Gβγ Isoforms Selectively Rescue Plasma Membrane Localization and Palmitoylation of Mutant Gaq and Gap

Received for publication, February 6, 2001
Published, JBC Papers in Press, April 9, 2001, DOI 10.1074/jbc.M101154200

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Mutations of Gaq or Gap N-terminal contact sites for Gβγ resulted in α subunits that failed to localize at the plasma membrane or undergo palmitoylation when expressed in HEK293 cells. We now show that overexpression of specific Gβ subunits can recover plasma membrane localization and palmitoylation of the Gβγ-binding-deficient mutants of αq or αq. Thus, the Gβγ-binding-defective α is completely dependent on co-expression of exogenous Gβγ for proper membrane localization. In this report, we examined the ability of β1−5 in combination with γ2 or γ5 to promote proper localization and palmitoylation of mutant αq or αq. Immunofluorescence localization, cellular fractionation, and palmitate labeling revealed distinct subtype-specific differences in the βγ interac-
tions with α subunits. These studies demonstrate that 1) α and Gβγ reciprocally promote the plasma membrane targeting of the other subunit; 2) βq, when co-expressed with γ2 or γ5, fails to localize to the plasma membrane or promote plasma membrane localization of mutant αq or αq; 3) β1 is deficient in promoting plasma membrane localization of mutant αq and αq, whereas β4 is deficient in promoting plasma membrane localization of mutant αq; 4) both palmitoylation and interactions with Gβγ are required for plasma membrane localization of α.

G proteins1 are found on the cytoplasmic face of the plasma membrane (PM) where they transduce signals from heptahelial receptors to effector proteins (1, 2). Activation of the receptor via agonist binding induces a conformational change in the G protein αβγ heterotrimer, which triggers its dissociation from the receptor in the form of a GTP-bound α subunit and a βγ complex. Each subunit is then capable of regulating the function of various effector proteins. Because proper membrane localization is a prerequisite for the correct functioning of this subtype, numerous studies have examined the requirements for targeting of the G protein α subunits to the plasma membrane. The two major requirements seem to be covalent lipid modifications (3, 4) and binding to the βγ complex (5, 6).

Two types of lipid modification of G protein α subunits have been described, both of which occur at the extreme N terminus of the protein. These subunits are either palmitoylated, in the case of αq, αq, α12, and α13, or myristoylated and palmitoylated in the case of αq and αq. Myristate, a 14-carbon fatty acid, is attached co-translationally and irreversibly, whereas palmitate, a 16-carbon fatty acid, is attached post-translationally and reversibly. These lipid modifications help anchor the G protein α subunit to the PM, but in the case of αq and αq, and presumably α12 and α13 as well, palmitoylation and proper localization of the α subunit at the PM requires stable binding between the α and Gβγ subunits (6). The βγ complex is anchored at the PM with the help of its own lipid modification, the post-translational attachment of either a farnesyl or geranylgeranyl moiety to a cysteine at a C-terminal CAAX box on the γ subunit.

Although formation of the αβγ heterotrimer is clearly essential for cell signaling, the selectivity of interactions among different subunit subtypes has not been well defined. Given that there are at least 23 different α subunits (including splice variants), 5 β subunits and 12 γ subunits, this allows for more than 1300 different heterotrimers. It has been previously documented that some G protein subunits are expressed in subtype-specific patterns in tissues and cells (7–11). This obviously determines the limits of subtype-specific heterotrimer formation in a particular cell but it does not address what βγ dimers and αβγ heterotrimers are actually formed. However, a number of reports have described subtype-specificity in βγ dimer and αβγ heterotrimer formation and function (12). Purification and identification of βγ dimers from tissues (13–15) and in vitro binding data (16, 17) have provided evidence for the existence of specific β and γ combinations. A variety of approaches, including overexpression of subunits, reconstitution studies, antisense, and ribozyme studies, have revealed subtype-specific differences in αβγ heterotrimer coupling to receptors (18–23) and subtype-specific differences in βγ heterodimer regulation of effectors (24–29). Yet, little information is available on differences in the abilities of specific α subtypes to interact with specific βγ dimers. It is not known if observed combinations are indicative of restricted functional associations between the α and βγ subunits, or whether all combinations are possible and selectivity is derived solely through variations in G protein expression and the kinetics of G protein coupling to receptors or effectors.

To begin to address questions of selectivity in αβγ heterotrimer formation and the role of such selectivity in proper subcel-
lular localization, we have expressed different combinations of β and γ subunits along with wild-type or βγ-binding-deficient mutants of αs or αq. We recently demonstrated that mutation of amino acids, which are predicted to contact βγ, in the N-terminal region of αs or αq resulted in α subunits that failed to localize to the plasma membrane or undergo palmitoylation when transiently expressed in HEK293 cells (6). Thus, it appeared that endogenous βγ was unable to effectively interact with the βγ-binding mutants of αs or αq. However, the mutants still contain a C-terminal region, which is known to contact βγ, and although this region seems to be insufficient for endogenous levels of βγ to functionally interact with the mutant α subunits, it may be possible to recover a functional interaction by overexpression of the βγ complexes. In this report, we test the hypothesis that overexpressed βγ can overcome the defects observed with the βγ-binding-defective mutants of αs and αq.

Furthermore, expression of unique βγ combinations would allow us to test different βγ complexes for their ability to recover the localization of the βγ-binding-defective mutants of αs and αq. Thus, rescue of PM localization could provide a model system for assaying subtype-specific differences in βγ interactions with α subunits that may be masked by the high levels of overexpressed protein displaying normal binding affinities. Herein, we present the first test of this system by examining the ability of β2γ3 in combination with γ5 or γ3 to promote plasma membrane targeting of the βγ-binding mutants of αs or αq. Importantly, this analysis revealed distinct subtype-specific differences in βγ interactions with α subunits.

We also tested the importance of α subunit palmitoylation in this system. We demonstrate that overexpression of βγ recovers palmitoylation of βγ-binding-defective mutants of αs and αq. Significantly, overexpression of βγ with cysteine to serine mutants of αs and αq, which are incapable of being palmitoylated, fails to recover membrane localization of the α subunits.

EXPERIMENTAL PROCEDURES

Materials—HEK293 cells were obtained from the American Type Culture Collection (CRL-1573). [9,10-3H]Palmitic acid was from PerkinElmer Life Sciences. 12CA5 mouse monoclonal antibody was a gift from Matthew Hart. All other primary antibodies were from Santa Cruz Biotechnology. Tissue culture reagents were from Life Technologies, Inc. Other reagents were from Fisher Scientific and Sigma Chemical Co.

Expression of Plasmids—The HA epitope (DVPDYA) tagged HA-α2pcDNA3 and HA-α3pcDNA3 βγ-binding-deficient constructs were described previously (6). In this paper α2IEK refers to HA-α2N23A, 126A, E27A, K28A, L30A, D33A-pcDNA3 and α2IE refers to HA-α2L25A, E26A-pcDNA3. The cDNA constructs encoding the human β1, β2, and β3 and the mouse β1 and β2 G protein subunits were subcloned with an N-terminal MycHis epitope tag into pcDNA3.1 (30). Bovine γ3 subunit cDNA in pcDNA1 was excised by digestion with BamHI and Xhol and ligated into pcDNA3. The γ3 subunit cDNA in pcB+ was obtained from N. Gautam.

Cell Culture and Transient Transfection—HEK293 cells were maintained in culture as previously described (6). Transfections were carried out in either 6-well cell culture plates with 1 µg of total plasmid DNA or 6-cm plates with 3 µg of total plasmid DNA using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals) according to the manufacturer’s protocol. Cells were transfected overnight, transferred to new plates the next day, and grown for 24–30 h prior to subsequent manipulations.

Immunofluorescence Localization—Immunofluorescence localization was performed as previously described (6). Briefly, HEK293 cells were transfected overnight in 6-well plates with the indicated amounts of each construct. The cells were washed and grown for 24 to 48 h before fixing with 3.7% formaldehyde in phosphate-buffered saline for 15 min. Cells were permeabilized and then incubated with the indicated primary antibodies for 1 h at the following dilutions: 12CA5 monoclonal (3 µg/ml), c-Myc monoclonal (1 µg/ml), HA-probe (Y-11) rabbit polyclonal (2 µg/ml), c-Myc-A14) rabbit polyclonal (2 µg/ml), Gq/11 (A-16) rabbit polyclonal (2 µg/ml) and Gq/11 (K-20) rabbit polyclonal (2 µg/ml). The cells were washed and incubated in a 1:100 dilution of the indicated secondary antibody: Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 488 goat anti-mouse, Alexa Fluor 594 goat anti-rabbit, and Alexa Fluor 594 goat anti-mouse (Molecular Probes, Eugene, OR), for 30 min. The coverslips were washed and mounted on glass slides equipped with Prolong Antifade reagent (Molecular Probes, Eugene, OR) and microscopy was performed with an Olympus BX60 microscope equipped with a Sony DRC-5000 digital camera. A minimum of 100 cells was examined for each transfection, and in most cases this number was at least 500. Many transfections were repeated once, and two or three representative pictures were taken of cells displaying a typical expression pattern for each transfection. Only cells displaying low to intermediate levels of expression were utilized. Similar or identical f-stops were used for imaging the fluorescence from the α or β subunits in each experiment to verify that imaged cells were expressing approximately equal amounts of each subunit. Images were processed with Adobe Photoshop.

Subcellular Fractionation—Soluble and particulate fractions were isolated as previously described (6). Briefly, HEK293 cells were transfected with the indicated amounts of each expression plasmid in 6-cm plates overnight, transferred to 10-cm plates, and grown for 48 h. The cells were washed with phosphate-buffered saline and lysed in 0.5 ml of lysis buffer by passage through a syringe. Nuclei and intact cells were removed by centrifugation, and the supernatant was centrifuged at 105,000 g to pellet the beads. The supernatant was discarded, and the beads were washed three times with 1 ml of radioimmune precipitation buffer. SDS-PAGE sample buffer containing 10% dithiothreitol was added to the washed beads, and the samples were heated at 65°C for 1 min. An aliquot was analyzed by 10% SDS-PAGE. Gels were incubated in 10 mM methanol/10% acetic acid, followed by 10% methanol/10% acetic acid for 20 min, and finally, Amplify (Amersham Pharmacia Biotech) for 20 min. Gels were dried and subjected to fluorography at ~80°C using Hyperfilm MP (Amersham Pharmacia Biotech).

RESULTS

Interdependence of α, β, and γ Subunits for Plasma Membrane Localization—To examine the intracellular targeting and localization of the G protein α and βγ subunits, these subunits were transiently expressed in HEK293 cells independently or with complementary subunits. As previously shown by us, αs expressed in the presence of only endogenous βγ is found predominately at the plasma membrane but some remains in the cytoplasm (6). Because βγ binding was determined to be essential for membrane localization of αs, it was reasonable to expect that overexpression of βγ complexes with the αs subunit would be able to target more αs to the PM than endogenous βγ alone. Co-expression of β2γ3 with αs in HEK293 cells resulted in prominent PM localization of αs (Fig. 1A). Comparison of these results with those of αs when expressed alone showed more pronounced PM localization in a larger number of cells. The β2γ3 subunits were also effective at promoting more pronounced PM localization of αs (Table I).
αs that was similar to αs expressed alone (Fig. 1B1). The presence of γs rather than γ2 in the βγ complex did not change the effectiveness of PM targeting for either β1 or β2 (Fig. 1, C1 and D1). Examination of the localization of the βγ complex in each case showed that it co-localized with the α subunit when the α subunit was exclusively at the PM (Fig. 1, A2 and C2) but failed to do so otherwise (Fig. 1, B2 and D2). In contrast to αs, αq displayed strong PM localization when expressed alone (6). As expected, αs retained a predominant PM localization when co-expressed with each of the βγ complexes, and the localization of the βγ complexes did not vary from the results with αs (Table I).

We have demonstrated that the βγ complex is critical for PM localization of αs and αq, but it is not clear how important the α subunit is for βγ complex localization. To help determine this, myc-tagged β subunits were expressed alone, co-expressed with γ subunits, or co-expressed with both α and γ, and visualized by immunofluorescence. The βs subunit displayed some plasma membrane staining but seemed to be most prominent in the cytoplasm or intracellular membranes (Fig. 2). Similar results were obtained with the β2 subunit (data not shown). β2, β3, and β4 displayed only intracellular staining, although β2 and β3 displayed some exclusion from the nucleus and β4 did not (data not shown). Co-expression with γs subunits resulted in little to no increase in PM localization for all β subunits despite the fact that the γ2 subunit should be prenylated and able to anchor to the PM (Fig. 2 and data not shown). Upon co-expression with αs and γs, essentially all of the β s subunits were targeted to the PM where they co-localized with αs (Figs. 1A2 and 2), as were βs-4 (Table I). Overlaying of the two pictures (Fig. 1, A1 and A2) showed conclusively that β2 co-localizes with αs at the plasma membrane, as demonstrated by the bright yellow plasma membrane coloring (Fig. 1A3). Similar to αs expression of αs induced increased PM localization of βs-4γs (Table I).

Although co-expression of the γ subunit with the β subunit did not seem to affect the localization of the β subunit, β subunit expression did affect the expression and localization of γ in our system. Because the γs antibody did not work reliably in our hands, we used the γ2 subunit for these experiments. Previous localization of endogenous γ2 in cardiac fibroblasts showed diffuse staining throughout the cell (32). Transient transfection of the γ2 subunit alone resulted in almost no detectable expression. The few cells that did express the protein displayed a small level of intracellular expression only (Fig. 2). Co-expression with the βs subunit resulted in expression in more cells with protein present both intracellularly and at the PM (Fig. 2). However, it was not until the αs subunit was also transfected that predominately PM localization was seen for the γ2 subunit and there was expression in a large number of cells (Fig. 2). Thus, it appears that expression and localization of each subunit of the heterotrimeric G protein is dependent in some way on the others and no single subunit determines the expression and localization of the others.

Unlike all the other β subunits, the β2 subunit never displayed any PM localization. When β2 was expressed alone or with the γs subunit, its expression was evenly distributed throughout the cell (Fig. 1D2). However, when it was co-expressed with the γ2 subunit, β2 expression was restricted to perinuclear and cytoplasmic membranes. Additional expression of αs (Fig. 1B2) or αs (Table I) did not change the localization of β2. This unique localization of the β2 subunit was found to be caused by co-expression of the γ2 subunit, which, when expressed alone, shows this same localization in perinuclear and cytoplasmic membranes (Ref. 33 and data not shown). These results suggest that β2 is able to co-localize and associate with γ2 but not γs. This is supported by studies that have

**Table I**

*Summary of immunofluorescent subcellular localization of α and βγ*

| β only | αs | αq | αs-IEK+ | αs-IEK |
|--------|----|----|---------|--------|
| α only | +  | +  | +       | +      |
| βγγ2   | +  | +  | +       | +      |
| βγγ2   | +  | +  | +       | +      |
| βγγ2   | +/−| +  | +       | +      |
| βγγ2   | +  | +  | +       | +      |
| βγγ2   | −/−| +  | +       | +      |
| βγγ2   | +/+| +  | +       | +      |
| βγγ2   | −/−| +  | +       | +      |
| βγγ2   | −/−| +  | +       | +      |
| βγγ2   | −/−| +  | +       | +      |
| βγγ2   | −/−| +  | +       | +      |

*αs* Perinuclear.

*βs* Cytoplasmic and bright intracellular aggregates.

*ND* not determined.
demonstrated signal transduction pathways utilizing overexpressed \( \beta_{1} \gamma_{2} \) subunits but not \( \beta_{2} \gamma_{3} \) subunits (24).

**\( \beta \) Subtype-specific Targeting of Mutant \( \alpha_{q} \)**—The results above demonstrated the ability of the \( \beta \gamma \) complex to promote PM binding of wild-type \( \alpha_{q} \). Because our previous results demonstrated that mutation of the N-terminal \( \beta \gamma \) contact region of \( \alpha_{q} \) resulted in its inability to localize at the plasma membrane, we wanted to test whether co-expression of \( \beta \gamma \) with a \( \beta \gamma \)-binding-deficient mutant of \( \alpha_{q} \) could overcome the effects of decreased \( \beta \gamma \) binding ability. The previously characterized \( \beta \gamma \)-binding mutant, \( \alpha_{sIEK}^{1} \), was co-transfected with \( \beta_{1-5} \) and \( \gamma_{2} \). The \( \beta \) subunits and \( \alpha_{sIEK}^{1} \) were then visualized by indirect immunofluorescence. As shown previously, \( \alpha_{sIEK}^{1} \) was totally cytoplasmic with no visible membrane staining (6). Co-expression of \( \beta_{1} \gamma_{2} \), \( \beta_{2} \gamma_{2} \), and \( \beta_{3} \gamma_{2} \) resulted in \( \alpha_{sIEK}^{1} \) localization at the plasma membrane as was the case with the wild-type \( \alpha \) (Fig. 3, A1, B1, D1). Visualization of \( \beta_{1} \), \( \beta_{2} \), and \( \beta_{4} \) demonstrated that all three \( \beta \) subunits co-localized with \( \alpha_{sIEK}^{1} \) at the plasma membrane (Fig. 3, A2, B2, D2). However, co-expression of \( \beta_{1} \gamma_{2} \) was unable to target \( \alpha_{sIEK}^{1} \) to the plasma membrane (Fig. 3C1). Visualization of the \( \beta_{3} \) subunit showed that at least some of it was in fact localized at the plasma membrane, and the merged photo clearly shows the red membrane staining of the \( \beta_{3} \) subunit and green cytoplasmic staining of the \( \alpha_{sIEK}^{1} \) (Fig. 3, C2 and C3). As would be expected from the results with the wild-type \( \alpha \) subunit (Fig. 1B), \( \beta_{3} \gamma_{2} \) was unable to target the \( \alpha_{sIEK}^{1} \) mutant to the plasma membrane (Fig. 3E1). Not only was \( \beta_{3} \gamma_{2} \) unable to target \( \alpha_{sIEK}^{1} \) to the plasma membrane, but the two subunits failed to co-localize within the cytoplasm. \( \alpha_{sIEK}^{2} \) displayed an even cytoplasmic distribution with no concentration in the perinuclear or Golgi regions where the \( \beta_{3} \) subunit was localized (Fig. 3E2).

As a complementary approach, we utilized cellular fractionation to determine the localization of the \( \alpha_{q} \) subunits in the entire population of expressing cells. HEK293 cells were transiently transfected with the various combinations of subunits and grown for 3 days. The cells were lysed, the soluble and particulate fractions representing the cytoplasmic and membrane cellular fractions were isolated, and the level of \( \alpha_{q} \) subunit in each fraction was determined. The results clearly show that co-expression of nearly every \( \beta \gamma \) complex combination tested was able to recover the membrane binding ability of \( \alpha_{sIEK}^{1} \) (Fig. 6A). The order of effectiveness was \( \beta_{1} \gamma_{2} = \beta_{2} \gamma_{2} = \beta_{3} \gamma_{2} > \beta_{4} \gamma_{2} \), which is exactly what was seen with immunofluorescence. Unexpectedly, fractionation results seem to indicate that \( \beta_{3} \gamma_{2} \) can recover minimal membrane binding of \( \alpha_{sIEK}^{1} \) even though immunofluorescence did not detect any \( \beta_{3} \gamma_{2} \)-dependent PM binding of \( \alpha_{sIEK}^{1} \). Unlike immunofluorescence, however, these fractionation experiments do not differentiate between PM and intracellular membrane binding. The only subunits that were completely ineffective at targeting \( \alpha_{sIEK}^{1} \) to the membrane fraction were the \( \beta_{3} \) subunits, which agrees with the immunofluorescence results.

**\( \beta \) Subtype-specific Targeting of Mutant \( \alpha_{q} \)**—The experiments above were repeated using \( \alpha_{q} \), a \( \beta \gamma \)-binding-defective mutant of \( \alpha \). Different \( \beta \gamma \)-binding-defective mutants of \( \alpha \) and \( \alpha_{q} \) were used in these experiments because we wanted to use \( \alpha \) subunits with the smallest number of mutations capable of blocking PM localization. Our previous results demonstrated that two mutations, I19A and E20A, are sufficient for \( \alpha \) to lose all PM localization, whereas \( \alpha_{q} \) required additional N-terminal mutations to lose PM binding (6). Co-expression of the \( \beta_{1} \) subunits with \( \alpha_{q} \) demonstrated a subtype-specific effect on targeting of \( \alpha_{q} \) to the plasma membrane (Fig. 4). Unlike \( \alpha \), we could not visualize the \( \alpha \) and \( \beta \) subunits in the same cells. This was because the polyclonal antibody against the HA tag could not detect the HA tag in \( \alpha \), thus we were forced to use monoclonal antibodies to visualize the \( \alpha \) and \( \beta \) subunits in different cells. \( \beta_{1} \) and \( \beta_{2} \gamma_{2} \) were each able to recruit \( \alpha_{q} \) to the PM as they were with \( \alpha_{sIEK}^{1} \) (Fig. 4). However, \( \beta_{2} \gamma_{2} \) and \( \beta_{3} \gamma_{2} \) differed in their ability to promote PM binding of \( \alpha_{q} \), compared with their effect on \( \alpha_{sIEK}^{1} \). Although \( \beta_{3} \gamma_{2} \) was unable to target \( \alpha_{sIEK}^{1} \) to the plasma membrane and co-localize with it (Fig. 4B), \( \beta_{3} \gamma_{2} \) was unable to target the \( \alpha_{sIEK}^{1} \) mutant to the plasma membrane (Fig. 4E).
3), it was able to promote weak PM staining of αIE (Fig. 4). The most profound difference involved βγ2γ. This subunit was poor at targeting αIE to the plasma membrane even though it was one of the most efficient subunits at targeting of αIEK+ to the PM. Examination of Fig. 4 indicates that βγ was, in fact, localized at the PM with seemingly the same efficiency as βγ and βγ but was unable to recruit αIE to the PM with the same efficiency (Fig. 4). βγ failed to promote PM targeting of αIE and did not co-localize with αIE, when co-expressed. βγ was localized to the perinuclear region and intracellular membranes while αIE remained cytoplasmic (Fig. 4).

Cellular fractionation results (Fig. 6B) from αIE paralleled the results of immunofluorescence. βγ was totally ineffecual at recovering membrane binding, and the other βγ dimers were more effective in order of βγ = βγ > βγ. Comparison of the αIE fractionation to that of αIEK+ indicates that βγ complexes are less efficient at recovering the plasma membrane binding of αIE than αIEK+, despite the fewer mutations in αIE.

Effect of γ Subunits on the Targeting and Co-localization of the α Subunits—So far our results have indicated a subtype-specific targeting of αs and αs mutants, which have had their N-terminal βγ-binding region disrupted. Although co-expression of βγ complexes is able to recover proper PM targeting and co-localization in most cases, βγ was unable to do so for αIEK+ and αIE. βγ was unable to do so for αIE, and no co-localization was ever seen between βγ and any α subunits (summarized in Table I). It is possible that such differences could be due to improper or inefficient binding between these particular β and γ subunits. One report demonstrated that the γ subunit associates weakly with the β, β, and β subunits but the γ subunit binds to all five β subunits with similar high affinities (17). To determine if this could be the cause of our β subtype-specific results, we repeated the experiments above using γ, βγ, and βγ (row 3) shows an overlay of α and β immunofluorescence images.

However, co-expression with the γ-binding mutants, αIEK+ and αIE, displayed interesting similarities and differences (Figs. 4 and 5). Immunofluorescence localization showed that βγ was unable to target αIEK+ to the PM just as βγ was unable to do so (Fig. 5C). Subcellular fractionation results indicate that βγ may be a little worse than βγ at PM targeting, but the deficiency seems to be largely a property of βγ in general and is not due to the complexed γ subunit. βγ functioned just as well as βγ at targeting and co-localization of αIEK+ (Figs. 5A and 5A). However, γ was less effective.
and-depennent (12CA5 monoclonal antibody. Levels of incorporated palmi-

ta pcDNA3 or 700 ng of m cells were transiently transfected with 2

show no significant difference between bg when co-expressed with g

In the plasma membrane are less efficiently palmitoylated (6). In

B

6

The results shown are the means ± S.D. for two experiments assayed

two times.

than γ2 at targeting the β2 and β4 subunits to the PM, and αIEK + was not effectively recruited to the PM, as determined by immuno- fluorescence (Fig. 5, B and D). The same results were observed when β2γ3 and β4γ3 were co-expressed with αIE

Fig. 6. Subcellular fractionation of βγ-binding mutants of αs and αq when co-transfected with different βγ complexes. A, HEK293 cells were transiently transfected with 2 μg of αs or αIEK + and 1 μg of pcDNA3 or 700 ng of βγ, and 300 ng of γs or γa (lane 2). HEK293 cells were transiently transfected with 2 μg of αs or αIE and 1 μg of pcDNA3 or 700 ng of βγ, and 300 ng of γs or γa. Soluble and particulate fractions were isolated as described under “Experimental Procedures.” The results shown are the means ± S.D. for two experiments assayed two times.

Palmitoylation—We previously demonstrated that βγ-binding-deficient α subunits of αs and αq, which are not targeted to the plasma membrane are less efficiently palmitoylated (6). In this report we have shown that co-expression of βγ complexes is able to recover the plasma membrane binding of α subunit mutants. We next wanted to determine whether βγ complex overexpression also restored normal levels of palmitoylation to βγ-binding-deficient α subunits of αs and αq, as determined by incorporation of radiolabeled palmitate. Because wild-type αs incorporates extremely low levels of palmitate when expressed in HEK293 cells, COS cells were used in these studies. Cells were transiently transfected with the HA epitope-tagged α subunits with and without various βγ complexes, metabolically labeled with [3H]palmitic acid and immunoprecipitated with the 12CA5 monoclonal antibody. Levels of incorporated palmitate were visualized by fluorography of immunoprecipitated proteins after SDS-PAGE fractionation. Co-expression of the β2–γ2 complexes resulted in increases of incorporation of radiolabeled palmitate into αIEK +, which corresponded quite well with their ability to promote membrane localization of the α subunit (Fig. 7A, lanes 5–10). Unexpectedly, β1γ2 promoted the incorporation of significantly more radiolabeled palmitate than β1γ3, β2γ2γ3, or β3γ2, despite the fact that all of these βγ complexes were just as efficient at targeting αIEK + to the PM (Fig. 7A, lane 3 and Fig. 6). Similar results were seen with αIE except that in this case it was the two βγ complexes that promoted the incorporation of more radiolabeled palmitate than expected based on the fractionation data (Fig. 6B). The only βγ complexes that were completely ineffective at promoting palmitoylation of the α subunit were those containing β5 (data not shown). With this only exception, co-expression of βγ complexes with the βγ-binding mutants of both αs and αq, was able to stimulate incorporation of more radiolabeled palmitate than seen with expression of the wild-type α subunits alone. This was particularly true for αIEK +, which labeled better than wt-αs when co-expressed with each βγ complex.

Because co-expression of βγ complexes is able to recover palmitoylation and membrane binding of α subunit mutants, this raises the question of whether palmitoylation is always required for membrane binding or if overexpression of βγ complexes can overcome this requirement. To examine this, we tested whether co-expression of βγ complexes can recover membrane binding of palmitoylation-deficient α subunits. αsC3S and αqC9S, C10S were previously shown to be non-palmitoy-

Fig. 7. Palmitoylation of βγ-binding mutants of αs and αq when co-transfected with different βγ complexes. A, COS7 cells were transiently transfected with 2 μg of αs and 1 μg of pcDNA3, or 2 μg of αIEK + and 1 μg of pcDNA3, or 2 μg of αIEK + and 700 ng of β1γ3, or 300 ng of αs or αIEK +, or 2 μg of αIE and 1 μg of pcDNA3, or 2 μg of αIE, or 700 ng of β1γ3, or 300 ng of γs or γa. B, COS7 cells were transiently transfected with 2 μg of αs and 1 μg of pcDNA3, or 2 μg of αIE and 1 μg of pcDNA3, or 2 μg of αIE and 700 ng of β1γ3, or 300 ng of γs or γa. The α subunits were metabolically labeled with [3H]palmitic acid (1 mCi for αs and 0.5 mCi for αq) and immunoprecipitated as described under “Experimental Procedures.” The upper panel for each subunit shows the radiolabeled palmitate incorporated by each subunit and visualized by fluorography (exposures: A, 10 days; B, 66 days). Western blot analysis (lower panels) of aliquots of each immunoprecipitation show the level of each α subunit.
and 150 ng of incubated with γ2, fails to localize to the PM or promote PM localization of mutant α5 or αβ5, while β5 is deficient in promoting PM localization of mutant α5 and αγ, while β5 is deficient in promoting PM localization of mutant α5, and αβ subunits.

The function of the βγ complex in helping to localize the α subunit to the PM has been demonstrated recently (6, 33, 37), but little is known about the localization of βγ itself except that it is considered to be anchored at the PM by prenylation (38). Here we have demonstrated that βγ localizes to the PM very poorly when overexpressed in HEK293 cells. However, co-expression with an α subunit leads to an increase in expression of βγ and greater PM localization. This was seen using antibodies directed at both the β and γ subunits. These results indicate that each of the subunits comprising the G protein heterotrimer are required for proper expression and targeting of the others. The βγ complex seems to be more sensitive to this than the α subunit, because overexpression of the α subunits results in significant levels of expression at the PM, while overexpression of βγ results in little PM localization. This difference between overexpressed α and βγ may reflect differences in degradation rates of the subunits or may reflect differences in the stoichiometry of αβγ inside a cell; little is known regarding either possibility. Defining such parameters as degradation rates and stoichiometry will be critical not only for understanding further the regulation of G protein heterotrimer formation and signaling, but will also be an important component of understanding specificity among all signaling pathways inside a cell.

Every pair of β and γ subunits examined, with the exception of the β3γ2 and β5γ3, displayed the ability to localize efficiently at the PM when co-expressed with wild-type αq and αq (Table I). Studies utilizing the β3γ2 complex have demonstrated its ability to form a functional dimer that interacts selectively with αq (31) and stimulates downstream effectors in a subtype-specific manner (24, 28, 39–41). Although the β3 subunit failed to localize at the PM under any of the conditions tested, it did co-localize with the γ2 subunit in perinuclear membranes, suggesting that it had dimerized with it (Fig. 1B2 and data not shown). β2 did not co-localize with γ2, indicating that it does not dimerize with this subunit (data not shown). In addition to the inability of the β3γ2 complex to co-localize with either αq or αq, results from other assays were also consistent with a lack of interaction between αq and β3. First, the β3γ2 complex was unable to promote the exclusion from the nucleus of the non-palmitoylated mutant of αq (Fig. 6L). When the non-palmitoylated mutant of αq was co-expressed with β5γ3, non-palmitoylated αq was excluded from the nucleus even though it could not localize at the PM (Fig. 6G). Presumably this was due to an interaction between αq and β5γ3. Second, co-expression of β5γ3 or β3γ2 with αq IE had no effect on the palmitoylation of the α subunit (data not shown), in contrast to the palmitoylation-inducing effect of other βγ combinations (Fig. 7). Taken together, our results fail to find any evidence of an αqβ5γ3 complex in transfected cells.

In fact, our studies are more consistent with the hypothesis that the main role of β3 is interaction with RGS proteins rather than γ subunit, and β2 may not participate in the formation of a classical G protein heterotrimer in vitro (42). Despite our results and those of others indicating that transfected β3 and γ2

**FIG. 8.** Immunofluorescence localization of non-palmitoylated mutants of αq and αq when co-transfected with different βγ complexes. HEK293 cells were transiently transfected with 600 ng of αg (A); 600 ng of αq C9S (B); 600 ng of αq C3S, 250 ng of myc-tagged β5, and 150 ng of γ2 (C and D); 600 ng of αq (E); 600 ng of αq C9S, C10S (F); 600 ng of αq C9S, C10S, 250 ng of myc-tagged β5 and 150 ng of γ2 (G); 600 ng of αq C9S, C10S, 250 ng of myc-tagged β5 and 150 ng of γ2 (H); and 600 ng of αq C9S, C10S, 250 ng of myc-tagged β5 and 150 ng of γ2 (I). The transfected proteins were fixed and then visualized by indirect immunofluorescence with Y-11 HA-polyclonal and Alexa 488 anti-rabbit for αq (A–C), 12CA5 and Alexa 488 anti-mouse for αq (E–I), or anti-myc and Alexa 594 anti-mouse antibodies for β5 (D) or β5 or β5 (not shown).
can dimerize, recent studies of the composition of \( \beta \) complexes in vivo and in vitro were unable to find \( \beta \) complexes with \( \gamma \) (43, 44). All of the \( \beta \) complexes examined contained RGS6, RGS7, or RGS9 rather than \( \gamma \). However, our results demonstrating a co-localization of \( \beta \) with \( \gamma \) raises the interesting possibility that there are, in fact, \( \beta \gamma \) complexes in the Golgi that form heterotrimers with \( \alpha \) subunits. A previous report suggested that there are more \( \alpha \) subunits in the Golgi than \( \beta \gamma \) subunits, which would result in excess \( \alpha \) subunits that do not form \( \alpha \beta \gamma \) heterotrimers (45). It is possible, given our results, that these extra \( \alpha \) subunits in the Golgi may be complexed with \( \beta \gamma \). This possibility will require further study to determine what the status of \( \beta \gamma \) is in the cell and whether it may function in the Golgi.

Excluding the \( \beta \) subunit, which shows no interaction with either \( \alpha \) or \( \alpha \) when expressed with either \( \gamma \) or \( \gamma \), the \( \beta \) subunit is the least efficient at translocating \( \alpha \) or \( \alpha \) to the PM. Importantly, this deficiency in \( \beta \) was only revealed through the use of the \( \beta \gamma \)-binding-deficient \( \alpha \) \( \alpha \) and \( \alpha \) \( \alpha \), validating the utility of this expression system for detecting subtype-specific differences in \( \alpha \beta \gamma \) complex formation. Although the inefficiency of \( \beta \) in promoting PM localization and palmitoylation of mutant \( \alpha \) and \( \alpha \) could be explained by the inability of \( \beta \) to bind \( \gamma \), several lines of evidence suggest the \( \beta \) subunit does complex with \( \gamma \): 1) \( \beta \gamma \) is able to promote PM localization of \( \alpha \) \( \alpha \) and co-localize with \( \alpha \) and \( \alpha \) at the PM (Table 1); 2) \( \beta \gamma \) complexes have been purified after co-expression (28); and 3) \( \beta \gamma \) can activate effectors after co-expression in mammalian cells (24, 26).

However, other reports have indicated that \( \beta \) and \( \gamma \) do not interact (16, 17, 46). Indeed, one report suggested that \( \gamma \) interacted with \( \beta \) better than \( \gamma \) did. However, in our system, \( \beta \gamma \gamma \), also inefficiently promoted PM localization and palmitoylation of \( \alpha \) \( \alpha \) and \( \alpha \) \( \alpha \). The lack of consensus as to the ability of \( \beta \) to interact with \( \gamma \) may relate to the different methods used to detect functional \( \beta \gamma \gamma \) complexes. A key component may be the presence of the \( \alpha \) subunit to aid in \( \beta \gamma \) complex formation. Thus, methods in which \( \alpha \) is absent would fail to detect \( \beta \gamma \) complexes (16, 17, 46), while methods, such as analyses of localization or function in cells, in which \( \alpha \) is present are consistent with \( \beta \gamma \gamma \) complex formation (24, 26). Little is known about the temporal relationship between \( \beta \gamma \) dimer formation and formation of the heterotrimer, but one report does suggest that there may be interactions between \( \beta \) and \( \gamma \) within a few minutes of translation, before the proteins reach the PM (47). It may be that the simple affinities of specific \( \beta \) and \( \gamma \) subunits to couple in vivo are not a good indication of what happens in vivo. The \( \alpha \) subunit as well as other factors may be critical in determining specificity of \( \beta \gamma \) dimer formation, as was previously suggested based on in vivo localization studies (10). Regardless of the exact mechanism, our results utilizing \( \beta \gamma \)-binding mutants of \( \alpha \) and \( \alpha \) demonstrate that \( \beta \gamma \gamma \) and \( \beta \gamma \gamma \gamma \) fail to promote PM localization of the mutant \( \alpha \) subunits. Importantly, the system described in this report will allow us to test other \( \gamma \) subunits. It is possible that \( \beta \gamma \) when expressed with a unique \( \gamma \) subtype, will be able to more efficiently recover PM localization of \( \alpha \) \( \alpha \) and \( \alpha \) \( \alpha \).

The use of \( \beta \gamma \)-binding-deficient \( \alpha \) \( \alpha \) and \( \alpha \) \( \alpha \) also revealed a striking difference in the ability of \( \beta \gamma \) to promote PM localization of mutant \( \alpha \) versus mutant \( \alpha \). The PM binding of \( \alpha \) \( \alpha \) was recovered with \( \beta \gamma \) dimers just as well as with \( \beta \gamma \) dimers (Fig. 6A). However, \( \beta \gamma \) dimers were unable to recover PM localization of \( \alpha \) \( \alpha \) (Fig. 6B). Although this was the only \( \beta \) subunit that displayed a profound difference in coupling between \( \alpha \) \( \alpha \) and \( \alpha \) \( \alpha \), it was not the only \( \beta \gamma \) dimer to preferentially target one \( \alpha \) subunit to the PM over another. \( \beta \gamma \) and \( \beta \gamma \) were much worse at targeting \( \alpha \) \( \alpha \) and \( \alpha \) \( \alpha \), respectively, to the PM than their \( \gamma \) containing counterparts. To our knowledge, this is the first demonstration of a \( \gamma \)-dependent modulation of the interaction between the \( \alpha \) and \( \beta \gamma \) subunits.

In a previous paper we showed that lack of membrane binding by \( \alpha \) or \( \alpha \) caused by lack of \( \beta \gamma \) binding resulted in a corresponding decrease in palmitoylation (6). In this report we examined this further by overexpression of \( \beta \gamma \) complexes to recover membrane binding of the \( \beta \gamma \)-binding-deficient \( \alpha \) subunits and assayed them for their ability to incorporate radio-labeled palmitate. We have found that recovery of PM localization does not occur without a corresponding increase in palmitate incorporation. However, it is possible to get a significant increase in incorporation of palmitate without a corresponding increase in PM localization. This is particularly true of \( \alpha \) \( \alpha \), which was able to incorporate more than 20 times the amount of radiolabeled palmitate when co-expressed with \( \beta \gamma \) compared with wt-\( \alpha \) expressed alone. \( \alpha \) \( \alpha \), co-expressed with wt-\( \gamma \) and wt-\( \alpha \) expressed alone, were assayed at similar levels and localized to the PM to similar degrees. Although co-expression of \( \beta \gamma \) with wt-\( \alpha \) was able to increase palmitate incorporation by the wild-type subunit, it was only increased 2-fold (data not shown). It seems unlikely that the steady-state level of palmitoylation is increased for these mutant \( \alpha \) subunits without a visible increase in PM localization. A more likely explanation is that the palmitate turns over more rapidly in these mutants due to their poor \( \beta \gamma \) binding. In this scenario, \( \beta \gamma \) promotes PM binding and palmitoylation of both wt-\( \alpha \) and the mutant \( \alpha \), but \( \alpha \) can more rapidly exchange unlabeled palmitate for the radiolabeled palmitate. This suggestion agrees with studies showing that \( \beta \gamma \) protects \( \alpha \) from depalmitoylation by thioesterases (48–50). This could also explain the curious result that \( \beta \) was able to stimulate as much palmitate incorporation in \( \alpha \) as \( \beta \) did, even though \( \beta \) was unable to promote efficient PM localization of \( \alpha \).

The importance of palmitoylation of \( \alpha \) and \( \alpha \) was highlighted further by our demonstration that \( \beta \gamma \) fails to induce any PM localization of \( \alpha \) \( \alpha \) or \( \alpha \) \( \alpha \), palmitoylation site mutants of \( \alpha \) and \( \alpha \) (Fig. 8). Previous studies of cysteine to serine palmitoylation site mutants of \( \alpha \) and \( \alpha \) have relied upon overexpression of the \( \alpha \) subunits alone in the absence of co-expressed \( \beta \gamma \), and differential effects on the \( \alpha \) subunits’ ability to bind membranes have been observed (4, 51). However, the results presented here clearly demonstrate that N-terminal cysteine residues, and by inference palmitoylation, are essential for PM localization of \( \alpha \) subunits. Under conditions in which overexpression of wt forms of \( \alpha \) or \( \alpha \) together with \( \beta \gamma \) results in efficient PM localization of all subunits, \( \alpha \) \( \alpha \) and \( \alpha \) \( \alpha \), C10S fail to display any PM localization. Thus, palmitoylation, in addition to interactions with \( \beta \gamma \), is required for stable binding of the \( \alpha \beta \gamma \) G protein heterotrimer to the PM.

In summary, the results presented in this report demonstrate not only the reciprocal importance of \( \alpha \) and \( \beta \gamma \) for PM targeting of the G protein heterotrimer, but also describe a novel system for detecting subunit subtype selectivity by employing \( \beta \gamma \)-binding mutants of \( \alpha \) and \( \alpha \). We predict that this system will be useful for defining further \( \alpha \beta \gamma \) subtype specificity. For example, \( \beta \gamma \)-binding mutants of other members of the \( \alpha \) subunit family can be created and assayed in a similar manner. In addition, this system allows us to test rapidly the ability of other \( \gamma \) subunits to complex with \( \beta \gamma \) and form functional complexes capable of promoting PM localization of mutant \( \alpha \) subunits. Finally, this analysis can be extended beyond subcellular localization, and it provides a means to ascer-
tain subunit subtype differences in a heterotrimer's ability to couple to unique receptors.

Acknowledgments—We thank Dr. N. Gautam for generously providing plasmids and Drs. Raja Bhattacharyya, Jeffrey Benovic, and Catherine Chen for critical reading of the manuscript.

REFERENCES

1. Neer, E. J. (1995) Cell 80, 249–257
2. Ham, H. E. (1998) J. Biol. Chem. 273, 669–672
3. Dunphy, J. T., and Linder, M. E. (1998) Biochim. Biophys. Acta 1436, 245–261
4. Wedegaertner, P. B. (1998) Biol. Signals Recept. 7, 125–135
5. Fishburn, C. S., Herzmark, P., Morales, J., and Bourne, H. R. (1999) J. Biol. Chem. 274, 18723–18800
6. Evanko, D. S., Thiyagarajan, M. M., and Wedegaertner, P. B. (2000) J. Biol. Chem. 275, 1327–1336
7. Brunk, I., Pahner, I., Maier, U., Jenner, B., Veh, R. W., Nurnberg, B., and Ahnert-Hilger, G. (1999) Eur. J. Cell Biol. 78, 311–322
8. Betty, M., Harnish, S. W., Rhodes, K. J., and Cockett, M. I. (1998) J. Biol. Chem. 273, 85, 475–486
9. Asano, T., Morishita, R., Ohashi, K., Nagahama, M., Miyake, T., and Kato, K. (1997) J. Biol. Chem. 272, 1327–1336
10. Schmidt, C. J., Thomas, T. C., Levine, M. A., and Neer, E. J. (1992) J. Biol. Chem. 267, 1267–1273
11. Liang, J. J., Cockett, M., and Khawaja, Z. X. (1998) J. Neurochem. 71, 345–355
12. Morishita, R., Saga, S., Kawamura, N., Hashizume, Y., Inagaki, T., Kato, K., and Asano, T. (1997) J. Neurochem. 68, 820–827
13. Asano, T., Morishita, R., Ueda, H., and Kato, K. (1999) J. Biol. Chem. 274, 14261–14268
14. Wilcox, M. D., Dingus, J., Balcueva, E. A., McIntire, W. E., Mehta, N. D., Schey, K. L., Robishaw, J. D., and Hildebrandt, J. D. (1995) J. Biol. Chem. 270, 4199–4205
15. Schmidt, C. J., Thomas, T. C., Levine, M. A., and Neer, E. J. (1992) J. Biol. Chem. 267, 13807–13810
16. Yan, K., Kalyanaraman, V., and Gautam, N. (1996) J. Biol. Chem. 271, 7141–7146
17. Kleuss, C., Scherubl, H., Hescheler, J., Schultz, G., and Wittig, B. (1992) Nature 358, 424–426
18. Kleuss, C., Scherubl, H., Hescheler, J., Schultz, G., and Wittig, B. (1993) Science 260, 832–834
19. Kalkbrener, P., Dippel, E., Wittig, B., and Schultz, G. (1996) Biochim. Biophys. Acta 1314, 125–139
20. Hou, H., Azpiazu, I., Smirca, A., and Gautam, N. (2000) J. Biol. Chem. 275, 30961–30964
21. Richardson, M., and Robishaw, J. D. (1999) J. Biol. Chem. 274, 13525–13533
22. Wang, Q., Mullah, B. K., and Robishaw, J. D. (1999) J. Biol. Chem. 274, 17365–17371
23. Zhou, J. Y., Siderovski, D. P., and Miller, R. J. (2000) J. Neurosci. 20, 7143–7148
24. Diverse-Pierluissi, M., McIntire, W. E., Myung, C. S., Lindorfer, M. A., Garrison, J. C., Guy, M. F., and Dunlap, R. (2000) J. Biol. Chem. 275, 28380–28385
25. Lei, Q., Jones, M. B., Tailey, E. M., Schrier, A. D., McIntire, W. E., Garrison, J. C., and Bayliss, D. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 9771–9776
26. Witherow, D. S., Levay, K., Cabrera, J. L., Chen, J., Williams, G. B., and Slepak, V. Z. (2000) J. Biol. Chem. 275, 24872–24880
27. Zhang, J. H., and Simonds, W. F. (2000) J. Biol. Chem. 275, 37435–37442
28. Clapham, D. E., and Neer, E. J. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 167–203
29. Watson, A. J., Katz, A., and Simon, M. I. (1994) J. Biol. Chem. 269, 22150–22156
30. Zhang, S., Caso, O. A., Lee, C., Gutkind, J. S., and Simonds, W. F. (1996) J. Biol. Chem. 271, 33575–33579
31. Lindorfer, M. A., Myung, C. S., Savino, Y., Yasuda, H., Khazan, R., and Garrison, J. C. (1998) J. Biol. Chem. 273, 34429–34436
32. Peters, R. D., and Siderovski, D. P. (2001) Biochem. Pharmacol. 61, 1329–1337
33. Withrow, D. S., Wang, Q., Levay, K., Cabrera, J. L., Chen, J., Williams, G. B., and Slepak, V. Z. (2000) J. Biol. Chem. 275, 24872–24880
34. Zhang, J. H., and Simonds, W. F. (2000) J. Neurosci. 20, RC59
35. Denker, S. P., McCaffery, J. M., Palade, G. E., Insel, P. A., and Farquhar, M. G. (1996) J. Cell Biol. 133, 1027–1040
36. Pronin, A. N., and Gautam, N. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6220–6224
37. Rehm, A., and Ploegh, H. L. (1997) J. Biol. Chem. 272, 305–317
38. Rehm, A., and Ploegh, H. L. (1997) J. Biol. Chem. 272, 305–317
39. Iiri, T., Backlund, P. S., Jr., Jones, T. L., Wedegaertner, P. B., and Bourne, H. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14592–14597
40. Wedegaertner, P. B., and Bourne, H. R. (1994) Cell 77, 1063–1070
41. Duncan, J. A., and Gilman, A. G. (1998) J. Biol. Chem. 273, 15830–15837
42. Mumby, S. M. (1997) Curr. Opin. Cell Biol. 9, 148–154