Heterotrimer Formation, Together with Isoprenylation, Is Required for Plasma Membrane Targeting of Gβγ

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Received for publication, December 27, 2002, and in revised form, February 8, 2003
Published, JBC Papers in Press, February 27, 2003, DOI 10.1074/jbc.M213239200

Nascent β and γ subunits of heterotrimeric G proteins need to be targeted to the cytoplasmic face of the plasma membrane (PM) in order to transmit signals. We show that β1γ2 is poorly targeted to the PM and predominately localized to endoplasmic reticulum (ER) membranes when expressed in HEK293 cells, but co-expression of a G protein α subunit allows strong PM localization of the β1γ2. Furthermore, C-terminal isoprenylation of the γ subunit is necessary but not sufficient for PM localization of β1γ2. Isoprenylation of γ2 and localization of β1γ2 to the ER occurs independently of α expression. Efficient PM localization of β1γ2 in the absence of co-expressed α is observed when a site for palmitoylation, a putative second membrane targeting signal, is introduced into γ2. When a mutant of αs is targeted to mitochondria, β1γ2 follows, consistent with an important role for α in promoting subcellular localization of βγ. Furthermore, we directly demonstrate the requirement for α by showing that disruption of heterotrimer formation by the introduction of α binding mutations into β1 impedes PM targeting of β1γ2. The results indicate that two membrane targeting signals, lipid modification and α binding, make concerted contributions to PM localization of βγ.

Heterotrimeric G proteins1 are composed of α and βγ subunits. The βγ complex only dissociates when denatured and hence is a functional monomer under physiological conditions. Upon receptor activation the βγ dimer is freed from GTP-bound α and relays signals to downstream molecules until it reassociates with GDP-bound α, re-forming the heterotrimer. To perpetuate this G protein cycle, the trimer must be tethered to the plasma membrane (PM) in order to transmit signals. We show that isoprenylation of the α subunit is necessary but not sufficient for PM localization of β1γ2. Isoprenylation of γ2 and localization of β1γ2 to the ER occurs independently of α expression. Efficient PM localization of β1γ2 in the absence of co-expressed α is observed when a site for palmitoylation, a putative second membrane targeting signal, is introduced into γ2. When a mutant of αs is targeted to mitochondria, β1γ2 follows, consistent with an important role for α in promoting subcellular localization of βγ. Furthermore, we directly demonstrate the requirement for α by showing that disruption of heterotrimer formation by the introduction of α binding mutations into β1 impedes PM targeting of β1γ2. The results indicate that two membrane targeting signals, lipid modification and α binding, make concerted contributions to PM localization of βγ.

This work was supported by National Institutes of Health Grant GM56444 (to P. B. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: G protein, guanine nucleotide-binding protein; PM, plasma membrane; ER, endoplasmic reticulum; HEK293 cells, human embryonic kidney cells; COS7, African green monkey kidney cells; HA, hemagglutinin; Ni-NTA, nickel-nitrilotriacetic acid; PBS, phosphate-buffered saline.

This paper is available online at http://www.jbc.org
Plasma Membrane Targeting of Gβγ

EXPERIMENTAL PROCEDURES

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.

Constructs—Wild-type αs (HA-tagged) and β-binding-deficient mutant αs(EK)24C (HA-tagged) were described previously (10). The expression vectors for β1 (Myc- and HA-tagged) was provided by David P. Siderovski (University of North Carolina) (9). α-Binding-defective β1 mutants, β1S8A/K98A, β1L117A, β1D228R, β1D246S, β1N88A/K98A/D246S (hereafter named β1NKD), β1N88A/K98A/D228R (named β1NKKD), and β1N88A/K98A/D228R/D246S (named β1NKDD), were created using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and Myc- and HA-tagged β1 in pcDNA3.1 as the template. Non-tagged γ1 was described previously (9). γ1(Myc-tagged) and γ1MITO in pcDNA3 (11) were provided by Henry R. Bourne (University of California, San Francisco). γ2F66C, γ2F67C, and γ2C68S were made from γ1C (no tag) using the QuickChange kit and so was Myc-tagged γ2C68S. A mitochondrial targeting sequence was excised from γ1MITO and inserted into wild type αs (HA-tagged) to create αs fused with mitochondrial targeting sequence (mito-αs). pcDNA3 containing His-tagged β1 was provided by Tohru Kozasa (University of Illinois, Chicago).

Transfection—Unless otherwise noted, cells were seeded 1 day before transfection, and 1 μg of total plasmid DNA at a 6:3:1 ratio of αs:β1:γ1 was transfected into the cells using FuGENE 6 (Roche Applied Science). Cells were incubated overnight, transfected to new plates, and grown for 24 h prior to subsequent manipulation.

Immunofluorescence Microscopy—Cells were fixed with 3% 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 Tris-buffered saline) for 20 min. Cells were then incubated with the indicated primary antibodies 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 Antifade reagent (Molecular Probes, Eugene, OR). Microscopy was performed with an Olympus BX60 microscope. Images were recorded with a Sony DRC-5000 digital camera and transferred to Adobe Photoshop for digital processing.

Confocal Microscopy—Coverslips were prepared for confocal microscopy as described above under “Immunofluorescence Microscopy.” Representative images were recorded by confocal microscopy at the Kimmel Cancer Center Bioimaging Facility using a Bio-Rad MRC-600 laser scanning confocal microscope running CoMOS 7.0a software and interfaced to a Zeiss Axiosvert 100 microscope with Zeiss Plan-Apo 63×/1.4 oil immersion objective. Dual-labeled samples were analyzed using simultaneous excitation at 488 and 568 nm. Images of “x-y” sections through the middle of a cell were recorded.

Ni-NTA Pull Down of β1—Transfected cells were washed once with ice-cold PBS and lysed in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 2.5 mM MgCl₂ supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin). After 1 h of incubation, the nuclei and insoluble material were removed by centrifugation. Ni-NTA magnetic agarose beads (Qiagen, Valencia, CA) were added to the clarified lysate, and the samples were tumbled for 2 h at 4 °C. The samples were washed three times and eluted with elution buffer containing 250 mM imidazole. Eluates were separated by SDS-PAGE followed by immunoblotting. Bands were visualized by chemiluminescence. These experiments utilized the hexahistidine tag of N-terminal Myc- or His-tagged β1.

Prenylation Assay—COS7 cells were seeded in 60-mm culture plates. 24 h later the cells were transfected with the indicated plasmids (Myc-His tagged β1 and non-tagged γ1 without or with HA-tagged αs). Cells were incubated overnight and labeled with 50 μCi/ml [3H]mevalonolactone (American Radiolabeled Chemical, St. Louis, MO) for another 18 h in the presence of 10 μM mevastatin (Biomol, Plymouth Meeting, PA). Cells were washed with ice-cold PBS and lysed. The βγ complex was pulled down and purified using Ni-NTA agrose beads as described above. Eluates were separated by SDS-PAGE and transferred onto polycrylidiene difluoride membrane. The membrane was sprayed with EnHance (PerkinElmer Life Sciences) and then exposed to Hyperfilm MP (Amersham Biosciences) at ~80 °C for 8–15 days. After fluorography, the γ1 subunit was detected by immunoblotting. Note that because an anti-γ2 monoclonal antibody did not recognize γ2C68S, His-tagged β1 (12) and Myc-tagged γ2C68S were used for the αsβ1γ1/γ2C68S control sample, and γ2C68S was detected on an immunoblot with an anti-Myc monoclonal antibody.

Cell Fractionation Assay—Soluble and particulate fractions were isolated as described previously (10). Briefly, 48 h after transfection HEK293 cells were washed in ice-cold PBS and lysed in hypotonic lysis buffer (50 mM Tris-HCl, pH 8, 2.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol) with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin). Cells were passed through a 27-gauge needle 10 times. Lysed cells were centrifuged at 400 × g for 5 min to remove nuclei and debris. The supernatant was centrifuged at 150,000 × g for 20 min at 4 °C. Fractions were analyzed by SDS-PAGE and immunoblotting using the indicated antibody.

Materials—pEYFP Mito vector (Clontech, Palo Alto, CA) was provided by Emad Alnemri (Thomas Jefferson University). pEYFP-IBV-M1 encoding an ER marker protein was a generous gift from Mark R. Phillips (New York University). 9E10 monoclonal antibody was from Covance (Berkeley, CA). 12CA5 monoclonal antibody was from Roche Applied Science. Anti-HA and anti-γ2C68S rabbit polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

In the present study, we focused on the αsβ1γ2 G protein heterotrimer. Immunofluorescence microscopy was utilized to examine the subcellular localization of the α and βγ subunits. It has been shown that αs is predominantly localized to the PM when expressed alone (9, 10). As described by us previously (10), when β1 and γ1 were expressed together in HEK293 cells very little PM localization was observed (Fig. 1, a and b). However, when αs was co-expressed with β1 and γ1, αs, γ1, and β1 strongly co-localized at the PM (Fig. 1, c and d). Expression of αs also strongly promoted PM localization of β1γ2 (not shown). γ2 displayed PM localization when αs, β1, and γ2 were all expressed together, but γ2 was also found intracellularly and not co-localizing with αs or β1 (Fig. 1, e–h). Apparently some of the γ2 did not form a dimer with β1. Because localization of β1 rather than γ2 appeared to be a better representative of the β1γ2 complex, most of the experiments described herein followed the localization of β1.

Replacement of Cysteine with Serine in the CAAAX Motif of the γ2 Subunit Resulted in Loss of PM Targeting but Not a Binding—the C terminus of the γ2 subunit contains the CAAAX motif, specifically the sequence of cysteine, alanine, isoleucine, and leucine. The cysteine is modified with a 20-carbon gerylnerylarni isoprenoid. We substituted a serine for the cysteine and transiently expressed γ2C68S in HEK293 cells in conjunction with wild type β1 in the presence and absence of wild type αs. In immunofluorescent staining, little β1γ2C68S was found at the PM regardless of αs expression (Fig. 2A). To test whether poor PM localization of β1γ2C68S, when expressed with αs, resulted from an inability to form a heterotrimer, the β1γ2 dimer was pulled down with Ni-NTA beads, taking advantage of an N-terminal hexahistidine tag on β1, and immunoblotted for the αs subunit. β1γ2 was able to efficiently pull down αs (Fig. 2B, lane 3). Similarly, the β1γ2C68S dimer pulled down the αs subunit (Fig. 2B, lane 6), implying that αs and β1γ2C68S are capable of assembling a heterotrimer. In this assay, efficient heterotrimer formation required co-expression of all three components. When lysates from cells expressing β1γ2 or β1γ2C68S were mixed with a lysate from cells expressing only αs heterotrimer formation was not detected (Fig. 2B, lanes 4 and 7). The results with γ2C68S indicate that non-prenylated γ2 can form a complex with wild type αs and β1, but the β1γ2C68S dimer was not localized at the PM.
The $\beta_1\gamma_2$ Complex Was Prenylated in the Absence of the $\alpha_s$ Subunit—To determine whether $\beta_1\gamma_2$ displayed very poor PM targeting without the co-expressed $\alpha_s$ because of inefficient lipid modification of the $\gamma_2$ subunit, a prenylation assay was carried out. Because HEK293 cells, in our hands, detached from the bottom of culture plates upon treatment with mevastatin, a hydroxymethylglutaryl-CoA reductase inhibitor, the experiments were carried out using COS7 cells. Similar to its subcellular localization in HEK293 cells, $\beta_1\gamma_2$ is poorly targeted to the PM in COS7 cells unless an $\alpha_s$ subunit is also co-expressed (13). The $\beta_1$ and $\gamma_2$ subunits were transiently transfected into COS7 cells in the absence or presence of the $\alpha_s$ subunit. The transfected cells were labeled with $[^3H]$mevalonolactone in the presence of mevastatin for 18 h. The $\beta_1\gamma_2$ complexes were isolated using Ni-NTA beads. Because the $\beta_1$ subunit contains the N-terminal hexahistidine tag, only $\gamma_2$ subunits that are bound to $\beta_1$ were pulled down in these experiments. The level of isoprenoid incorporated into $\gamma_2$ was visualized by fluorography and that of expression of $\gamma_2$ was assessed by Western blotting. Mock/pCDNA3 transfection showed no nonspecific uptake of the radioactivity (Fig. 2C, lane 1), and as expected, $\gamma_C68S$ failed to incorporate radioactivity (Fig. 2C, lane 4). The $\beta_1\gamma_2$ efficiently incorporated radioactive isoprenoid in the presence of the $\alpha_s$ subunit, and virtually no difference was seen in incorporation of radioactivity into the $\beta_1\gamma_2$ without the $\alpha_s$ subunit (Fig. 2C, lanes 2 and 3). Therefore, the defect in the PM localization of $\beta_1\gamma_2$ when expressed in the absence of $\alpha_s$ was not due to failure of the $\gamma_2$ subunit to be prenylated. In other words, the $\beta_1\gamma_2$ dimer is capable of being modified with the isoprenoid group in the absence of the $\alpha_s$ subunit. It is therefore conceivable that lipid modification takes place prior to trimer formation.

Prenylated $\beta_1\gamma_2$ Dimer Was Membrane-bound—Without co-expressed $\alpha_s$, the $\beta_1\gamma_2$ complex is prenylated but localized to the PM very poorly. Next we examined the subcellular localization of the prenylated $\beta_1\gamma_2$ by a cell fractionation assay. After transient transfection, cells were lysed in hypotonic buffer, and the soluble and particulate fractions, representing cytoplasmic and membrane fractions, were separated by ultracentrifugation. Proteins in each fraction were analyzed by Western blotting using an anti-HA monoclonal antibody and an Alexa 594 anti-mouse antibodies (c) or anti-HA monoclonal and Alexa 488 anti-rabbit antibodies (d). Cells were fixed, and immunofluorescent staining for $\beta_1$ was carried out using an anti-Myc monoclonal antibody and an Alexa 594 anti-mouse antibody. B, HEK293 cells transiently expressing $\alpha_s$ (lane 1), $\beta_1$, and $\gamma_2$ (lane 2), $\alpha_s$, $\beta_1$, and $\gamma_2$ (lane 3), $\beta_1$, and $\gamma_C68S$ (lane 5), or $\alpha_s$, $\beta_1$, and $\gamma_C68S$ (lane 6) were lysed and subjected to a Ni-NTA pull-down assay, utilizing an N-terminal hexahistidine tag on $\beta_1$, as described under “Experimental Procedures.” In some cases, lysates were combined as indicated before the Ni-NTA pull-down assay (lane 4 and 7). Eluates were analyzed for the presence of $\alpha_s$ by SDS-PAGE and Western blotting using an anti-HA monoclonal antibody. C. COS7 cells were transiently transfected with pCDNA3 alone (lane 1) or with expression vectors encoding $\beta_1$ and $\gamma_2$ (lane 2), $\alpha_s$, $\beta_1$, and $\gamma_2$ (lane 3), or $\alpha_s$, $\beta_1$, and $\gamma_C68S$ (lane 4). After labeling for 18 h with $[^3H]$mevalonolactone, cells were lysed, and $\beta_1\gamma_2$ was pulled down with Ni-NTA beads. Proteins were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was exposed to a film at $-80^\circ$C (upper panel). Subsequently, the membrane was subjected to Western blotting for the $\gamma_2$ subunit (lower panel).

TABLE 1

| Expression Vectors | Lane 1 | Lane 2 | Lane 3 | Lane 4 |
|--------------------|--------|--------|--------|--------|
| pcDNA3             | 0.1    | 0.1    | 0.1    | 0.1    |
| $\alpha_s$         | 0.1    | 0.1    | 0.1    | 0.1    |
| $\beta_1$          | 0.1    | 0.1    | 0.1    | 0.1    |
| $\gamma_2$         | 0.1    | 0.1    | 0.1    | 0.1    |
| $\alpha_s$, $\beta_1$, and $\gamma_2$ | 0.1 | 0.1 | 0.1 | 0.1 |
| $\alpha_s$, $\beta_1$, and $\gamma_C68S$ | 0.1 | 0.1 | 0.1 | 0.1 |
| $\alpha_s$, $\beta_1$, and $\gamma_C68S$ | 0.1 | 0.1 | 0.1 | 0.1 |

Fig. 2. Prenylation of $\gamma_2$ is required for $\alpha_s$-dependent PM targeting of $\beta_1\gamma_2$, but $\alpha_s$ does not affect isoprenylation of $\gamma_2$. A, HEK293 cells were transfected with a plasmid encoding $\gamma_C68S$ in conjunction with expression vectors for $\alpha_s$ and $\beta_1$, 48 h after transfection, cells were fixed, and immunofluorescent staining for $\beta_1$ was carried out using an anti-HA monoclonal antibody and an Alexa 594 anti-mouse antibody. B, HEK293 cells transiently expressing $\alpha_s$ (lane 1), $\beta_1$, and $\gamma_2$ (lane 2), $\alpha_s$, $\beta_1$, and $\gamma_2$ (lane 3), $\beta_1$, and $\gamma_C68S$ (lane 5), or $\alpha_s$, $\beta_1$, and $\gamma_C68S$ (lane 6) were lysed and subjected to a Ni-NTA pull-down assay, utilizing an N-terminal hexahistidine tag on $\beta_1$, as described under “Experimental Procedures.” In some cases, lysates were combined as indicated before the Ni-NTA pull-down assay (lane 4 and 7). Eluates were analyzed for the presence of $\alpha_s$ by SDS-PAGE and Western blotting using an anti-HA monoclonal antibody. C. COS7 cells were transiently transfected with pCDNA3 alone (lane 1) or with expression vectors encoding $\beta_1$ and $\gamma_2$ (lane 2), $\alpha_s$, $\beta_1$, and $\gamma_2$ (lane 3), or $\alpha_s$, $\beta_1$, and $\gamma_C68S$ (lane 4). After labeling for 18 h with $[^3H]$mevalonolactone, cells were lysed, and $\beta_1\gamma_2$ was pulled down with Ni-NTA beads. Proteins were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was exposed to a film at $-80^\circ$C (upper panel). Subsequently, the membrane was subjected to Western blotting for the $\gamma_2$ subunit (lower panel).
When expressed alone, the \( \beta_1\gamma_2 \) dimers, which localized very poorly at the PM (Fig. 1, a and b) were also found mostly in the particulate fraction (Fig. 3A, lane 4). This suggests that the prenylated \( \beta_1\gamma_2 \) was targeted to membranes other than the PM. To examine the intracellular localization of \( \beta_1\gamma_2 \) more closely, we compared the subcellular localization of \( \beta_1\gamma_2 \) with an ER marker protein using confocal microscopy. \( \beta_1\gamma_2 \) when expressed alone, exhibited a subcellular distribution virtually identical to the ER marker (Fig. 3B, a and b), whereas \( \beta_1\gamma_2 \) when expressed with \( \alpha_c \), displayed PM localization that was clearly distinct from the ER (Fig. 3B, c and d). These results are thus consistent with a model in which the \( \beta_1\gamma_2 \) dimer is geranylgeranylated in the cytosol by a cytoplasmic geranylgeranyltransferase (14) and then targeted to ER, prior to \( \alpha_c \)-dependent transit to the PM.

Introduction of a Palmitoylation Site into \( \gamma_2 \) Allows \( \alpha_c \)-independent PM Targeting of \( \beta_1\gamma_2 \)—It has been shown that isoprenylation is necessary but not sufficient for PM targeting of the Ras family of small GTPase, and a so-called second membrane targeting signal is required for PM targeting (6, 7). H-Ras and N-Ras have been shown to be palmitoylated at cysteines upstream of their CAAX boxes. K-Ras possesses polybasic lysines flanking the prenylcysteine. Of interest, Ste18p, the yeast \( \gamma \) subunit, also is palmitoylated at a cysteine next to the prenylcysteine, and palmitoylation is necessary for avid PM membrane binding (8, 15). We tested the possibility that the \( \beta_1\gamma_2 \) dimer becomes able to localize to the PM if \( \gamma_2 \) is bestowed with a “second” membrane targeting signal by constructing \( \gamma \) mutants with a potential palmitoylation site. A phenylalanine residue at the 66- or 67-position of \( \gamma_2 \) is shown in boldface. B, \( \gamma_2 \) (a), \( \gamma_2\)F66C (b), or \( \gamma_2\)F67C (c) were transiently expressed in HEK293 cells in conjunction with \( \beta_1 \). Immunofluorescence staining for \( \beta_1 \) was carried out using anti-Myc monoclonal and Alexa 594 anti-mouse antibodies.
mitochondria, as demonstrated by the yellow color in Fig. 5f. When β1 and γ2 were expressed with mito-αN, the β1·γ2 dimer accompanied mito-αN to mitochondria (Fig. 5, j and k), as a superimposed image clearly demonstrates co-localization (Fig. 5, l). In contrast, the β1·γ2 complex was found at the PM when expressed with wild type αN (Fig. 5, g–i).

**Impaired Interaction of βγ with a Prevented PM Localization of βγ**—To address more directly the requirement for assembly of the αβγ heterotrimer in βγ localization, we tested the subcellular localization of α-binding-deficient β mutants. Two surfaces of β, the α switch interface and the α N-terminal interface, contain important residues for interaction with the α subunit (17, 18). Mutation of the putative α-contacting residues in the β subunit resulted in a decreased affinity for the α subunit (12, 19). Based on previous findings, we introduced the mutations, I80A, N88A/K89A, L117A, D228R, or D246S (note that Ile-80, Asn-88, and Lys-89 are in the α N-terminal interface, and Leu-117, Asp-228, and Asp-246 are in the α switch interface), into Myc-His-tagged β1, and transiently expressed them in conjunction with wild type γ2 in HEK293 cells. All mutant β1·γ2 showed measurer PM localization, similar to wild type β1·γ2 (Fig. 6A, a). Just as wild type β1·γ2 displayed much greater PM localization when co-expressed with wild type αN, the mutant β1·γ2 complex exhibited stronger PM membrane targeting when expressed with αN (Fig. 6A, b). The ability of αN to promote PM localization of the α-binding-deficient mutants of β1 suggests that the β1 mutants are not completely unable to interact with α. Consistent with this interpretation, others (12, 19) using these β1 mutants observed varying degrees of loss of α binding, depending upon the assay used.

Next, we expressed the β1 mutants and wild type γ2 in conjunction with an αN mutant, αN IEK+, that contains mutations to five N-terminal amino acids at the βγ binding interface. Our previous work (10) demonstrated that this mutant lost its ability to localize to the PM when expressed alone, but co-expression of wild type βγ restored the PM localization of the αN IEK+ mutant. It was expected that a combination of the βγ-binding-deficient mutant of αN and an α-binding-deficient mutant of β1 would result in more impaired heterotrimer formation. Consistent with this prediction, a β1·γ2 complex containing β1 D246S (Fig. 6A, c) and, to lesser extent, ones with β1 D228R and β1 N88A/K89A (not shown) were poorly localized at the PM when co-expressed with αN IEK+.

With these results, we sought to construct β mutants with more severe mutations, to generate ones that are less capable of binding to wild type α. Three mutants were created by combining mutations in both interfaces. β1 NKD contains the
mutations N88A, K89A, and D246S; β1NKDD is like β1NKD with an additional D228R mutation, and β1IN KD is further mutated at 180A. To allay concerns that multiple mutations impede proper folding of the β1 protein, we checked their ability to bind the γ2 subunit. The β1NKD and β1NKDD mutants showed γ2 binding similar to wild type β1 as assessed by Ni-NTA pull-down assay (Fig. 6B, lanes 1–3). Interestingly, β1IN KD, containing one additional mutation, failed to associate with the γ2 subunit (Fig. 6B, lane 4), and thus β1IN KD was not analyzed further. The ablated capability of the mutants to interact with α was also confirmed by a Ni-NTA pull-down assay (Fig. 6B, lanes 6 and 7). Collectively, the β1N KD and β1NKDD mutants are correctly folded yet substantially defective in association with α. When these two mutants were expressed with wild type γ2, the β1γ2 complex was localized to the PM poorly, similar to wild type β1 (Fig. 6C, a and c). Importantly, co-expression of wild type αd did not promote PM localization of the dimer containing either mutant (Fig. 6C, b and d). Expression of the αd subunit was confirmed by double staining of the subunit in the same cells. Collectively, impaired interaction of the βγ dimer with the α subunit resulted in poor PM targeting of the dimer. These results underscore the significance of proper heterotrimer formation and indicate that the α subunit plays an important role in PM targeting of the βγ dimer.

Discussion

Data presented here refine the requirements for PM targeting of the G protein βγ complex. In addition to demonstrating that isoprenylation of γ is required but not sufficient, our results reveal a crucial role for the G protein α subunit. Thus, both heterotrimer assembly and lipid modifications function together to promote proper PM localization of βγ.

Substitution of cysteine 68 with serine in the C terminus of γ2 prevented attachment of isoprenoid to it, and β1γ2C68S exhibited virtually no PM localization, consistent with earlier immunofluorescence observations in COS cells (20). The additional co-expression of αd failed to promote PM localization of β1γ2C68S (Fig. 2A), although αd strongly promoted PM localization of β1γ2 (Fig. 1d) (9). In addition, we demonstrated that the γ2C68S mutant can form a heterotrimer with co-expressed αs and β1 subunits as assessed by Ni-NTA pull-down assay (Fig. 2B). Although prenylation of the βγ complex has been reported to increase its affinity for the α subunit (14, 21), prenylation is not a strict requirement for heterotrimer formation. Consistent with this, none of the subunits in a crystalized aβγ complex contained lipid modifications; the C68S γ mutant produced in SF9 cells was able to assemble with α and β subunits (17). The inability of the β1γ2C68S complex to localize to the PM when co-expressed with αs, even though it is capable of binding αs, implies that heterotrimer formation alone is not sufficient for PM targeting of the βγ dimer.

Heterotrimer formation, however, appears to be necessary for localization of βγ, and several lines of evidence are consistent with a role for α in PM targeting of βγ. First, we demonstrated previously that transiently expressed βγ was poorly targeted to the PM (9) and was found predominantly at intracellular membranes (Fig. 1, a and b, Fig. 3B, b, and Fig. 4B, a). However, co-expression of an α subunit promoted strong PM localization of the βγ dimer (9) (Fig. 1, d and g, and Fig. 3B, d). A recent report (13) confirmed these results using a green fluorescent protein-tagged γ in COS cells. Second, when αd was targeted to mitochondria the β1γ2 subunits followed (Fig. 5). The ability of αd in this case misdirected to mitochondria, to mistarget βγ is consistent with a prominent role for α subunits in guiding βγ to its appropriate cellular destination.

Third, we directly tested the effects of impaired aβγ assembly in the subcellular localization of the βγ dimer by mutating putative a-binding residues in the β subunit. Currently available crystal structure models of the aβγ dimer indicate that the β subunit contacts the α subunit at two surfaces, termed the α N-terminal interface and the α switch interface (17, 18). Others reported (12, 19) that introduction of a mutation into the β subunit in either interface resulted in reduced ability to form a heterotrimer properly. However, when we examined subcellular localization of mutant β1γ2 complexes in which the β1 contained single mutations, or the double N88A/K89A mutation, αd was able to promote efficient PM localization of the mutant βγ. This is consistent with demonstrations that such β1 mutants still retain some ability to interact with α (12, 19).

Nonetheless, a defect in PM localization of mutant β1γ2 was revealed when an α-binding defective β1 mutant was expressed with γ2 and a previously described βγ-binding defective αd mutant αdIEK + (9) (Fig. 6A, c). Combination of the βγ-binding defective α and the α-binding defective β impeded proper heterotrimer assembly, resulting in poor PM targeting. We further constructed the mutants β1N KD and β1NKDD with combined mutations in both α-binding interfaces to achieve more impaired interaction with wild type α subunit. Importantly, co-expression of αd failed to promote PM localization of β1N KD or β1NKDD (Fig. 6C, b and d). The β1 mutants were capable of binding γ2 (Fig. 6B), and thus the failure of PM targeting was not due to misfolding of the mutant β1 subunit. Collectively, the results with α-binding defective β1 mutants clearly demonstrate that interaction of the βγ subunit with the α subunit is critical in PM targeting of the βγ dimer. To our knowledge, these results are the first to show explicit evidence of the significance of heterotrimer assembly by βγ localization at the PM.

Fourth, studies in model organisms indicate a role for α in targeting βγ. In the yeast S. cerevisiae, βγ is defective in localizing at the PM in an α subunit (Gpa1) null mutant (22). Moreover, expression of the yeast α Gpa1p rescues PM localization of a cytoplasmic βγ mutant in which the palmitoylation site in γ is mutated (15). In addition, a recent study of G protein localization in Caenorhabditis elegans showed that deletion of an α subunit resulted in failure of the βγ subunits to localize properly (23). In C. elegans embryos, GBP-1, the β subunit, and GOA-1, the α subunit, were found at the cell PM and on microtubule asters. When expression of GOA-1 and GPA-16, the widely expressed α subunit with redundant functions to GOA-1 in C. elegans, was abrogated by RNA interference, GBP-1 lost its ast and PM localization (23). The orientation role of the α subunit in βγ localization may be widespread.

Recent findings (24, 25) revealed that Ras undergoes prenylation and then transits via intracellular membranes to the PM rather than moving directly from the cytosol to the PM as was once thought. G protein βγ subunits may take a similar pathway to the PM. The enzymes that catalyze proteolytic cleavage of the last three amino acids and methylation of the carboxyl group of the prenylcysteine have been cloned recently and identified as membrane-bound proteins at the ER (26, 27). Thus, Ras is prenylated in the cytosol and then targeted to the cytoplasmic face of the ER where additional C-terminal processing takes place. The βγ dimer is similarly prenylated in the cytoplasm and then presumably targeted to the ER to undergo subsequent CAAX processing. Interaction with an α subunit does not appear to be required for the initial step in βγ trafficking. βγ is localized to intracellular membranes in the absence of α co-expression (Fig. 3), and βγ undergoes prenylation equally well in the absence or presence of α expression (Fig. 2C). It has been known that, in addition to the CAAX processing, a second signal in the hypervariable region is required for
PM targeting of Ras (6, 7); H-Ras and N-Ras become palmitoylated at cysteines upstream of the prenylcysteine, whereas K-Ras contains a polylysine sequence adjacent to the prenylcysteine. Unlike Ras, none of the human γ subunits contain potential palmitoylation residues or a stretch of basic residues adjacent to the prenylcysteine. However, we demonstrate that introduction of a potential palmitoylation site into γ2 resulted in PM localization of β1γ2 and obviated the requirement for co-expression of αs (Fig. 4B, b and c). This indicates that if the γ subunit is conferred a second signal, the dimer becomes able to transit to the PM alone. Furthermore, it is conceivable that the α subunit may function as a “provider” of the second signal, rendering its palmitate to the βγ complex. Consistent with this proposal, β1γ2 fails to localize efficiently at the PM when co-expressed with palmitoylation-deficient mutants of α (Fig. 3E, f) (13).

Not only does βγ require α, as described here, but several reports (28, 29) have demonstrated that α depends on binding to βγ for its PM localization. Expression of βγ recovers palmitoylation and PM localization of non-myo-rastisoylated G2A mutants of αs and αt. Furthermore, inhibiting α and βγ interaction by expression of a βγ-sequestering protein (30) or by mutating βγ contact sites in α subunits decreases palmitoylation and PM localization of αt (11) or αt (10). Targeting of β1γ2 to mitochondria via a mito-γ2 mutant also directs co-expressed αt to mitochondria (11). This last result, taken together with our reciprocal results showing that mito-αt can direct β1γ2 to mitochondria, is consistent with the idea that both α and βγ rely on heterotrimer formation to reach their destination.

Demonstrations of a role for βγ in the PM targeting of α are not incompatible with the results presented in this report. The reciprocal requirement for heterotrimer assembly is most consistent with a model in which heterotrimer formation occurs intracellularly prior to transit of αβγ to the PM. Thus, if heterotrimer formation must occur before α or βγ can proceed to the PM, both α and βγ would exhibit a requirement for binding to their partner in order to achieve PM localization. On the other hand, the reciprocal requirement for α and βγ interaction is more difficult to understand in the context of a model in which α and βγ traffic separately to the PM and is stably anchored there independent of binding to its partner. Although our results are more consistent with the proposal that heterotrimer formation occurs prior to PM localization, we cannot rule out that α and βγ traffic separately to the PM but are each rapidly recycled to intracellular membranes unless heterotrimer formation occurs at the PM.

Where do α and βγ initially interact? Recently, heterotrimer assembly at Golgi was suggested based on co-localization at the Golgi of βγ and a palmitoylation-defective αtγ2 (13). However, in the rat exocrine pancreas the β subunit was not found on Golgi membranes, whereas various α subunits were detected there (31). Meanwhile, a palmitoyltransferase for Ras was found in ER membranes (32, 33). The involvement of the Golgi in G protein trafficking and the location of a relevant palmitoyltransferase remain to be further investigated.

An emerging model of heterotrimer trafficking to the PM proposes the following. Newly synthesized β and γ rapidly form a dimer, and the C terminus of the γ subunit is modified with an isoprenyl group in the cytosol. Subsequently, the dimer transits to the ER where its prenylated CAAX sequence is further processed. Finally, at intracellular membranes, the βγ and α subunits form a heterotrimeric complex, and then αβγ traffic to the PM together, utilizing at least palmitate and isoprenoid as two-plateau membrane targeting signals. Forming the heterotrimer prior to reaching the PM may confer “heterotrimer-specific” localization and may be crucial to maintain the proper stoichiometry of α to βγ.

Acknowledgments—We thank Janice Buss and John Stickney for valuable advice on the prenylation assay, and Maurine Linder, Daniel Evanko, and Manimekalai Thiagajaran for critical reading of the manuscript and helpful comments.

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