THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 33, pp. 24099–24108, August 17, 2007

A Family of G Protein $\beta\gamma$ Subunits Translocate Reversibly from the Plasma Membrane to Endomembranes on Receptor Activation*[S]

Received for publication, February 8, 2007, and in revised form, April 13, 2007 Published, JBC Papers in Press, June 20, 2007, DOI 10.1074/jbc.M701191200

Deepak Kumar Saini[‡], Vani Kalyanaraman[‡], Mariangela Chisari[‡], and Narasimhan Gautam[‡§1]

From the [‡]Department of Anesthesiology and [§]Genetics, Washington University School of Medicine, St. Louis, Missouri 63110

The present model of G protein activation by G protein-coupled receptors exclusively localizes their activation and function to the plasma membrane (PM). Observation of the spatiotemporal response of G protein subunits in a living cell to receptor activation showed that 6 of the 12 members of the G protein γ subunit family translocate specifically from the PM to endomembranes. The γ subunits translocate as $\beta\gamma$ complexes, whereas the α subunit is retained on the PM. Depending on the γ subunit, translocation occurs predominantly to the Golgi complex or the endoplasmic reticulum. The rate of translocation also varies with the γ subunit type. Different γ subunits, thus, confer distinct spatiotemporal properties to translocation. A striking relationship exists between the amino acid sequences of various γ subunits and their translocation properties. γ subunits with similar translocation properties are more closely related to each other. Consistent with this relationship, introducing residues conserved in translocating subunits into a non-translocating subunit results in a gain of function. Inhibitors of vesicle-mediated trafficking and palmitoylation suggest that translocation is diffusion-mediated and controlled by acylation similar to the shuttling of G protein subunits (Chisari, M., Saini, D. K., Kalyanaraman, V., and Gautam, N. (2007) *J. Biol. Chem.* 282, 24092–24098). These results suggest that the continual testing of cytosolic surfaces of cell membranes by G protein subunits facilitates an activated cell surface receptor to direct potentially active G protein $\beta\gamma$ subunits to intracellular membranes.

GPCR[2] stimulation results in the activation of G protein α and $\beta\gamma$ subunit complexes which modulate the function of downstream effector molecules that function on the cytosolic surface of the PM (1–4). The classic model of GPCR action, thus, restricts the activation of G proteins and consequently their effectors to the two-dimensional plane of the PM (1–4). Intracellular effects have been thought to occur through second messengers released through the activation of effector molecules such adenylyl cyclase, phospholipase Cβ, and ion-conducting channels. Direct communication of a GPCR with intracellular membranes through the G protein subunits has not been anticipated. We have used live cell imaging methods to examine the spatiotemporal dynamics of the localization of components of a GPCR-mediated signaling pathway when the pathway is activated and deactivated.

In mammalian cells similar to GPCRs the G protein subunits are also families of proteins. Based on the presence of distinct genes, there are 16 α subunits, 5 β subunit, and 12 γ subunit types. The α subunit types appear to possess distinctly different properties (3, 4). Although evidence exists for the differential activity of $\beta\gamma$ subunit types in terms of their role in receptor activation of a G protein and modulation of effector function, these differences have been subtle and quantitative (5–12). One potential reason for a lack of evidence for qualitative differences in the properties of these diverse proteins is that most assays used so far to measure G protein function have used techniques that lead to disruption of cells. The possibility that these proteins are involved in spatially distinct functions has, thus, remained unexplored.

Here we examined the entire family of γ subunit types complexed with different β subunit types for potential translocation in response to GPCR activation in various cell lines. There have been previous indications from our laboratory that the $\beta_1\gamma_{11}$ and $\beta_1\gamma_5$ subunit complexes translocate away from the PM on receptor activation (13). To identify the mechanistic basis, the translocation properties were examined in the presence of inhibitors of vesicle-mediated trafficking, an acylation inhibitor, and after introducing mutations into the γ subunit C-terminal domain previously shown to interact with receptors (14). We identified potential $\beta\gamma$ complexes capable of translocation in a live cell by examining fluorescence resonance energy transfer (FRET) between fluorescent protein-tagged subunits. We examined the impact of cell lines of different origins and various α subunit types and receptors on the translocation of $\beta\gamma$ complexes. Although it has been known that a large and diverse family of γ subunits with highly conserved structures exists (12, 15, 16), striking differences in their signaling properties have not been found. Results here show that the γ subunits control the spatiotemporally distinct translocation of a large variety of $\beta\gamma$ complexes from the PM to endomembranes, thus making it

* This work was supported by National Institutes of Health Grant GM 69027 and an American Heart Association post-doctoral fellowship (to M. C.). 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] To whom correspondence should be addressed: Box 8054, Washington University School of Medicine, St. Louis, MO 63110. Tel.: 314-362 8568; Fax: 314-362-8571; E-mail: gautam@wustl.edu.

[2] The abbreviations used are: GPCR, G protein-coupled receptor; PM, plasma membrane; FRET, fluorescence resonance energy transfer; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; 2BP, 2-bromopalmitate; mCh, mCherry.

possible for GPCRs on the cell surface to direct an active component of a G protein to intracellular membranes.

MATERIALS AND METHODS

Chemicals and Expression Constructs—All chemicals were purchased from Sigma unless otherwise indicated. α_o-cyan fluorescent protein (CFP), yellow fluorescent protein (YFP)-β_1 and CFP- or YFP-tagged γ_5 and γ_{11} constructs have been described previously (13). The citrine mutant of YFP and the non-oligomerizing forms of both CFP and YFP (17) were engineered by mutagenesis and used in all experiments. The γ_1, γ_2, γ_3, γ_4, γ_7, γ_8 (γ_{olf}), γ_9 (γ_{cone}), γ_{10}, γ_{12}, γ_{13}, β_1, β_2, β_3, and β_4 cDNAs were introduced downstream of YFP or mCherry (18) (from R. Tsien) in the pCDNA 3.1 or pDest vectors (Invitrogen). All constructs were checked by determining the nucleotide sequence. Red fluorescent protein DsRed2-KDEL marker and DsRed monomer galactosyl transferase (Clontech). Transferrin-Texas Red was from Molecular Probes.

Cells and Transfection—Native CHO cells and those stably expressing the M2 muscarinic receptor (M2-CHO) (19) and the M3 muscarinic receptor (M3-CHO) (20) have been described previously. CHO cells were grown in CHO IIIa medium (Invitrogen) containing dialyzed fetal bovine serum (Atlanta Biologicals), methotrexate (for M2-CHO, M3-CHO), penicillin, streptomycin, glutamine, and Fungizone. HeLa and J774.1 cell lines were obtained from American Type Culture Collection and were grown in specific media recommended by the ATCC. All the transfections were performed using Lipofectamine 2000 (Invitrogen). M2-CHO cells stably expressing α_o-CFP, β_1, and YFP-γ_{11} were generated using standard procedures, screened with flow cytometry, and cultured in CHO-IIIa medium containing methotrexate and Geneticin (G418).

Treatment of Cells—To disrupt Golgi in the cells, 10 μM brefeldin A was used in the culture medium 6–10 h before cell imaging. For treatment with nocodazole, transfected or stable cells were cooled on ice for 5 min, then nocodazole (5 μg/ml) was added, and cells were further incubated for 15 min on ice followed by incubation at 37 °C for 5 min. As a control, 5 μg/ml transferrin-Texas Red conjugate was then added to the cells for 15 min at 37 °C after which the cells were visualized to monitor vesicular trafficking. The imaging was done in the presence of nocodazole. For monensin treatment the cells were treated with 50 μM monensin for 1 h at 37 °C before imaging. For cycloheximide treatment the cells were treated with 50 μg/ml cycloheximide for 6–8 h at 37 °C before imaging. For treatment with 2-bromopalmitate (2BP), the media was removed from the cells, and fresh Hanks' buffer saline solution containing 50 μM 2BP (stock prepared in Me_2SO (21)) was added to the cells, and the cells were incubated for a further 30 min at 37 °C before imaging.

Imaging of Live Cells—Cells were cultured on acid-washed glass coverslips and transiently transfected with appropriate combinations of different G protein subunits and GPCRs as described in the text and figure legends. After 16–24 h post-transfection the cells were processed for imaging as follows. The coverslips were washed with Hanks' buffer saline solution supplemented with 10 mM HEPES, pH 7.4, and mounted on an imaging chamber with an internal volume of 25 μl (RC-30 chamber, Warner Instruments). For live cell imaging, a fluid delivery system including a programmable valve controller and Teflon valves (10-ms open/closure time) (Automate Scientific) was used to deliver the buffer with or without agonist or antagonist through the chamber at a rate of 0.5 ml/min with a regulated flow controller. The cells were visualized with a Zeiss Axioskop fluorescent microscope using a 63× oil immersion objective (1.4 NA) and 100-watt mercury lamp with a Hamamatsu CCD Orca-ER camera. The shutter and emission and excitation filter wheels were controlled by a Sutter Lambda 10–2 optical filter changer (Sutter Instrument Co.) run by MetaMorph 6.3.7 (Molecular Devices) software. The filter and beam splitter combinations (Chroma Technology) were as follows; for CFP, D436/10 excitation, D470/30 emission; for YFP, D500/20 excitation, D535/30 emission; for mCherry or DsRed D580/20 excitation, D630/60 emission, a polychroic beam splitter (Chroma 86002BS) and 25 or 10% neutral density filters. In those cases where both CFP and YFP fusions were co-expressed in cells, the cells expressing relatively similar levels of the two fusions were selected based on emission intensities. Images were acquired at 20- or 10-s intervals, and the exposure times were between 0.1 and 1.0 s. Details of the FRET analysis are described in Chisari *et al.* (66). FRET analysis was performed not only with YFP- and CFP-tagged proteins but also mCherry (mCh) and YFP combinations as described. Details of the experiments performed with confocal microscopy are provided in Chisari *et al.* (66).

RESULTS

Receptor Induced βγ Subunit Translocation—G protein signaling has been thought to be exclusively localized to the PM with heterotrimers being activated by transmembrane receptors and the activated subunits acting on PM-associated effectors. We used CHO cells stably expressing M2 receptors (M2-CHO) transiently transfected with α_o, β_1, and different γ subunits tagged with YFP to evaluate the effect of receptor activation on various G protein γ subunits. We have previously demonstrated that β and γ subunits with an N-terminal fluorescent protein fusion localize normally on the PM and support normal activation of the G protein by a receptor (22). Images of the cells were captured at defined intervals of time, whereas they were sequentially exposed first to a muscarinic receptor agonist, carbachol, and then to an antagonist, atropine. Emission intensities from the intracellular membranes were plotted as a function of time to quantitate potential translocation of the fluorescent protein-tagged γ subunit. The γ subunits which translocated were initially clearly localized to the PM (Fig. 1). When various members of the γ subunit family were examined, it was observed that six different γ subunit types translocated to specific intracellular membranes in a receptor-mediated manner. On antagonist addition they translocated back to the PM. The γ_1, γ_5, γ_9, and γ_{10} translocated to a focused intracellular region, which was similar to the cellular localization of the translocated γ_{11} subunit previously reported (13) (Fig. 1*A*, *top panel*, Fig. 1*B*, and supplemental Figs. 1 and 2). In striking contrast, the γ_{13} subunit translocated to endomembranes with a more diffuse spatial distribution (Fig. 1*C*, *top panel*). Corresponding to the changes in endomembrane fluorescence inten-

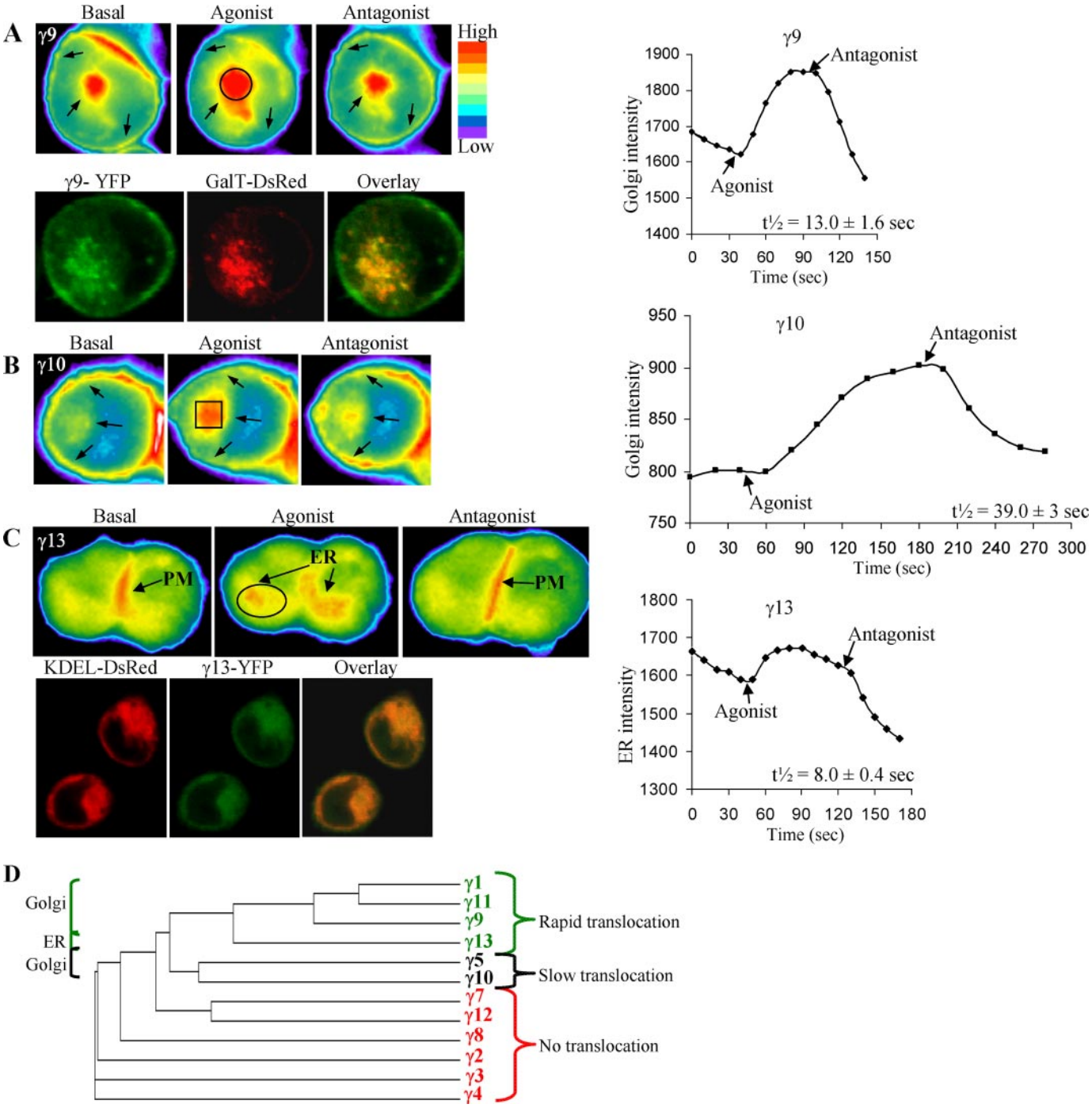
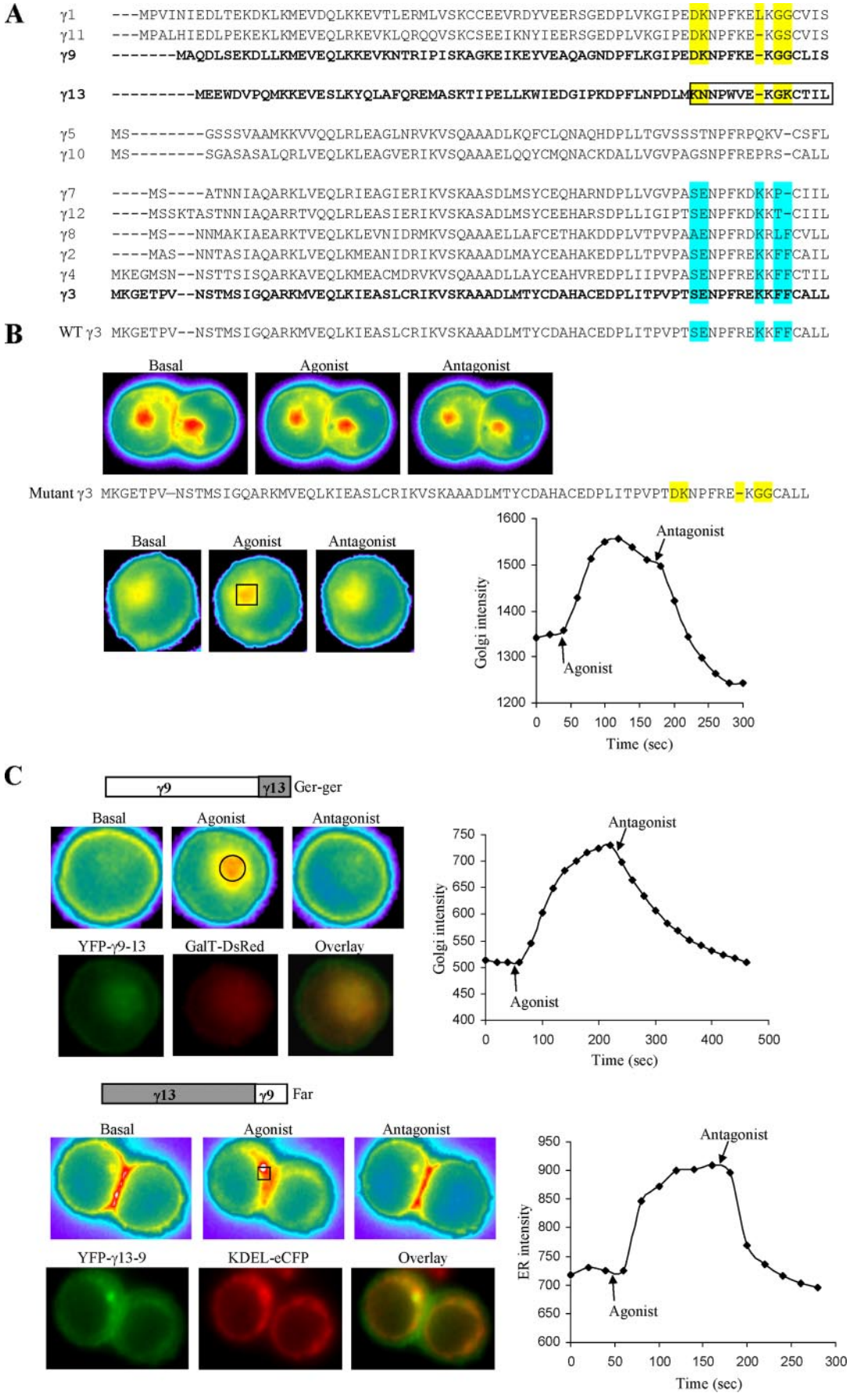


FIGURE 1. **Receptor-mediated translocation of G protein βγ complexes.** M2-CHO cells transfected with α_o, mCherry-β_1, and YFP-tagged γ subunits as indicated were used. Images of YFP-γ subunits from transfected cells were captured every 10 or 20 s. Cells were exposed to 100 μM carbachol (*Agonist*) followed by 100 μM atropine (*Antagonist*) at the indicated time points. YFP intensity changes over time in Golgi or ER were plotted. Plots are of similar scales for comparison. Representative images are shown. Mean pixel intensity measurements for plots were from regions enclosed in *black circles* or *squares*, indicated in agonist-treated images. The decrease in fluorescence intensity over time is due to photobleaching. *A*, translocation of YFP-γ_9 to Golgi ($n > 20$). Shown are confocal images of M2-CHO cells expressing α_o, β_1, YFP-γ_9, and galactosyl transferase (*GalT*)-DsRed monomer (*DsRed*) after exposure to carbachol ($n = 8$). *B*, translocation of YFP-γ_{10} to the Golgi ($n = 10$). *C*, translocation of YFP-γ_{13} to ER ($n > 20$). Cells were treated with brefeldin A to eliminate the Golgi. Shown are confocal images of carbachol-exposed M2-CHO cells expressing α_o, β_1, YFP-γ_{13}, and KDEL-DsRed, an ER marker ($n = 10$). Note that the PM distribution of γ_{13} compared with the ER marker is visible (*green* peripheral signal) in the overlay. *D*, cladogram shows the relationship between primary structures and translocation properties. The predominant endomembrane targets after translocation of γ subunit types are shown.

sity in response to the agonist and antagonist, corresponding changes in the opposing direction were observed in the PM (supplemental Fig. 3). In addition, a plot of the intensity of the Golgi marker, galactosyl transferase-DsRed, after exposure to agonist and antagonist did not show significant changes like the intensity plot for YFP-γ9 (supplemental Fig. 4). These results confirmed that the increase in endomembrane fluorescence intensity is not due to a change in Golgi size or shape.

The G protein γ subunits are modified with a prenyl moiety. γ_1, γ_9, and γ_{11} are farnesylated, whereas all other γ subunits are



geranylgeranylated (23). The translocation process by itself is not dependent on the type of prenyl moiety attached to a γ subunit type because γ_5, γ_{10}, and γ_{13} are capable of translocation, although they are geranylgeranylated.

Endomembranes as Target Membranes for Translocating βγ Complexes—To identify the membranes to which the γ_9 and γ_{13} subunits translocate, markers tagged with fluorescent proteins specific to the Golgi and the ER were used. A trans-Golgi marker, galactosyl transferase-tagged with monomeric DsRed (24, 25) and YFP-γ_9 were coexpressed in M2-CHO cells and imaged in a confocal microscope after M2 activation. Overlaying the images of galactosyl transferase-DsRed monomer and YFP-γ_9 showed that YFP-γ_9 localized specifically to the endomembranes (predominantly Golgi complex) after translocation (Fig. 1*A*, *bottom panel*). To further confirm this localization, we treated the transfected M2-CHO cells with brefeldin A, which is known to disrupt the Golgi complex (26). When these cells were examined after agonist treatment, translocation of YFP-γ_9 to the Golgi region could no longer be detected (not shown). These results indicated that γ_9 translocates primarily to the Golgi region.

To identify the membranes to which the G protein β_1 γ_{13} complex translocated, we coexpressed the ER retention KDEL sequence of calreticulin, a specific marker for the ER tagged with DsRed (27–29) with YFP-γ_{13} in M2-CHO cells and imaged the cells after M2 activation. To examine whether γ_{13} translocates to the Golgi also apart from the ER, we treated the transfected M2-CHO cells with brefeldin A. On agonist addition γ_{13} translocated to endomembranes. Overlaying the images of KDEL-DsRed and translocated YFP-γ_{13} showed that YFP-γ_{13} was localized to the ER (Fig. 1*C*, *bottom panel*). When antagonist was added, γ_{13} reverse-translocated back to the PM. The γ_{13} subunit was initially localized to the PM and ER (Fig. 1*C*, *top panel*). When cells expressing γ10 were similarly examined with a Golgi marker, the target of the translocation was predominantly the Golgi complex (not shown).

We examined whether reverse translocation on the addition of an antagonist subsequent to agonist addition was due to an effect independent of the receptor. Instead of antagonist addition, agonist was withdrawn by introducing buffer. The γ_9, γ_{11}, and γ_{13} subunits reverse-translocated in these cells, similar to the cells treated with antagonist, although the $t_{1/2}$ for reversal was comparatively slower (not shown). The slower kinetics would be anticipated because the antagonist would be able to shift the receptor to its inactive state more rapidly by displacing agonist binding directly. This experiment indicated that reverse translocation is the outcome of switching the activated state of the receptor to its inactive state.

Subfamilies of γ Subunits with Distinct Kinetics of Translocation—We measured the rate of translocation of the various γ subunits by measuring the proportion of the fluorescent protein-tagged γ subunit that translocated from PM to intracellular membranes at various time points. When the rate of translocation of the different γ subunits was compared, distinct differences were seen. γ_{10} and γ_5 translocated relatively slowly ($t_{1/2} \sim 39-85$ s) (Fig. 1*B*, *plot*, and supplemental Fig. 2), whereas the other subunits, γ_1, γ_9, γ_{11}, and γ_{13}, translocated rapidly ($t_{1/2} \sim 6-13$ s) (Fig. 1, *A* and C, supplemental Fig. 1). To examine the relationship between the translocating and nontranslocating γ subunits, the human γ subunit amino acid sequences were aligned and analyzed for their evolutionary relationship. The resulting phylogenetic tree shows that the γ subunits that translocate in a receptor induced fashion, γ_1, γ_5, γ_9, γ_{10}, γ_{11}, and γ_{13}, show a strikingly closer relationship to each other compared with other γ subunits (Fig. 1*D* and Fig. 2*A*). The two classes of γ subunit types based on the rate of translocation, relatively slow and rapid, also show a closer relationship within a subfamily (Fig. 1*D*). Furthermore, γ_{13} is distinct compared with the other rapidly translocating γ subunits. These results suggest that translocation is dependent on the amino acid sequences of the γ subunits. This is an inference that is consistent with the nature of the prenyl moiety not having any discernible effect on translocation (below). The differential translocations of the βγ complexes are, thus, both qualitative and quantitative. Such differences in the properties of G protein βγ subunits have not been noted before.

The G Protein γ Subunit Mediates Translocation of a βγ Complex—The α subunit types, α_o, αi, or α_q, coexpressed with a translocating γ subunit did not translocate (supplemental Fig. 5*A*). This result suggested that translocation is restricted to the βγ complex because the γ subunit is known to be tightly bound to the β subunit (12, 15, 16). To confirm that the γ subunits translocate as βγ complexes, untagged γ subunits were expressed with the β_1 subunit tagged with YFP or mCh (18) in M2-CHO cells. In the presence of agonist, translocation of the YFP-β_1 or mCh protein was observed (supplemental Fig. 5*B*). This result confirmed (i) γ subunit translocation occurs as a βγ complex, (ii) that the translocation process is directly influenced only by the type of γ subunit, and (iii) the γ subunit fluorescent protein tag does not have an effect on the translocation. To rule out the possibility that β subunit translocation is a result of interaction with a cellular protein other than the γ subunit, which is capable of translocation, we evaluated receptor-mediated translocation in M2-CHO cells transfected with the fluorescent protein-tagged β_1 subunit without a translocating γ subunit. In such cells significant translocation of the β_1 subunit was not detected on receptor activation (data not shown). This result indicated that the γ subunits directly mediate translocation of the βγ complex. Furthermore, YFP or mCh-β_1 not only cotranslocated with CFP- or YFP-tagged translocation-competent γ subunits, but more important, acceptor (YFP or mCh) photobleaching indicated measurable FRET between YFP or mCh-β_1 and CFP or YFP-γ subunits as

FIGURE 2. **Translocation of γ subunit chimeras and mutants.** *A*, the γ subunit family is divided as in the cladogram (Fig. 1*D*) into four groups based on the spatiotemporal properties of translocation. Amino acids mostly conserved within the C-terminal domain of the subunits are *highlighted*. *B*, translocation of a wild type and mutant γ_3 subunit. Mutated residues are *highlighted* in both the wild type and mutant γ_3. Transfected cells were assayed for translocation, and the data are plotted as described in Fig. 1. Shown are representative data ($n \geq 4$). *C*, translocation of chimeric molecules of γ_9 and γ_{13}. Transfected cells were assayed for translocation, and the data are plotted as described in Fig. 1. The images of cells after translocation were overlaid with corresponding images of the same cells expressing Golgi or ER marker. Representative data are shown ($n \geq 5$).

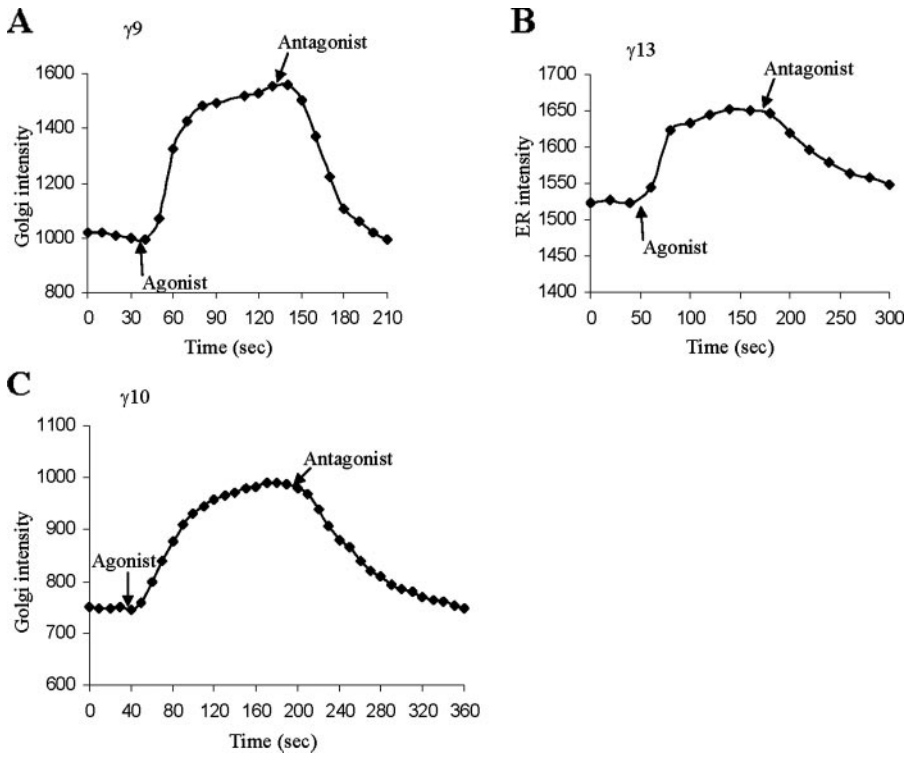


FIGURE 3. **Effect of different α subunits on translocation of different γ subunits.** Plots of emission intensities from the intracellular membranes (Golgi or ER) as a function of time in CHO cells expressing M3 receptor, α_q, mCh-β_1, and YFP-tagged γ subunits as indicated to quantitate potential translocation. Transfected cells were assayed for translocation, and the data are plotted as described in Fig. 1. Representative data are shown ($n \geq 4$). *A*, translocation plot for YFP-γ_9 subunit. *B*, translocation plot for YFP-γ_{13} subunit. *C*, translocation plot for YFP-γ_{10} subunit.

described below. These results confirm that translocation occurs as a βγ complex.

Mutation of Residues at the C-terminal Domain of a Non-translocating Subunit Results in the Gain of Translocating Ability—The ability of the G protein γ subunit to influence receptor-mediated translocation was reminiscent of the role of the γ subunit in receptor interaction of a G protein that we first showed in the rhodopsin-transducin system and later with ligand binding receptors (5, 6, 19, 30). It raised the possibility that receptor contact with the γ subunit is a critical mediator of translocation. Previous evidence from the rhodopsin and muscarinic receptor systems has suggested that an 11 amino acid domain at the C terminus of the processed γ subunit contacts receptors (19, 30–33). Two-dimensional NMR has shown that a peptide encompassing this domain directly interacts with activated but not inactive rhodopsin (14, 34). To examine this possibility we compared the amino acid sequences of the human γ subunits. Fig. 2*A* shows an alignment of the γ subunit primary structures. In Fig. 2*A* residues in this C-terminal domain conserved within the subfamily of subunits that translocate rapidly and the subfamily of subunits that do not translocate are *highlighted*. The conservation of these residues within the subfamilies with distinct translocation properties suggested that they may play a role in regulating translocation. We examined a hypothesis that the affinity of this C-terminal domain of a γ subunit for a receptor determines whether it will translocate. To examine whether conserved residues that are strikingly different between the non-translocating and translocating subunits played a role in the translocation, we mutated five residues in the γ_3 subunit that does not translocate (Fig. 2*B*). The γ_3 mutant was expressed in M2-CHO cells, and receptor-induced translocation was examined. Results in Fig. 2*B* show that the γ_3 mutant has gained the ability to translocate due to the alterations in the C-terminal domain. This result shows that C-terminal residues of the γ subunit are critical for translocation, consistent with a model that non-translocating γ subunits have a high affinity for the activated receptor, whereas translocating γ subunits have distinctly lower affinity (32).

Translocation to Different Endomembranes Is Not Dependent on the C-terminal Domain of the γ Subunit or the Type of Prenyl Moiety—γ_{13} is targeted predominantly to the ER, unlike γ_1, γ_9, and γ_{11}, which are targeted to the Golgi, although they also translocate relatively rapidly. We examined whether the C-terminal domains of the G protein γ subunits are involved in the differential targeting of the translocating γ subunit types. Because this domain is also post-translationally modified with different prenyl moieties (farnesyl or geranylgeranyl), these experiments also examined whether the prenyl moiety influences differential targeting. The C-terminal 14 residues of γ_9 were substituted with the corresponding sequence of the γ_{13} subunit (Fig. 2, *A* and *C*). This domain has been shown to be involved in receptor interaction previously (14, 19, 30, 31). Although γ_9 is farnesylated, the C-terminal residues of γ_{13} (-CTIL; Fig. 3*A*) will ensure that the chimera is geranylgeranylated in the cell as demonstrated before for such γ subunit mutants using high performance liquid chromatograph separation and mass spectrometry (35, 36). When the YFP-γ_9–γ_{13} chimera was expressed in M2-CHO cells and its receptor-mediated translocation was examined, it translocated predominantly to the Golgi complex (Fig. 2*C*). In a similar experiment, a YFP-γ_{13} chimera made up of the C-terminal 14 residues of γ_9 which will be farnesylated, was targeted predominantly to the ER (Fig. 2*C*). These results indicated that the targeting of the γ subunits to different membranes is not influenced by the C-terminal domain of the γ subunit. The results also show that the prenyl moiety does not play a role in the differential targeting.

Effect of Different α Subunit Types, Receptors, and Cell Types on Translocation—The α subunits of G proteins are the most diverse among the G protein subunit families and are known to be specific in their interaction with GPCRs (4). To evaluate the effect of different α subunits on receptor-mediated translocation of βγ complexes, we examined translocation in the pres-

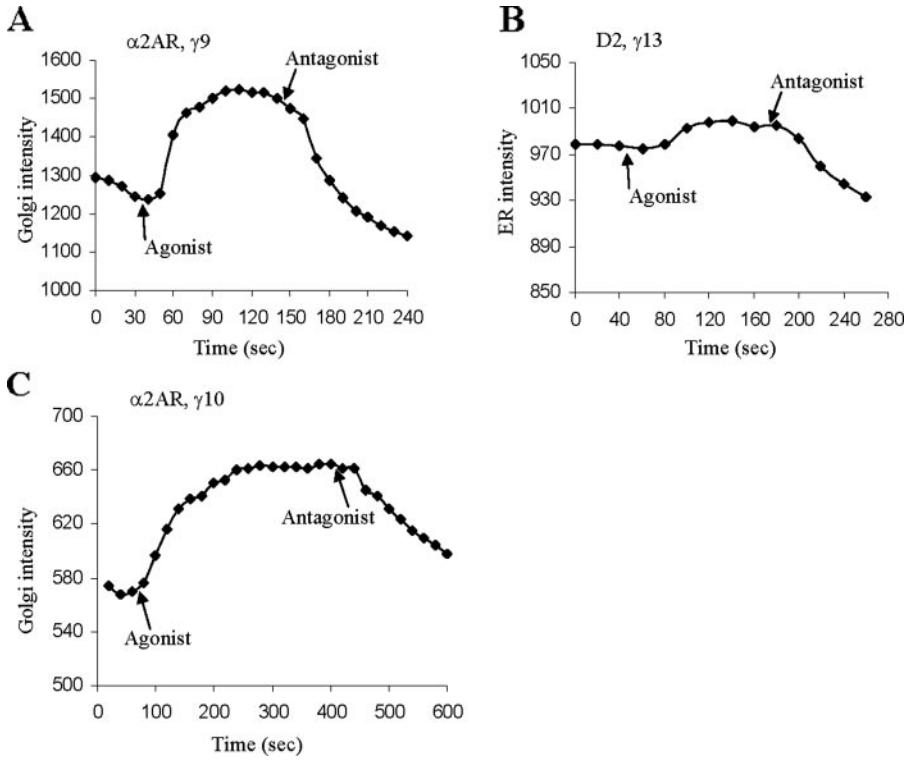


FIGURE 4. **Effect of different receptors on translocation of γ subunits.** Plots of emission intensities from the intracellular membranes (Golgi) as a function of time in CHO cells expressing different receptors as indicated to quantitate potential translocation of YFP-tagged γ subunits as indicated. Transfected cells were assayed for translocation, and the data are plotted as described in Fig. 1. Representative data are shown ($n \geq 4$). *A*, translocation plots in CHO cells transfected with α_2 adrenergic receptor (*α2AR*) tagged with CFP, α_o, mCh-β_1 and YFP-γ9 subunits. Transfected cells were exposed to 100 μM norepinephrine, and images were captured at the indicated time points followed by exposure to 20 μM yohimbine. *B*, translocation plots in CHO cells transfected with D2 dopamine receptor tagged with CFP, α_o, mCh-β_1 and YFP-γ_{13} subunits. Transfected cells were exposed to 10 μM quinpirole, and images were captured at indicated time points followed by exposure to 100 nM sulpiride. *C*, translocation plots in CHO cells transfected with α_2 adrenergic receptor tagged with CFP, α_o, mCh-β_1 and YFP-γ_{10} subunits. Transfected cells were exposed to 100 μM norepinephrine, and images were captured at the indicated time points followed by exposure to 20 μM yohimbine.

ence of α_q, which couples with M3 receptors (20). M3-CHO cells showed receptor-induced translocation of different γ subunits (Fig. 3). Similarly different GPCRs also induced βγ translocation (Fig. 4). Translocation occurred in a variety of cell lines (Fig. 5, *A* and *B*). γ_9 and γ_{11} translocation was also detected in COS 7 cells, and γ_{11} was detected in human lung epithelial cells (not shown). These results showed that receptor-induced βγ translocation is a general phenomenon. We then examined whether receptors endogenous to a cell induce translocation of γ_{13}, which is targeted to the ER. α2-adrenergic receptors endogenous to HeLa cells (11) induced the translocation of $\beta_1\gamma_{13}$ complexes (Fig. 5*C*), and hence, translocation is not a consequence of heterologous expression of a receptor.

Identification of the βγ Complexes Capable of Translocation in a Live Cell—To identify the β subunit types capable of forming a complex with the translocating γ subunits in a live cell, we used two approaches. We examined the cotranslocation of YFP or mCh-β subunit types in the presence of various translocating γ subunit types tagged with YFP or CFP. Secondly, we examined FRET between the various members of the translocating family of γ subunits and the four members of the β subunit family known to function as complexes with γ subunits. mCh- or YFP-tagged β subunits were used as acceptors, and YFP- or CFP-tagged γ subunits were used as donors. Acceptor photobleaching experiments were used to examine for the presence of a FRET signal as we and others have done (22, 37–39). We obtained a measurable FRET signal between some combinations of β and γ subunits but not all, indicating which combinations bound effectively with each other (supplemental Fig. 6 and Table 1). The cotranslocation of mCh-β subunit types was completely consistent with the results of the FRET experiments; only those γ subunits that provided FRET with a β subunit type induced co-translocation of that β subunit type. Together these experiments showed that β_1 and β_3 subunits form a complex with all the translocating γ subunits, whereas β_2 and β_4 form a complex with only γ_5, γ_9, and γ_{13} subunits (Table 1). We and others have shown selective association of G protein β and γ subunit types, and these results both emphasize such selectivity and also expand the examination of association between members of these families to the entire family of potential βγ complexes (40–44). Furthermore, the results show that even if a cell contains γ subunits that are capable of translocating, translocation of a βγ complex can occur only if the appropriate β subunit is expressed in that cell.

Translocation Process Is Controlled by Acylation and Is Likely Diffusion-mediated—Evaluation of the kinetics of translocation and reverse translocation of γ_1, γ_9, γ_{11}, and γ_{13} indicated that these subunits respond swiftly to both receptor-dependent agonist activation and antagonist inactivation as shown above (Fig. 1 and supplemental Fig. 1). This rapidity of translocation in both directions suggested that the translocation process is most likely diffusion-mediated. To examine whether the translocation was vesicle-mediated or diffusive, we observed the receptor-induced translocation of the γ_{11} subunit at 16 °C. At this temperature, vesicle-mediated transport is inhibited completely (45). Translocation induced by M2 receptor activation was unaffected by lowering the temperature (data not shown), indicating that the translocation is most likely diffusion-mediated. To further confirm that the translocation is independent of vesicle-mediated trafficking, translocation of YFP-γ_{11} was examined in M2-CHO cells after treating them with a microtubule disrupting agent, nocodazole, which blocks vesicular trafficking occurring through Golgi, and also monensin, which blocks the trafficking of proteins from the Golgi to PM (26). Each of these treatments did not have an effect on βγ translo-

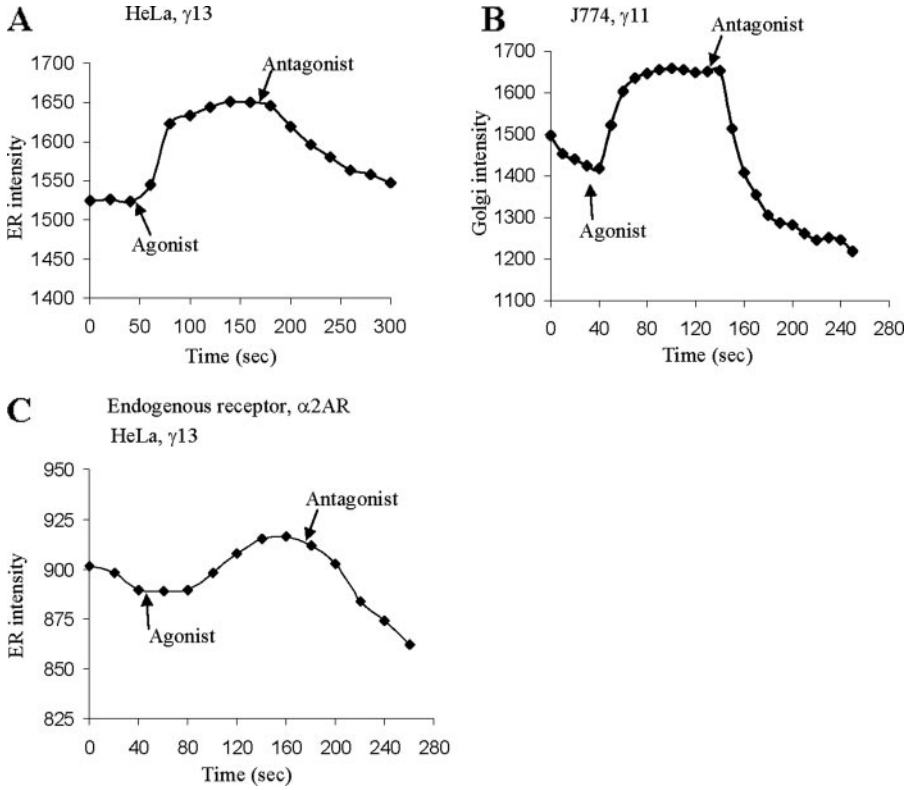


FIGURE 5. **Translocation of different γ subunits in different cell lines.** Plots of emission intensities from the intracellular membranes (Golgi or ER) as a function of time. Different cell lines (as indicated) were transfected with M3 receptor, α_q, β_1, and YFP-tagged γ subunits as indicated to quantitate potential translocation. Transfected cells were assayed for translocation, and the data are plotted as described in Fig. 1. Representative data are shown ($n \geq 4$). *A*, translocation plots of γ_{13} in HeLa cells transfected with M3 receptor, α_q, mCh-β_1, and YFP-γ_{13} subunits. *B*, translocation plots of γ_{11} in J774 cells transfected with M2 receptor, α_o, mCh-β_1, and YFP-γ_{11} subunits. *C*, translocation plots of γ_{13} in HeLa cells transfected with α_o, mCh-β_1, and YFP-γ_{13} subunits stimulated through the endogenous α_2 adrenergic receptor (*α2AR*).

TABLE 1
Interaction between G protein β subunits and translocating γ subunits ($n \geq 4$)
Cotransl, cotranslocation; ND, not determined.

	β_1		β_2		β_3		β_4	
	Cotransl	FRET	Cotransl	FRET	Cotransl	FRET	Cotransl	FRET
γ_1	+	+	−	ND	+	+	−	ND
γ_5	+	ND	+	+	+	+	+	+
γ_9	+	+	+	ND	+	+	+	+
γ_{10}	+	+	−	ND	+	+	−	−
γ_{11}	+	+	−	−	+	ND	−	−
γ_{13}	+	+	+	ND	+	+	+	+

cation, confirming the earlier observation that the translocation process is most likely diffusion-mediated (not shown). Receptor-mediated translocation of the βγ subunits was also unaffected by inhibition of protein synthesis with cycloheximide (not shown). This result showed that the translocation process does not require newly synthesized proteins. Furthermore, we observed antagonist-mediated reverse translocation of βγ in the presence of monensin, showing that receptor-governed reverse translocation of the βγ complex to the PM is independent of forward vesicular trafficking of proteins from Golgi to PM. Although the trafficking pathway that determines G protein localization on the PM originating from the Golgi has been shown to be both vesicle-mediated and non-vesicle-mediated (46, 47), the results here show that, similar to forward translocation, reverse translocation is also likely to be entirely diffusion-mediated. These findings are consistent with a prediction that translocating membrane-binding proteins are likely to move rapidly back and forth through the cytosol diffusively (48).

We determined that shuttling of G protein subunits between the PM and endomembranes is likely regulated by an acylation/deacylation cycle because it is inhibited by 2BP (66), a well characterized inhibitor of palmitoyl transferases (49). When M2-CHO cells stably expressing α_o-CFP, β_1, and YFP-γ_{11} were exposed to 2BP and receptor-induced translocation was examined, translocation was significantly inhibited in most cells (Fig. 6). In addition, 2BP inhibited $\beta\gamma_{11}$ translocation by a different receptor, M3, in cells coexpressing α_q (not shown). A FRET signal was detected in the PM of the cells stably expressing α_o-CFP, β_1, and YFP-γ_{11}, and FRET was abrogated on receptor activation with an agonist showing that the G protein present on the PM in 2BP-treated cells was capable of getting activated (66). This result showed that activation of the G protein is not sufficient for translocation and an acylation dependent mechanism underlies this process.

DISCUSSION

Overall these results indicate that free βγ subunits generated as a result of G protein activation by a GPCR are directed to endomembranes from the PM. This spatial dislocation of activated G protein molecules uncovers an unanticipated process in cellular regulation mediated by G proteins. As shown in the Chisari *et al.* (66), the inactive heterotrimeric G proteins shuttle between the PM and endomembranes. Because heterotrimers are inactive, the shuttling will not have on effect on downstream effectors. The striking effect of 2BP on both shuttling and translocation and the possible diffusion-dependent movement in both cases suggests that the shuttling process is harnessed for receptor-mediated translocation of βγ subunits. Thus, inactive heterotrimer shuttling is converted to the translocation of a potentially active βγ complex to specific endomembranes at different rates.

Two broad roles for the translocation of βγ subunits can be hypothesized. One possibility is that the translocation will reduce the βγ available to act on effectors and to support receptor activation of an α subunit. We predicted that the γ subtype constitution of a cell type can, thus, regulate signal amplification in a cell (13). Consistent with this prediction, a recent report shows that translocation of $\beta\gamma_1$, which is specific to rod

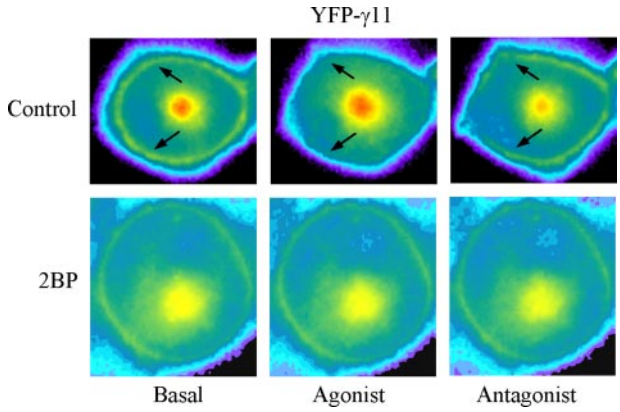


FIGURE 6. **Images of translocation of YFP-γ_{11} in the presence and absence of 2BP.** M2-CHO cells stably expressing α_o-CFP, β_1, and YFP-γ_{11} were treated with 2BP, and translocation was assayed as in Fig. 1 under "Materials and Methods" ($n \geq 10$).

photoreceptors, helps photoreceptors adapt to light by reducing the G_t available for activation by rhodopsin (50). The mechanisms at the basis of the translocation are, however, quite distinct from the translocation detected here. The translocation of γ_1 occurs along with the α_t rod subunit, is comparatively very slow ($t_{½}$), and has been shown to be dependent on the nature of the prenyl moiety. Translocation occurs only when the γ_1 subunit is modified with farnesyl but not when it is modified with geranylgeranyl. A related possibility is that βγ translocation can regulate unwanted cross-talk. In a variety of cell types, G protein activation leads to activation of a Gα-mediated pathway but not the βγ-mediated pathway. In heart cells Gs activates adenylyl cyclase (through the α_s subunit) but not K^+ channels, although βγ is released and is known to be capable of acting on the channel (51). A second potential function for the translocated βγ subunits is regulation of unknown effectors in the Golgi or ER. Recent reports show that Ras isoforms are present in endomembranes and are capable of getting activated at that location (52). There is extensive evidence of biologically significant cross-talk between GPCR and receptor-tyrosine kinase-mediated pathways (53). It is known that one mechanism for the activation of Ras and MAPK by the G proteins is through the βγ complex (54). Although there are suggestions that βγ regulation of Ras activity may occur through transactivation of a receptor-tyrosine kinase (55) or through direct action on Ras through Ras guanine nucleotide exchange factors (56), the mechanisms at the basis of GPCR and receptor-tyrosine kinase pathways is still unclear, especially in intact cells. The GPCR-mediated translocation of a variety of βγ complexes to endomembranes provides a mechanism for regulating Ras activity directly or indirectly. Additionally and importantly, the translocated βγ may act on proteins that regulate trafficking of proteins through the endomembrane system. G protein subunits have been shown to be associated with the Golgi complex, and evidence has been presented to show that they are capable of regulating trafficking of proteins through the Golgi (57–62), but there has been little direct evidence indicating that these functions are executed in a regulated manner (61). It has not been clear whether the G protein subunits identified remain transiently in the endomembranes on their way to the plasma membrane or are resident proteins of the endomembrane system. Mechanisms that would allow these subunits to associate with endomembranes and function there in a controllable manner have not been found. Such a mechanism is provided by the constant testing of the surfaces of the PM and intracellular membranes by a G protein (66), facilitating the translocation of a potentially active βγ complex on receptor stimulation. The large family of γ subunits with diverse sequences confers spatiotemporal complexity to this unanticipated signaling mechanism. βγ complexes are targeted differentially to intracellular membranes, $\beta\gamma_{13}$ predominantly to the ER and the others predominantly to the Golgi. The temporal kinetics of translocation differs; $\beta\gamma_1$, $\beta\gamma_9$, $\beta\gamma_{11}$, and $\beta\gamma_{13}$ are rapid, $\beta\gamma_5$ and $\beta\gamma_{10}$ are slow, and the remaining βγ subunits do not translocate. Although the existence of a large family of mammalian G protein γ subunits has been known for a long time (2, 63, 64), such striking distinctions in properties among members of the family have not previously been identified. The ability to alter a non-translocating subunit to a translocating subunit by directed mutagenesis in a domain previously shown to interact with receptors further emphasizes the importance of the differences in the primary structures. It is also consistent with the high degree of evolutionary conservation of the γ subunit-type amino acid sequences (12, 65). To our knowledge this is the only instance of activation-dependent reversible translocation of a family of proteins between the PM and endomembranes.

REFERENCES

1. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56,** 615–649
2. Simon, M. I., Strathmann, M. P., and Gautam, N. (1991) *Science* **252,** 802–808
3. Neves, S. R., Ram, P. T., and Iyengar, R. (2002) *Science* **296,** 1636–1639
4. Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R., and Hamm, H. E. (2003) *Endocr. Rev.* **24,** 765–781
5. Kisselev, O., and Gautam, N. (1993) *J. Biol. Chem.* **268,** 24519–24522
6. Hou, Y., Azpiazu, I., Smrcka, A., and Gautam, N. (2000) *J. Biol. Chem.* **275,** 38961–38964
7. Hou, Y., Chang, V., Capper, A. B., Taussig, R., and Gautam, N. (2001) *J. Biol. Chem.* **276,** 19982–19988
8. McIntire, W. E., MacCleery, G., and Garrison, J. C. (2001) *J. Biol. Chem.* **276,** 15801–15809
9. Wang, Q., Jolly, J. P., Surmeier, J. D., Mullah, B. M., Lidow, M. S., Bergson, C. M., and Robishaw, J. D. (2001) *J. Biol. Chem.* **276,** 39386–39393
10. Lim, W. K., Myung, C. S., Garrison, J. C., and Neubig, R. R. (2001) *Biochemistry* **40,** 10532–10541
11. Gibson, S. K., and Gilman, A. G. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 212–217
12. Yang, W., and Hildebrandt, J. D. (2006) *Cell. Signal.* **18,** 194–201
13. Akgoz, M., Kalyanaraman, V., and Gautam, N. (2004) *J. Biol. Chem.* **279,** 51541–51544
14. Gautam, N. (2003) *Structure* (*Camb.*) **11,** 359–360
15. Downes, G. B., Copeland, N. G., Jenkins, N. A., and Gautam, N. (1998) *Genomics* **53,** 220–230
16. Robishaw, J. D., and Berlot, C. H. (2004) *Curr. Opin. Cell Biol.* **16,** 206–209
17. Zhang, J., Campbell, R. E., Ting, A. Y., and Tsien, R. Y. (2002) *Nat. Rev. Mol. Cell Biol.* **3,** 906–918
18. Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E., and Tsien, R. Y. (2004) *Nat. Biotechnol.* **22,** 1567–1572
19. Azpiazu, I., Cruzblanca, H., Li, P., Linder, M., Zhuo, M., and Gautam, N. (1999) *J. Biol. Chem.* **274,** 35305–35308
20. Carroll, R. C., and Peralta, E. G. (1998) *EMBO J.* **17,** 3036–3044
21. Kenworthy, A. K. (2006) *Methods* **40,** 198–205
22. Azpiazu, I., and Gautam, N. (2004) *J. Biol. Chem.* **279,** 27709–27718

23. Escriba, P. V., Wedegaertner, P. B., Goni, F. M., and Vogler, O. (2007) *Biochim. Biophys. Acta* **1768,** 836–852
24. Storrie, B., White, J., Rottger, S., Stelzer, E. H., Suganuma, T., and Nilsson, T. (1998) *J. Cell Biol.* **143,** 1505–1521
25. Chiu, V. K., Bivona, T., Hach, A., Sajous, J. B., Silletti, J., Wiener, H., Johnson, R. L., Jr., Cox, A. D., and Philips, M. R. (2002) *Nat. Cell Biol.* **4,** 343–350
26. Dinter, A., and Berger, E. G. (1998) *Histochem. Cell Biol.* **109,** 571–590
27. Pelham, H. R. (1990) *Trends Biochem. Sci.* **15,** 483–486
28. Lewis, M. J., and Pelham, H. R. (1990) *Nature* **348,** 162–163
29. Sbalzarini, I. F., Mezzacasa, A., Helenius, A., and Koumoutsakos, P. (2005) *Biophys. J.* **89,** 1482–1492
30. Kisselev, O., Pronin, A., Ermolaeva, M., and Gautam, N. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 9102–9106
31. Kisselev, O. G., Ermolaeva, M. V., and Gautam, N. (1994) *J. Biol. Chem.* **269,** 21399–21402
32. Akgoz, M., Kalyanaraman, V., and Gautam, N. (2006) *Cell. Signal.* **18,** 1758–1768
33. Azpiazu, I., and Gautam, N. (2001) *J. Biol. Chem.* **276,** 41742–41747
34. Kisselev, O. G., and Downs, M. A. (2003) *Structure* (*Camb.*) **11,** 367–373
35. Lindorfer, M. A., Sherman, N. E., Woodfork, K. A., Fletcher, J. E., Hunt, D. F., and Garrison, J. C. (1996) *J. Biol. Chem.* **271,** 18582–18587
36. Akgoz, M., Azpiazu, I., Kalyanaraman, V., and Gautam, N. (2002) *J. Biol. Chem.* **277,** 19573–19578
37. Miyawaki, A., and Tsien, R. Y. (2000) *Methods Enzymol.* **327,** 472–500
38. Mochizuki, N., Yamashita, S., Kurokawa, K., Ohba, Y., Nagai, T., Miyawaki, A., and Matsuda, M. (2001) *Nature* **411,** 1065–1068
39. Sekar, R. B., and Periasamy, A. (2003) *J. Cell Biol.* **160,** 629–633
40. Pronin, A. N., and Gautam, N. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6220–6224
41. Schmidt, C. J., Thomas, T. C., Levine, M. A., and Neer, E. J. (1992) *J. Biol. Chem.* **267,** 13807–13810
42. Yan, K., Kalyanaraman, V., and Gautam, N. (1996) *J. Biol. Chem.* **271,** 7141–7146
43. Hynes, T. R., Tang, L., Mervine, S. M., Sabo, J. L., Yost, E. A., Devreotes, P. N., and Berlot, C. H. (2004) *J. Biol. Chem.* **279,** 30279–30286
44. Dingus, J., Wells, C. A., Campbell, L., Cleator, J. H., Robinson, K., and Hildebrandt, J. D. (2005) *Biochemistry* **44,** 11882–11890
45. Punnonen, E. L., Ryhanen, K., and Marjomaki, V. S. (1998) *Eur. J. Cell Biol.* **75,** 344–352
46. Michaelson, D., Ahearn, I., Bergo, M., Young, S., and Philips, M. (2002) *Mol. Biol. Cell* **13,** 3294–3302
47. Takida, S., and Wedegaertner, P. B. (2004) *FEBS Lett.* **567,** 209–213
48. Teruel, M. N., and Meyer, T. (2000) *Cell* **103,** 181–184
49. Resh, M. D. (2006) *Methods* **40,** 191–197
50. Kassai, H., Aiba, A., Nakao, K., Nakamura, K., Katsuki, M., Xiong, W. H., Yau, K. W., Imai, H., Shichida, Y., Satomi, Y., Takao, T., Okano, T., and Fukada, Y. (2005) *Neuron* **47,** 529–539
51. Clapham, D. E., and Neer, E. J. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37,** 167–203
52. Bivona, T. G., Perez De Castro, I., Ahearn, I. M., Grana, T. M., Chiu, V. K., Lockyer, P. J., Cullen, P. J., Pellicer, A., Cox, A. D., and Philips, M. R. (2003) *Nature* **424,** 694–698
53. Luttrell, L. M. (2005) *J. Mol. Neurosci.* **26,** 253–264
54. Gutkind, J. S. (1998) *J. Biol. Chem.* **273,** 1839–1842
55. Luttrell, L. M. (2003) *J. Mol. Endocrinol.* **30,** 117–126
56. Mattingly, R. R., and Macara, I. G. (1996) *Nature* **382,** 268–272
57. Ercolani, L., Stow, J. L., Boyle, J. F., Holtzman, E. J., Lin, H., Grove, J. R., and Ausiello, D. A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4635–4639
58. Leyte, A., Barr, F. A., Kehlenbach, R. H., and Huttner, W. B. (1992) *EMBO J.* **11,** 4795–4804
59. Pimplikar, S. W., and Simons, K. (1993) *Nature* **362,** 456–458
60. Stow, J. L., and Heimann, K. (1998) *Biochim. Biophys. Acta* **1404,** 161–171
61. Le-Niculescu, H., Niesman, I., Fischer, T., DeVries, L., and Farquhar, M. G. (2005) *J. Biol. Chem.* **280,** 22012–22020
62. Sato, M., Blumer, J. B., Simon, V., and Lanier, S. M. (2006) *Annu. Rev. Pharmacol. Toxicol.* **46,** 151–187
63. Gautam, N., Downes, G. B., Yan, K., and Kisselev, O. (1998) *Cell. Signal.* **10,** 447–455
64. Hildebrandt, J. D. (1997) *Biochem. Pharmacol.* **54,** 325–339
65. Downes, G. B., and Gautam, N. (1999) *Genomics* **62,** 544–552
66. Chisari, M., Saini, D. K., Kalyanaraman, V., and Gautam, N. (2007) *J. Biol. Chem.* **282,** 24092–24098