Localization of a peripheral membrane protein: G$\beta\gamma$ targets Gα_Z

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To explore the relative roles of protein-binding partners vs. lipid modifications in controlling membrane targeting of a typical peripheral membrane protein, Gα_z, we directed its binding partner, $\beta\gamma$, to mislocalize on mitochondria. Mislocalized $\beta\gamma$ directed wild-type Gα_z and a palmitate-lacking Gα_z mutant to mitochondria but did not alter localization of a Gα_z mutant lacking both myristate and palmitate. Thus, in this paradigm, a protein–protein interaction controls targeting of a peripheral membrane protein to the proper compartment, whereas lipid modifications stabilize interactions of proteins with membranes and with other proteins.

Targeting a peripheral membrane protein to the right subcellular compartment is thought to depend on a combination of signals, of which the best studied are posttranslational lipid modifications, polybasic domains, and protein-binding partners. Previous work from our (1, 2) and other (3–5) laboratories suggested that palmitate acts as a membrane targeting signal by trapping proteins at organelles containing a palmitoyl transferase. Specific membrane localization requires targeting signals in addition to palmitate, however, because mutants lacking palmitoylation sites undergo only partial or no mislocalization (2, 6–8) and some proteins are palmitoylated en route rather than at their final subcellular destination (9, 10). For this study, we used subunits of a heterotrimeric G protein, G_z, to test the hypothesis that protein partners direct the targeting of peripheral membrane proteins. We find that specific membrane localization of one subunit of this protein is determined by its interaction with the other, whereas posttranslational lipid modifications seem to stabilize the interaction of the subunits with each other and with membranes.

Heterotrimeric G proteins, which are signal transducers located on the cytoplasmic leaflet of the plasma membrane (PM), are composed of two functional subunits, a guanine nucleotide-binding α-subunit and a tightly bound $\beta\gamma$-heterodimer. The membrane attachment of $\beta\gamma$ depends on the prenyl group attached to the C terminus of the γ-polypeptide (11). Although fatty acids attached at or near the N termini of Gα-subunits clearly tether them to membranes (12–15), other evidence suggests that $\beta\gamma$ can play a controlling role in directing them specifically to the PM. For example, in cultured cells, overexpressed $\beta\gamma$ can recruit to the PM an α_z mutant lacking any lipid attachment (1), and pure $\beta\gamma$ can recruit α_o to phospholipid vesicles *in vitro* (16). Moreover, $\beta\gamma$ cooperates with palmitate to bind α-subunits at the PM (1), and sequestration of $\beta\gamma$ by the β-adrenergic receptor kinase impairs association of α_z with the PM (2).

Materials and Methods

Expression Constructs. cDNA constructs expressing α_zEE, α_z-C3A-EE and α_z-G2AC3A-EE in pcDNA3 were generated as described (12). α_zMUT (α_zEE containing the mutations I19A, D20A, and E26A) was generated by using the Quickchange site-directed mutagenesis kit (Stratagene). γ_2MITO was generated by three successive PCRs on myc-tagged γ_2 (1) by using the primers shown below (5′ primers 1–3 and 3′ primers 4 or 5) in standard PCR procedures. The product of the reaction with primers 1 and 4 served as the template for the reaction with primers 2 and 4. Likewise, the product of this reaction served as the template for the reaction with primers 3 and 4. The final product was subcloned into the *Eco*RI and *Xba*I sites of pcDNA3.1 (Invitrogen). γ_2C68S-MITO was produced the same way, with myc-tagged γ_2C68S as the template, by using primer 5 instead of primer 4. (Primer 1, 5′-GGAATTC-GCTATCGGAGCCTACTATTACTACGGAGCCGAACAAA-AACTCATCTCAGAAGAGG-3′; primer 2, 5′-GGAATTCTA-TCCTCGCTACCGTGGCTGCAACAGGAACAGCTATCGG-AGCCTACTATTACTACGG-3′; primer 3, 5′-GAATTCATG-A AGTCCTTCATCACCAGAAACAAGACCGCTATCCTCG-CTACCGTGGCTGCAACAGG-3′; primer 4, 5′-CCTCTAGAT-TACCAGGATAGCACAGAAAAAAC-3′; primer 5, 5′-GGTC-TAGATTAAAGGATAGCACTGAAAAAC-3′.)

Immunofluorescence and Microscopy. Cells were transfected by the adenovirus-DEAE dextran method (17), plated onto glass coverslips after 24 h, and fixed in 3.7% (vol/vol) formaldehyde/PBS 48 h after transfection. Immunofluorescence was performed as described (1). The primary antibodies used were as follows: anti-EE mouse monoclonal antibody (Onyx Pharmaceuticals, Richmond, CA; 20 μg/ml), mouse monoclonal anti-Hsp60 antibody (StressGen Biotechnologies, Victoria, Canada; 1:200), and rabbit polyclonal anti-γ_2 (Santa Cruz Biotechnology; 1:100). Primary antibodies were followed by secondary antibodies: FITC-conjugated goat anti-mouse (1:100 dilution) and Texas Red-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch; 1:100 dilution). Cells were examined with a DeltaVision Nikon TE200 microscope equipped with a cooled charge-coupled device CH350L camera. Images were taken at nine z levels, deconvolved, and analyzed with DELTA VISION software. The figures show the deconvolved pattern corresponding to z level five.

Quantitation of Immunofluorescence. Locations of mitochondria were determined from deconvolved images of the mitochondrial stain (hsp60; see Fig. 2) or γ_2MITO (see Figs. 3–5). For α_z tagged with the EE epitope and expressed in the absence of γ_2MITO, cells were costained with rabbit polyclonal anti-hsp60 (StressGen Biotechnologies; 1:200) and mouse monoclonal anti-EE antibodies (image not shown). In all cases, the locations of mitochondria were defined from the image showing the relevant stain by creating polygons at each of nine z levels in regions greater than 10 pixels that contained a fluorescence intensity above a threshold value. These polygons were then copied onto the image of the same cell representing the second stain, and the fluorescence intensities contained by the polygons, through all nine z levels, were calculated. The proportion of a G protein subunit located in mitochondria was calculated by comparing fluorescence intensity from that subunit contained within the polygons to the fluorescence intensity of the same stain associated with the whole cell.

Abbreviations: PM, plasma membrane; MAPK, mitogen-activated protein kinase; CHO, Chinese hamster ovary.

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Mitogen-Activated Protein Kinase (MAPK) Assays. To assay the activity of transfected α_z without interference from endogenous α_i-subunits, we treated cells with pertussis toxin, which does not act on α_z but inactivates all other α_i family members. MAPK assays were performed on Chinese hamster ovary (CHO) cells transfected 48 h earlier with the D_2 dopamine receptor and hemagglutinin epitope-tagged MAPK (HA-MAPK) together with wild-type α_z, α_zMUT, or α_zMUT plus β_1 and wild-type γ_2 as described (1). MAPK activity was determined after a 4-h treatment with pertussis toxin (100 ng/ml) and a 7-min exposure to the D_2 agonist quinpirole (10 μM) or no agonist.

Results

Mislocalization of α_z Caused by Impaired Binding to $\beta\gamma$. To determine whether $\beta\gamma$ is required for targeting Gα specifically to the PM, we constructed α_zMUT, a recombinant α_z carrying alanine substitution mutations designed to specifically impair its ability to bind $\beta\gamma$. To impair $\beta\gamma$ binding without affecting the ability of α_z to fold properly or to bind guanine nucleotides, we substituted alanines for three residues (I19, D20, and E26) in the N terminus that are likely to interact with $\beta\gamma$, as determined by inspection of the crystal structures of $\alpha_{t/i}/\beta\gamma$ and $\alpha_i/\beta\gamma$ heterotrimers (18, 19). Throughout this study, we used epitope-tagged α_z (α_zEE), a myristoylated and palmitoylated member of the G_i family, in which the Glu–Glu epitope had been shown not to disrupt signaling or PM targeting (12).

Unlike recombinant wild-type α_z, α_zMUT mislocalizes substantially to intracellular membranes, as indicated by immunofluorescence microscopy of CHO cells (Fig. 1*A*). Immunofluorescence of wild-type α_z (Fig. 1*Aa*) fits the pattern characteristic of a protein located exclusively at the PM of CHO cells (1, 2)—that is, a uniform intensity of stain extending to the edge of the cell where it is sometimes more intense. Mislocalization of α_zMUT to intracellular membranes, however, is partial (Fig. 1*Ab*)—that is, a portion of the fluorescence shows a pattern characteristic of PM localization. We imagine that mislocalization is incomplete, because the affinity of α_zMUT for $\beta\gamma$ is reduced but not abolished. In keeping with this interpretation, α_zMUT does not mediate receptor activation of the MAPK pathway unless coexpressed with excess $\beta\gamma$ (Fig. 1*B*). Because this MAPK response requires receptor-dependent release of $\beta\gamma$ from $\alpha_z/\beta\gamma$ heterotrimers (1, 20), we infer that α_zMUT is properly folded in cells but forms functional heterotrimers only when supplied with sufficiently high levels of $\beta\gamma$ to overcome its diminished ability to bind $\beta\gamma$.

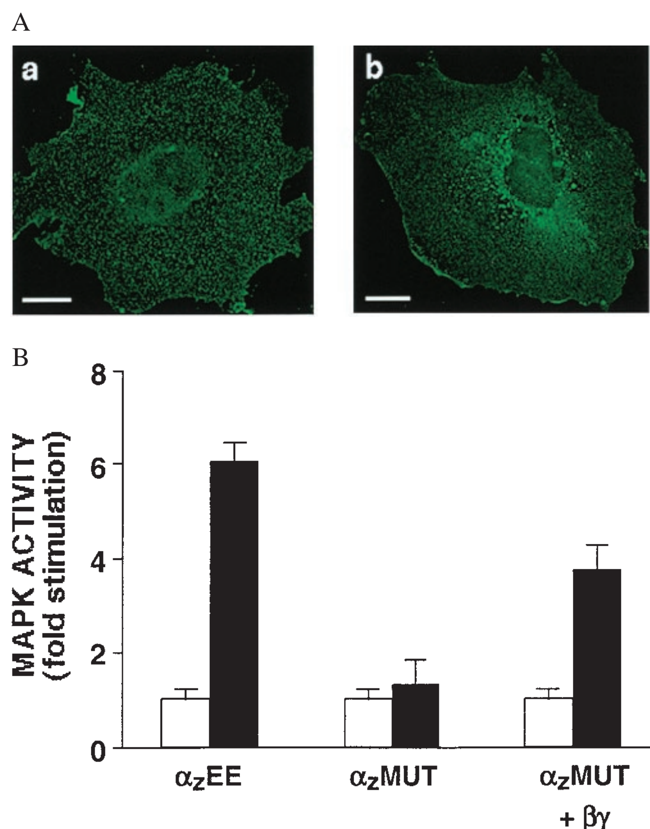

Fig. 1. Alanine mutations at a $\beta\gamma$-binding interface of α_z cause the mutant protein, α_zMUT, to mislocalize to intracellular membranes. (*A*) Immunofluorescence microscopy of CHO cells transfected with wild-type α_z (*a*) or α_zMUT (*b*). Although the fluorescent signal for wild-type α_z (*a*) is not confined to the outer perimeter of the cell, the staining pattern indicates localization at the PM. This pattern is due to the extremely flat and thin shape of CHO cells under our culture conditions; using confocal microscopy, we previously observed the same pattern of fluorescence for PM-localized proteins in CHO cells (1). (Bar = 20 μm.) (*B*) MAPK assays were performed on CHO cells transfected 48 h earlier with the D_2 dopamine receptor and HA-MAPK, together with wild-type α_z, α_zMUT, or α_zMUT plus β_1 and wild-type γ_2. MAPK activity was determined after a 4-h treatment with pertussis toxin (100 ng/ml) and a 7-min exposure to the D_2 agonist (10 μM quinpirole, black bars) or no agonist (white bars). Data shown are the means $\pm$2 SEM of four experiments.

α_z Follows Mistargeted $\beta\gamma$ to the Mitochondria. To ask whether $\beta\gamma$ is *sufficient* for targeting α_z, we applied a strategy that may be generally useful for studying targeting of peripheral membrane proteins: we assessed the ability of a misdirected protein to induce its partner to accompany it into the "wrong" cellular compartment—in this case, mitochondria. To do so, we used a well characterized mitochondrial targeting signal from the yeast protein Mas70p (21), which causes cytosolic proteins to translocate to the mitochondrial outer membrane without triggering import into the mitochondrial matrix (22) and thus anchors proteins on the cytosolic surface of the mitochondrial outer membrane. Attached to the N terminus of the γ_2 polypeptide, Mas70p caused the fusion protein γ_2MITO (Fig. 2*A*) to localize exclusively to mitochondria, as assessed by immunofluorescence (Fig. 2*B*). and quantitative analysis (Fig. 2*C*) γ_2MITO immunofluorescence overlaps almost perfectly (81%; Fig. 2*C*) with that of the mitochondrial marker Hsp60, in marked contrast to that of wild-type γ_2, which is seen at the PM and in perinuclear membranes (Fig. 2*B*). A limited overlap (24%; Fig. 2*C*) of wild-type γ-subunits with mitochondria was also detected, most probably reflecting nonspecific interaction with mitochondrial membranes mediated by the hydrophobic prenyl group. We (1) and others (11) have observed localization of wild-type γ-subunits on intracellular membranes after overexpression in transfected cells. This localization is clearly different, however, from the almost exclusive mitochondrial localization of γ_2MITO.

Coexpression of wild-type α_z with γ_2-MITO causes the α-subunit to colocalize at the mitochondria, indicating that mistargeted $\beta\gamma$ can direct α_z to a new subcellular location (Fig. 3). In cells coexpressing epitope-tagged α_z, β_1, and γ_2MITO, α_z immunofluorescence substantially overlaps that of γ_2MITO (Fig. 3 *a–c*). Even in the presence of mistargeted $\beta\gamma$, some α_z immunofluorescence appears to target to the PM, presumably because it associates there with endogenous $\beta\gamma$. Nonetheless, coexpression with γ_2MITO and β_1 markedly increases localization of α_z to mitochondria (compare Fig. 1*Aa* to Fig. 3*b*); the quantitative increase is highly significant: 39 $\pm$ 12% vs. 7 $\pm$ 0.4%, with and without misdirected $\beta\gamma$, respectively (Fig 2*C*). As compared with α_z, an even higher proportion (77 $\pm$ 7%) of epitope-tagged β_1 colocalizes with γ_2-MITO at the mitochondria (Fig. 3 *d–f*); this difference probably reflects different trafficking pathways taken by α- and β-subunits (23).

Lipid Modifications Stabilize Interaction of α_z with $\beta\gamma$. The idea that $\beta\gamma$ directs targeting of α-subunits contrasts with an earlier view

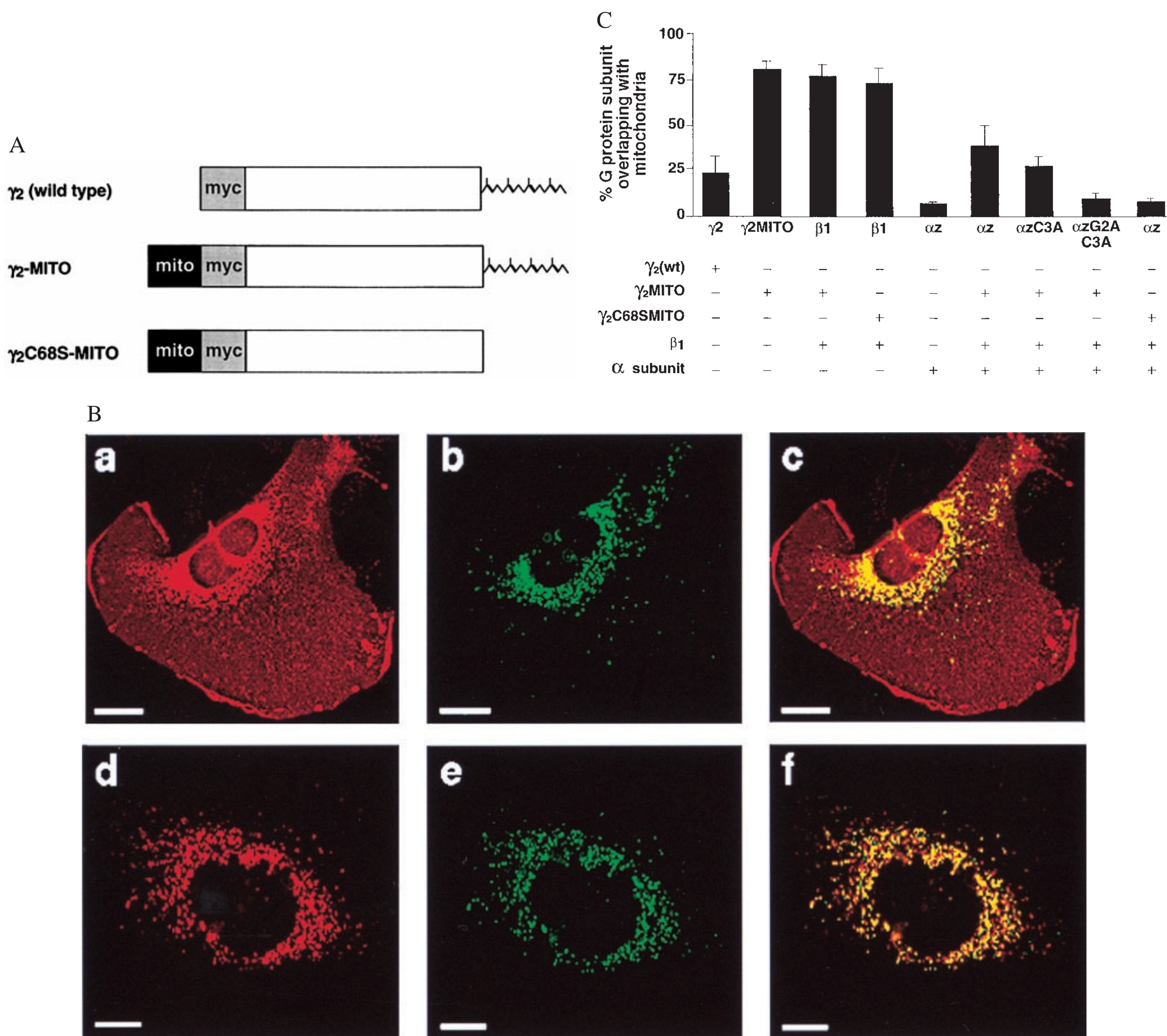

Fig. 2. Mitochondrial targeting of proteins in cells expressing γ_2MITO. (*A*) Diagrammatic representation of γ-subunit constructs, showing N-terminal mitochondrial targeting signal (mito), myc epitope tag (myc), and C-terminal prenyl modification. (*B*) Immunofluorescence microscopy of CHO cells transfected with wild-type γ_2 (*a–c*) or γ_2-MITO (*d–f*). Cells were costained with antibody to γ_2 (*a* and *d*) and mitochondrial Hsp60 (*b* and *e*). *c* and *f* show the overlap (yellow) of the two antibody stains. (*C*) Quantitative analysis of the calculated percentage of overlap between immunofluorescent stain for the appropriate mitochondrial probe (anti-γ_2 or anti-Hsp60, as indicated in the figure legends) and the stain for the indicated G protein subunit in cells cotransfected with no γ-subunit, β_1 plus γ_2MITO, or β_1 plus γ_2C68S-MITO. Bars represent means ± 2 SEM (n = 4).

(1, 24), that palmitate confers membrane specificity. Several mitochondrial proteins are palmitoylated (25), suggesting that the two views might be reconciled by postulating that palmitoylation and γ_2MITO act in combination to recruit α_z to mitochondria. Mitochondrial targeting by γ_2MITO of an α_z mutant lacking the palmitoylation site (α_z-C3A,; ref. 12) indicates, however, that $\beta\gamma$ can direct membrane localization of α-subunits in the absence of palmitate (Fig. 4 *a–d*). When coexpressed with β_1 and γ_2MITO, α_zC3A and wild-type α_z localize at mitochondria with similar efficiencies (28 ± 5% vs. 39 ± 12%; not significantly different) (Fig. 2*C*). As is the case with wild-type α_z, targeting of α_z-C3A to mitochondria by γ_2MITO requires coexpression of α_2 with $\beta_1\gamma_2$MITO (Fig. 4, compare *b* and *d*).

Although palmitate *per se* is not required, similar experiments showed that association of α_z with $\beta\gamma$ at the mitochondria does require two lipid attachments: a myristate at the N terminus of α_z (Fig. 4 *e–h*) and a prenyl group at the C terminus of γ_2MITO (Fig. 5). In contrast to α_z-C3A, which contains myristate, a mutant α_z carrying no lipid modification (α_zG2AC3A; ref. 12) did not colocalize with γ_2MITO and β_1 at the mitochondria. Instead, α_z-G2AC3A is distributed through the cytoplasm and in nuclei, showing little or no association with any cellular membrane (Fig. 4 *e–g*); only 11 ± 4% of this mutant overlapped with the γ_2 fluorescence (Fig. 2*c*). The distribution of α_z-G2AC3A was therefore not altered by overexpression of $\beta\gamma$ at the mitochondria (Fig. 4, compare *f* and *h*), which suggests that $\beta\gamma$ requires assistance from myristate (and/or palmitate) to hold α_z stably on the mitochondrial membrane. It is likely that palmitate by itself could play a similar role to myristate; however, because mutation of the myristoylation site prevents both myristoylation

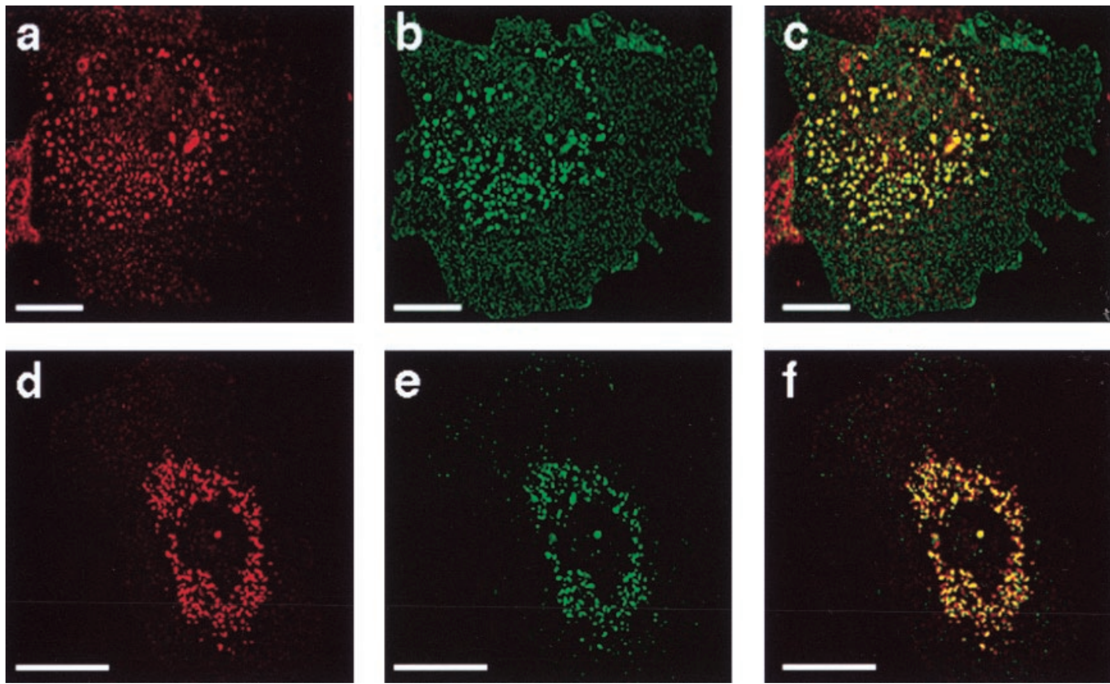

Fig. 3. α_z follows γ_2MITO to mitochondria. Cells were transfected with γ_2MITO, β_1, and EE-tagged wild-type α_z (*a–c*) or γ_2MITO plus β_1 tagged with the EE epitope (*d–f*). Cells were costained with anti-γ_2 (*a* and *d*) and anti-EE antibodies (*b* and *e*). *c* and *f* show the overlap (yellow) of the two antibodies. (Bars = 20 μm.)

and palmitoylation of α_z (1, 12), we could not assess the ability of $\beta\gamma$ to target α_z containing palmitate alone.

The C-terminal prenylation site of γ_2 is exposed to the cytoplasm in γ_2MITO, which is attached to the mitochondrial outer membrane via a hydrophobic N-terminal targeting signal. Prenylation at the C terminus of γ_2MITO is required for targeting α_z, but not β_1, to membranes (Fig. 5 and Fig 2*C*). Addition of a point mutation that prevents prenylation of γ_2 (C68S; ref. 11) creates a mutant protein, γ_2C68S-MITO, that does not differ from γ_2MITO in its exclusive targeting to mitochondria (Fig. 5) or in its ability to induce overexpressed β_1 to accompany it to mitochondria (Fig. 5). In contrast to γ_2MITO, however, γ_2C68S-MITO induced little or no mislocalization of α_z to mitochondria (compare Fig. 5 *b* and *c* to Fig. 3 *b* and *c*). Quantitation showed that overlap of α_z fluorescence with

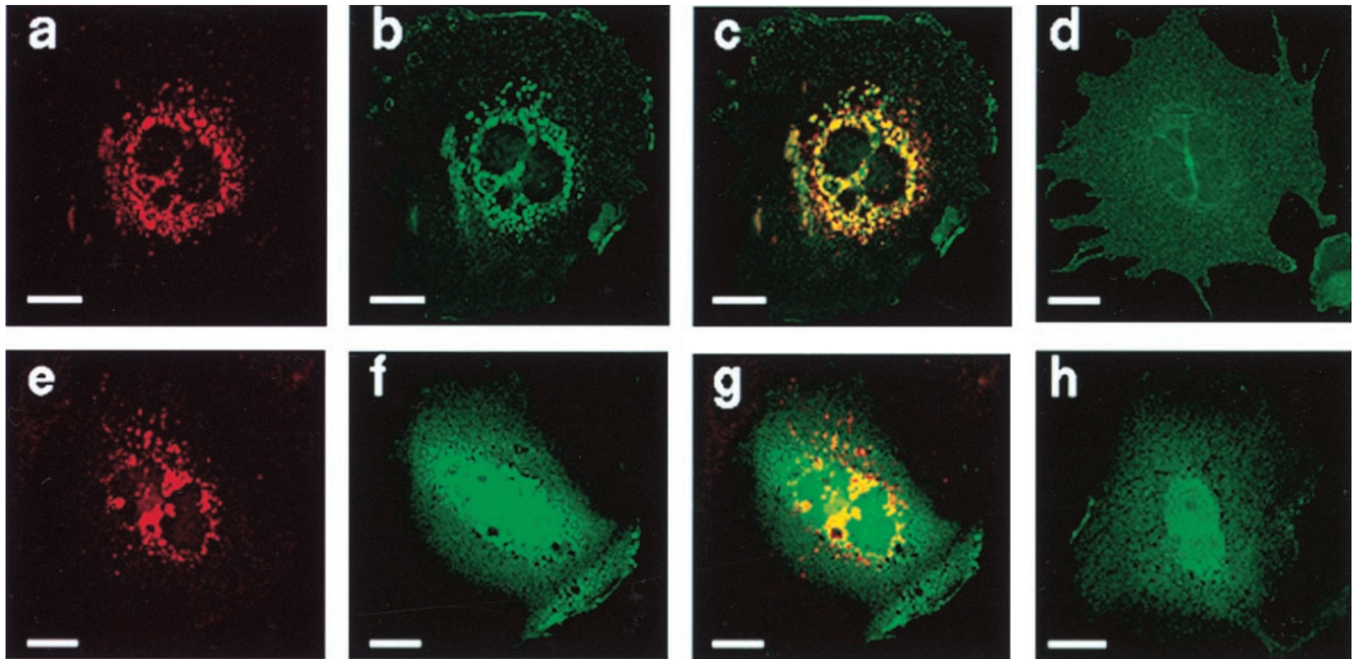

Fig. 4. Effect of removing lipid modification sites on PM and mitochondrial targeting of α_z. Cells were transfected with α_zC3A, which lacks the palmitoylation site (*a–d*), or with α_zG2AC3A, which lacks both myristoylation and palmitoylation sites (*e–h*), either alone (*d* and *h*) or in combination with γ_2MITO and β_1 (*a–c* and *e–g*). Cells were stained with anti-EE antibody (*b*, *d*, *f*, and *h*; to detect epitope-carrying α_z mutants) and anti-γ_2 antibody (*a* and *e*). *c* and *g* show the overlap (yellow) of the antibody stains (*c* shows overlap between *a* and *b*; *g* shows overlap between *c* and *f*). (Bar = 20 μm.)

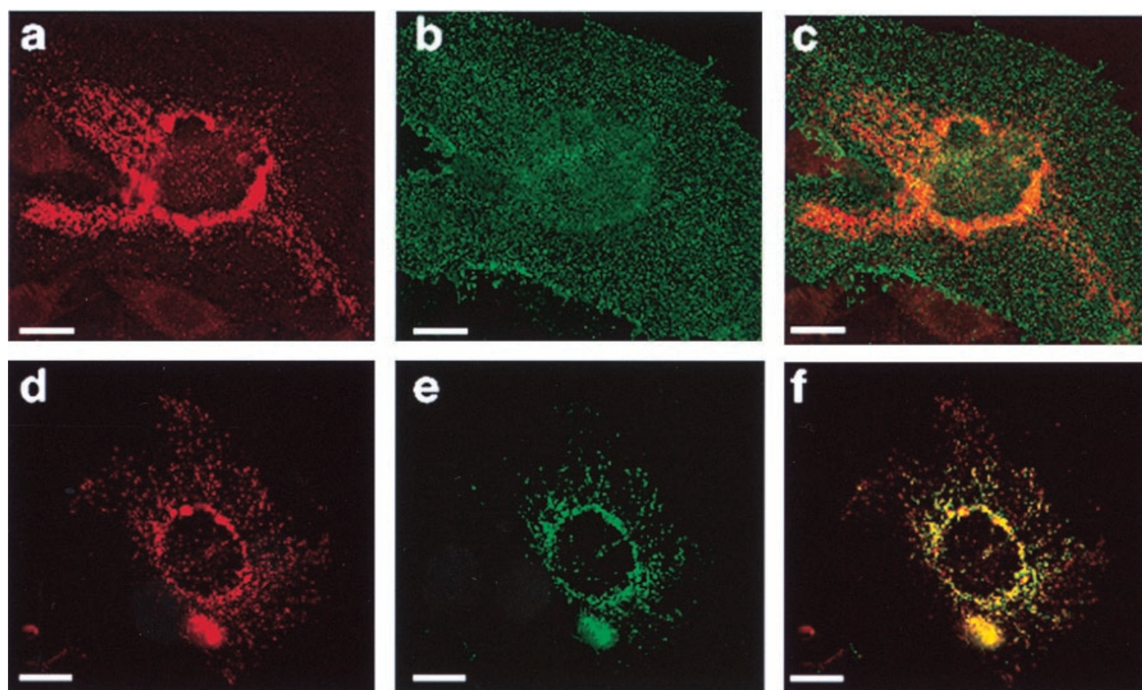

Fig. 5. Removal of the prenylation site prevents association of α_z with $\beta\gamma$ at the mitochondria. Immunofluorescence microscopy of cells expressing γ_2C68S-MITO, β_1, and EE-tagged α_z (*a–c*) or γ_2C68S-MITO and EE-tagged β_1 (*d–f*). Cells were costained with anti-γ_2 (*a* and *d*) and anti-EE (*b* and *e*) antibody. *c* and *f* show the overlap of the two antibodies. (Bar = 20 μm.)

mitochondria was small and virtually the same in cells coexpressing α_z with γ_2C68S-MITO vs. α_z with no γ-subunit. Prenylation of the γ-subunit plays an essential role, therefore, in the association of α_z with $\beta\gamma$ at the mitochondria.

Discussion

The present results provide strong evidence that the G protein $\beta\gamma$-subunit, rather than palmitate, directs specific targeting of G protein α-subunits to membranes. Mislocalization of α_zMUT to intracellular membranes (Fig. 1) suggests that association with $\beta\gamma$ is *necessary* for targeting an α-subunit to the PM, and the experiments with mistargeted γ_2MITO (Figs. 3 and 4) indicate that $\beta\gamma$ can *suffice* for targeting an α-subunit to a membrane-bound organelle.

These results require that we modify and extend a recently proposed general model proposed (2, 24, 26) to account for the specific targeting of dually lipidated peripheral membrane proteins. In this model, attachment of a single lipid (in this case myristate) endows the protein with sufficient hydrophobicity to associate randomly and reversibly with cellular membranes. At its correct target membrane, however, the protein encounters a second signal, which anchors the protein stably to the membrane and specifically retains it on the correct organelle. Previous studies (reviewed in ref. 24) focused on attachment of palmitate as the targeting signal, in part because palmitoylation takes place at the protein's final subcellular destination. In this study of G protein α-subunits, however, we demonstrate that subcellular location is primarily dictated by the protein's binding partner ($\beta\gamma$), rather than by palmitate. Considerable evidence indicates that palmitoylation of α-subunits and their binding to $\beta\gamma$ are closely associated (1, 27, 28), suggesting a "dock-and-lock" modification of the previous model for targeting peripheral membranes at the PM: α-subunits first dock on $\beta\gamma$ at the PM and then undergo rapid palmitoylation, which locks them in place. Indeed, localization of other peripheral membrane proteins to the correct membrane or organelle may likewise depend principally on association with specific protein partners. In keeping with this idea, stable membrane association of two palmitoylated proteins, SNAP25 and GAD65, seems to require additional proteins not yet identified (6, 7, 9).

The requirement for lipids attached to both α_z and γ_2MITO for targeting α_z to mitochondria (Figs. 4 and 5) suggests that hydrophobic interactions between the two lipid groups enhance the affinity of α_z for $\beta\gamma$. *In vitro* experiments comparing functional activities of acylated vs. nonacylated α-subunits (28, 29) and of prenylated vs. nonprenylated γ-subunits (30–32) do not agree with respect to the relative importance of these lipid groups for the interaction of α and $\beta\gamma$. Our *in vivo* experiments indicate that lipid modifications on both α and $\beta\gamma$ are involved in assembling $\alpha\beta\gamma$ heterotrimers at membranes. The idea that these lipid attachments enhance association of α and $\beta\gamma$ by a direct lipid–lipid interaction idea accords with the likely proximity of lipid groups at the N termini of α-subunits and the C termini of γ-subunits, based on three-dimensional crystal structures of G protein trimers (18, 19). Alternatively, or in addition, the prenyl group of $\beta\gamma$ may associate with the hydrophobic mitochondrial outer membrane and orient α_z such that its myristoyl group interacts more effectively with the membrane, thereby stabilizing the association of the heterotrimer with the membrane.

Taken together, our data indicate a prominent role for $\beta\gamma$ as a targeting signal for α-subunits. A related riddle—how $\beta\gamma$ itself is targeted to PM—remains unsolved. Finally, by extension, our results suggest that investigators should look for accessory proteins that direct other peripheral membrane proteins to their correct locations in cells.

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