. 2B shows that the PM is predominantly enriched in the later, light sucrose fractions (Fig. 2C), whereas the earlier, denser fractions contain mainly endoplasmic reticulum and some Golgi membranes, but are relatively devoid of PM (Fig. 2D, E, and F). We used presence or absence of immunoreactivity to the PM marker, Na\(^+\)/K\(^-\)-ATPase, to distinguish fractions enriched, respectively, for PM or for intracellular membranes (IMs). After a 2.5 h chase, \(^{35}\)S-methionine-labeled \( \alpha_s \) shows the same distribution as the \( \alpha_s \)EE protein detected by Western analysis (Fig. 2A, A and B) and cofractionates with the PM marker, Na\(^+\)/K\(^-\)-ATPase (Fig. 2C). Thus, by 2.5 h following synthesis, \( \alpha_s \)EE has reached its steady-state distribution, in which it is predominantly located in the PM; this agrees with previous observations of steady-state, by indirect immunofluorescence, of \( \alpha \) subunits expressed in cultured cells (6, 24). Experiments using shorter chase periods showed that \( \alpha_s \)EE reached its steady-state distribution within 30 min following a 10-min pulse (see Fig. 6).

To determine whether newly synthesized \( \alpha_s \) associates di-

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**FIG. 1. Movement of newly synthesized \( \alpha \) subunits from cytosol to membranes.** A, pulse-labeling and crude fractionation of CHO cells stably expressing \( \alpha_s \)EE (left) or \( \alpha_s \)HA (right). Cells were incubated with 350 \( \mu \)Ci/ml \(^{35}\)S-methionine for the times indicated and then separated into soluble (S, black columns) and particulate (P, white columns) fractions as described under "Experimental Procedures." \( \alpha_s \)EE and \( \alpha_s \)HA were immunoprecipitated from detergent extracts of each fraction, subjected to SDS-PAGE, and quantitated by PhosphorImager analysis. B, appearance of newly synthesized \( \alpha \) subunits in the particulate fraction with time. Cells were pulse-labeled for 10 min and incubated in chase medium for the indicated times before being subjected to crude fractionation as in A. \( \alpha_s \)EE (black circles) and \( \alpha_s \)HA (white circles) were immunoprecipitated, run on gels, and quantitated as described. Values represent the means \( \pm \) 2 S.E. of three independent experiments.

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**FIG. 2. \( \alpha_s \) co-fractionates with the PM on sucrose gradients.** Pulse-chase labeling of CHO cells stably expressing \( \alpha_s \)EE. Cells were labeled with \(^{35}\)S-methionine for 10 min and then incubated in chase medium for 2.5 h and fractionated on sucrose gradients as described under "Experimental Procedures." Fractions (\( \sim 1.2 \) ml) were collected from bottom to top (shown left to right) from each fraction, 0.8 ml was taken for immunoprecipitation of radiolabeled \( \alpha_s \)EE with EE mAb, and 0.2 ml was subjected to trichloroacetic acid precipitation and Western blotting onto Immobilon P\(^{3}54\) membranes. Following immunoprecipitation, radiolabeled \( \alpha_s \)EE was subjected to SDS-PAGE and quantitated by PhosphorImager analysis. Subcellular organelles were detected using either antibodies against marker enzymes or by assaying enzyme activity (see under "Experimental Procedures"). A, distribution of radiolabeled \( \alpha_s \)EE on a sucrose gradient. B, distribution of total \( \alpha_s \)EE, detected by immunoblotting with the EE mAb. C, Na\(^+\)/K\(^-\)-ATPase (PM). D, p58 (cis-Golgi). E, \( \alpha \) glucosidase I (endoplasmic reticulum). F, protein concentration. These results are representative of six similar experiments.
directly with the PM or whether it first associates with other intracellular membranes, we compared the subcellular distribution of αEE at a short time after synthesis (10-min pulse, no chase) with that at steady-state (10-min pulse, 2.5-h chase). αEE associated indiscriminately with cellular membranes shortly after synthesis and was present in both IM and PM fractions (Fig. 3). Following a 10-min pulse, 35% of membrane-bound αEE was localized in intracellular membranes (Fig. 3, A and C). This proportion fell to 10% with time, as αEE moved to the PM (Fig. 3, B and C) and then remained at this level with chase periods of up to 4 h (data not shown).

**α Does Not Travel to the Plasma Membrane via the Exocytic Pathway**—The results of Fig. 3 suggest that immediately after synthesis αEE associates with various intracellular membranes, and with time accumulates at the PM. To test whether αEE travels to the PM through the exocytic pathway (hypothetically attached to the cytoplasmic face of trafficking vesicles), we treated αEE-expressing cells with Brefeldin A (BFA), which disrupts endoplasmic reticulum-Golgi transport and prevents proteins that use the exocytic pathway from reaching the PM (25). As a control for the effect of BFA, we performed a parallel experiment on CHO cells stably expressing the μ-opioid receptor with a FLAG epitope tag at the N terminus. This receptor is an integral membrane protein, the transport of which to the PM should be inhibited by BFA. Fig. 4 shows that BFA failed to prevent αEE from reaching the PM; that is, the distribution of αEE is not affected by BFA (Fig. 4, A and B). In contrast, the newly synthesized μ-opioid receptor remains in the early, heavy sucrose fractions following treatment with BFA (Fig. 4, C and D). BFA treatment caused the Golgi marker p58 to localize in the heavy sucrose fractions, and its distribution no longer overlapped partially with that of the PM marker Na+/K+-ATPase (data not shown). However, because BFA acts early in the secretory pathway, we cannot rule out the possibility that αEE associates with membranes in the trans-Golgi network or the endosomes. These observations therefore confirm that (with or without BFA treatment) the αEE in light sucrose fractions is located in membranes derived from the PM or organelles of the late secretory pathway, rather than in co-fractionating cis-Golgi membranes.

Thus αEE reached the PM efficiently despite disruption of the structure of the intracellular organelles by BFA, which was confirmed by immunocytochemistry on marker enzymes (data not shown). Thus passage of αEE to the PM does not depend on vesicular transport or even on properly organized intracellular organelles.

**Palmitoylated α Co-fractionates with the Plasma Membrane**—In the absence of vesicular transport, the most likely route for α to reach the PM is that α uses the limited membrane affinity of its myristoyl attachment to sample different membrane populations until the protein eventually is trapped

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**Fig. 3.** Newly synthesized α moves from intracellular membranes to the PM with time. αEE-expressing cells were pulse-labeled for 10 min with 350 μCi/ml [35S]methionine and then either harvested immediately (A) or incubated for 2.5 h in chase medium before harvest (B). Cells were then fractionated on a sucrose gradient, and radiolabeled αEE (solid line) and Na+/K+-ATPase (not radiolabeled) (dotted line) were detected as for Fig. 2. The amount of αEE in each fraction was determined as a percentage of the total membrane αEE, C, relative proportion of αEE in the IMs (black columns) and PM (white columns); values were calculated by pooling the content of αEE in the fractions according to the presence (PM) or absence (IM) of Na+/K+-ATPase immunoreactivity. Bars indicate the means ± 2 S.E. of three independent experiments.

**Fig. 4.** Brefeldin A does not inhibit the transport of αEE to the PM. CHO cells stably expressing αEE (A and B) or the FLAG-tagged μ opioid receptor (μ-Opi-R) (C and D) were labeled for 10 min with 350 μCi/ml [35S]methionine and then incubated for 2.5 h in chase medium in the absence (A and C) or presence (B and D) of 10 μg/ml Brefeldin A. Cells were fractionated on sucrose gradients, and αEE or FLAG μ opioid receptor was immunoprecipitated as described under “Experimental Procedures.” Radiolabeled immunoprecipitates were subjected to SDS-PAGE and quantitated by PhosphorImager analysis. The distribution of Na+/K+-ATPase is shown by a dotted line. The percent total membrane α or μ-Opi-R is represented by a solid line.
specifically at the PM. Several studies have suggested that palmitate traps α subunits at the PM (6, 26, 27), where enzymatic palmitoylation of α subunits is proposed to take place (28). If palmitate acts to trap α subunits at the PM, then palmitoylated αs should be present only at the PM. To test this, we labeled cells expressing αEE with [3H]palmitate and fractionated them on a sucrose gradient (Fig. 5). Palmitoylated αEE was detected in PM-enriched fractions, co-fractionating precisely with Na\(^{+}/K\)^-ATPase, and was not found in the heavier IM fractions.

If, following synthesis, α needs only to be palmitoylated to localize specifically at the PM, then the palmitoylation-defective mutant αC3A should not be enriched at the PM, but should instead associate indiscriminately with membranes via its myristoyl anchor. Fractionation of αC3A-EE-expressing cells following metabolic labeling shows that in the absence of palmitoylation, the mutant protein associates less efficiently and more slowly with the PM than does wild-type α (Fig. 6). Shortly after synthesis (Fig. 6A), only 52% of membrane-bound αC3A-EE bound to PM, compared with 65% for wild-type αEE (Fig. 3). After 30 min (Fig. 6B), 63% of the radiolabeled αC3A-EE localized in PM-enriched fractions, compared with 90% for wild-type αEE; this proportion remained constant for longer chase periods (data not shown). This agrees with our previous observations by immunofluorescence microscopy (6): although a considerable portion of the palmitoylation-defective mutant αC3A was detected at the PM, a significant fraction was mistargeted to intracellular membranes, where it clustered around Golgi-like and other intracellular organelles.

Role of βγ in Targeting α to the Plasma Membrane—Because the PM constitutes only ~10% of total cellular membranes (29), αC3A-EE clearly must be enriched in this domain. Even at early times after synthesis, significant proportions of both wild-type αEE and αC3A-EE stably associate with the PM. It follows that a second targeting factor for α subunits must play a role in specific anchoring to the PM. As we previously postulated (6), the most likely candidate for an α subunit targeting protein is the βγ subunit, the partner of Ga in the G protein heterotrimer; the βγ subunit localizes exclusively in PM fractions upon density gradient fractionation of CHO cells (30).

To determine whether βγ plays a role in the specific targeting of αs to the PM, we transiently expressed βARK-ct in cells stably expressing αEE or αC3A-EE. βARK-ct sequesters free βγ and prevents βγ-mediated regulation of effectors (31). A recombinant adenovirus vector encoding βARK-ct (20) (or encoding LacZ as a control) produced highly efficient expression of βARK-ct in αEE- or αC3A-EE-expressing cells (more than 80% of cells transfected). After transfection cells were metabolically labeled to steady-state and fractionated, as before (Fig. 7). Sequestering free βγ in this way impaired the association of both αEE and αC3A-EE with the PM, reducing the amount of each present in the PM by more than 20%. Removal of both

C. S. Fishburn, P. Herzmark, J. Morales, and H. R. Bourne, unpublished data.
Impaired PM localization of \( \alpha_s \) after sequestration of endogenous free \( \beta\gamma \) by \( \beta\text{ARK-ct} \) suggests a possible role for \( \beta\gamma \) in targeting newly synthesized \( \alpha_s \) to the PM. In the absence of assistance from palmitate, \( \beta\gamma \) co-operates with myristate to tether \( \alpha_s\text{C3A} \) to the PM, enabling approximately 65% of it to localize there (Fig. 6). If myristate alone were sufficient to hold \( \alpha_s\text{C3A} \) in place, the distribution of this protein would not be affected by expression of \( \beta\text{ARK-ct} \). If palmitate and myristate could confer PM-localization on \( \alpha_s \) without requiring \( \beta\gamma \), targeting of wild-type \( \alpha_s \) would not be affected by \( \beta\text{ARK-ct} \). Because palmitate and myristate together generally provide enough free energy for stable association of proteins with membranes (33, 34), we question the notion that \( \alpha_s \) uses \( \beta\gamma \) simply as a third tether, and we suggest that \( \beta\gamma \) plays an additional and essential role in the PM-targeting of \( \alpha_s \). We propose that \( \alpha_s \) requires \( \beta\gamma \) to bind it at the PM and to promote its subsequent palmitoylation. We imagine that \( \beta\gamma \) stabilizes the \( \alpha_s \) subunit at the PM, positions it correctly for palmitoylation by a palmitol transferase, and/or prevents access of an acyl protein thioesterase. Myristate accelerates targeting by providing sufficient hydrophobicity for random but transient membrane association of \( \alpha_s \), increasing the probability that \( \alpha_s \) will associate with the PM and encounter \( \beta\gamma \).

Although the evidence presented here strongly supports a role for \( \beta\gamma \) in targeting \( \alpha_s \) subunits to the PM, it does not preclude the requirement for additional factors. Indeed the inability of \( \beta\text{ARK-ct} \) to reduce the amount of \( \alpha_s\text{C3A} \) associated with the PM below 45% (Fig. 7) may indicate a requirement for a third targeting signal, in addition to \( \beta\gamma \) and palmitate, for plasma membrane localization of \( \alpha_s \).

Although further experiments will be required to confirm the hypothesis that \( \beta\gamma \) acts as a targeting signal for \( \alpha_s \) subunits, three lines of evidence from earlier studies suggest that \( \beta\gamma \) and palmitate co-operate to target and stabilize \( \alpha_s \) subunits at the PM. First, overexpression of \( \beta\gamma \) causes nonmyristoylated (G2A) mutants of \( \alpha_s \) and \( \alpha_i \) to translocate from the cytosol to the PM and to become palmitoylated (6, 26), indicating that \( \beta\gamma \) can substitute for myristate in attracting or retaining the \( \alpha_s \) subunit at the PM; the effectiveness of \( \beta\gamma \) suggests that \( \beta\gamma \) binding may in fact play a necessary role in promoting PM-localization and palmitoylation of the myristoylated normal \( \alpha_s \) subunit. Second, the \( \alpha\beta\gamma \) heterotrimer serves as a better substrate for a partially purified palmitoyl transferase activity than does the \( \alpha_s \) subunit alone (28), suggesting that \( \beta\gamma \) may actually promote palmitoylation of \( \alpha_s \) subunits by positioning the \( \alpha_s \) subunit correctly for the palmitol transferase. Third, \( \beta\gamma \) inhibits depalmitoylation of \( \alpha_s \) in vitro (18), whereas removal of palmitate from \( \alpha_s \) in vivo causes it to dissociate from membranes and move to the cytosol (22, 35), suggesting that \( \beta\gamma \) may also protect \( \alpha_s \) subunits from cellular thioesterases and thus stabilize their attachment to membranes.

**Targeting of other \( \alpha \) Subunits (\( \alpha_s \), \( \alpha_p \), and \( \alpha_i \))—Extending the hypothesis, we propose that \( \beta\gamma \) serves as a general (albeit not exclusive) targeting signal for PM-localization of \( \alpha \) subunits. For \( \alpha_s \), the lag in initial membrane (P100) association (Fig. 1) may reflect the time \( \alpha_s \) takes to find \( \beta\gamma \) without the increased membrane avidity generated by myristate. A recent report documents a second unidentified covalent modification on \( \alpha_s \) (36), which, like myristate, may provide additional hydrophobicity. The modification is likely to be less hydrophobic than myristate because mutational removal of palmitate prevents \( \alpha_s \) but not \( \alpha_i \) or \( \alpha_p \), from binding to membranes (22, 26, 35). This difference in hydrophobicity is consistent with the slower membrane association of \( \alpha_s \) compared with \( \alpha_i \) that we observed.

In addition to containing the palmitoylation sites, their N termini form part of the \( \alpha \) subunit surface that binds \( \beta\gamma \) (37, 38). Previous studies inferred a key role of lipid modifications in the association with the PM-specific signals, palmitate and \( \beta\gamma \), halved the amount of \( \alpha_s \) in the PM—reduced it, that is, from 90% to 45%. \( \beta\text{ARK-ct} \) did not eliminate all PM-directed bias in the localization of \( \alpha_s\text{C3A} \), for one or more of several reasons, including: (a) limited ability of \( \beta\text{ARK-ct} \) to sequester all the free \( \beta\gamma \); (b) unimpaired localization of the recombinant \( \alpha_s \) in a subpopulation of \( \alpha_s\text{C3A} \)-expressing cells that failed to internalize the \( \beta\text{ARK-ct} \)-adenovirus during transfection; and (c) operation at the PM of a third \( \alpha_s \)-targeting mechanism, in addition to palmitoylation and association with \( \beta\gamma \).

**DISCUSSION**

Previous studies have identified palmitate as a key PM-targeting factor for a number of peripheral membrane proteins, including \( \alpha \) subunits, Ras and 5′c family kinases (reviewed in Ref. 32), and we previously demonstrated that palmitate acts as a PM-specific anchor for \( \alpha_s \) (6). Here we extend and modify this inference, presenting data that suggest that \( \beta\gamma \) participates in the specific PM-targeting of newly synthesized \( \alpha \) subunits. We propose a hypothetical model in which \( \beta\gamma \) acts as a PM-targeting signal for \( \alpha_s \), operating in concert with palmitate to localize the \( \alpha \) subunit at the PM, and myristate serves to increase the efficiency of targeting, acting as a nonspecific hydrophobic anchor. By extension, we propose an important role for protein partners, in addition to lipid modifications, in subcellular targeting of all peripheral membrane proteins.

**\( \beta\gamma \) Binds a Subunits at the PM and Promotes Their Palmitoylation**—Previous studies inferred a key role of lipid modifications in the subcellular distribution of \( \alpha \) subunits from assessing differences between steady-state localization of wild-type and mutant \( \alpha \) subunits in different compartments. Here, using a kinetic approach, we followed the subcellular distribution of newly synthesized \( \alpha_s \) at different times following synthesis, in order to understand the sequence of events that results in targeting of \( \alpha \) subunits to the PM.

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**Fig. 7.** \( \beta\text{ARK} \) limits the ability of \( \alpha_s \) EE and \( \alpha_s \) C3A-EE to associate with the PM. CHO cells stably expressing \( \alpha_s \) EE (A) or \( \alpha_s\text{C3A-EE} \) (B) were transiently transfected with adenovirus vectors encoding the lacZ gene (control) or \( \beta\text{ARK-ct} \) minigene, as indicated. Bars indicate means \( \pm 2 \) S.E. of three independent experiments.
furnished both a site for myristoylation and a sequence confluences of fluorescent protein, associated with the PM and underwent pathway, there is clearly no single pathway for peripheral proteins share a common route to the PM in the exocytic Similar Pathways?—Whereas all secreted and transmembrane PM lipid bilayer (45). microdomains of different lipid composition do exist within the cascades (41–44). Despite controversy regarding the size, nature may act as focal points for concentrating members of signaling molecules in detergent-resistant subdomains of the PM, which consequently contain the photoreactive membranes constitute the vast majority of cellular membranes. Myristate may provide sufficient hydrophobicity for initial membrane association, and the predominance of the disc membranes together with the abundance of βγ may be sufficient to ensure efficient targeting of αs in these cells. In addition, the extremely high concentration of rhodopsin in these cells (~3 mM) binds, albeit loosely, the αβγ heterotrimer and secures it in place, mirroring the action of palmitate.

Role of Palmitate in Targeting—If βγ is a PM-targeting signal for α subunits, how does its role in targeting differ from that of palmitate? Clearly, palmitate acts to stabilize or trap α subunits at the PM, because α3C3a, which is still myristoylated and capable of binding βγ, associates less efficiently with the PM than wild-type αs. Possibly, in addition to cooperating with βγ to secure α subunits at the PM, palmitoylation is added as a means of controlling their more precise localization within the PM, in order to regulate their availability for signaling. Several studies indicate that palmitoylation is required for (or associated with) localization of α subunits and other signaling molecules in detergent-resistant subdomains of the PM, which may act as focal points for concentrating members of signaling cascades (41–44). Despite controversy regarding the size, nature, and role of such subdomains, a consensus is growing that microdomains of different lipid composition do exist within the PM lipid bilayer (45).

Do Other Peripheral Membrane Proteins Reach the PM by Similar Pathways?—Whereas all secreted and transmembrane proteins share a common route to the PM in the exocytic pathway, there is clearly no single pathway for peripheral membrane proteins. p59gsn and SNAP-25 utilize different targeting mechanisms, and both differ from the path taken by αs. Fyn, a Src family kinase, may use a “shuttle” to convey it rapidly and specifically to the PM, similar to the complex of two heat-shock proteins, pp50 and pp90, which were postulated to transport Src to the PM (46). Fyn is both myristoylated and palmitoylated and is synthesized in the cytosol (16), like αs. However, Fyn associates with membranes far more rapidly than αs and accumulates in the membrane fraction within 5 min after synthesis (16), compared with 30 min for αs (Fig. 1B). In addition, within minutes of its synthesis, Fyn appears to associate directly with the PM rather than to sample intracellular membranes first, as does αs. Although a clear role for the pp50–pp90 complex in trafficking of Src kinases has yet to be demonstrated, the complex does associate with Fyn (16) and may act as its shuttle.

SNAP-25, a palmitoylated member of the SNARE receptor family, plays a role in neurotransmitter release from synaptic terminals. Like αs, SNAP-25 requires another protein, possibly syntaxin (which is also a SNARE) for stable membrane association (47). Unlike αs, however, SNAP-25 does not reach the PM when treated with Brefeldin A, indicating that it travels via the secretory pathway, and palmitoylation of SNAP-25 occurs on intracellular organelles rather than at the PM (47). Although α subunits, Src kinases, and SNAP-25 follow different paths to the PM, each class of proteins appears to require the assistance of one or more specific proteins (βγ, pp50–pp90 complex, or syntaxin) in addition to their lipid modifications.

Taken together, these results suggest that the proper intracellular location of many similarly lipid-modified proteins depends upon an additional targeting factor(s), probably protein-binding partners. These partners, in combination with the lipid anchors, determine the location of a protein, thus allowing similarly modified proteins to display different subcellular distributions and to use different targeting mechanisms.

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