Seven Transmembrane Receptor Core Signaling Complexes Are Assembled Prior to Plasma Membrane Trafficking*†‡

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Much is known about β2-adrenergic receptor trafficking and internalization following prolonged agonist stimulation. However, less is known about outward trafficking of the β2-adrenergic receptor to the plasma membrane or the role that trafficking might play in the assembly of receptor signaling complexes, important for targeting, specificity, and rapidity of subsequent signaling events. Here, by using a combination of bioluminescence resonance energy transfer, bimolecular fluorescence complementation, and confocal microscopy, we evaluated the steps in the formation of the core receptor-G protein heterotrimer complex. By using dominant negative Rab and Sar GT Pases constructs, we demonstrate that receptor dimers and receptor-GFβy complexes initially associate in the endoplasmic reticulum, whereas Ga subunits are added to the complex during endoplasmic reticulum-Golgi transit. We also observed that G protein heterotrimers adopt different trafficking itineraries when expressed alone or with stoichiometric co-expression with receptor. Furthermore, deliberate mistargeting of specific components of these complexes leads to diversion of other members from their normal subcellular localization, confirming the role of these early interactions in targeting and formation of specific signaling complexes.

The regulation of heptahelical receptor (7TM-R)3 activity is intimately connected with receptor trafficking events that are important for decisions involving the internalization, recycling, or degradation of receptors. Although the signal termination processes have been extensively studied (1, 2), much less is known about receptor (or for that matter its signaling partners) ontogeny and intracellular trafficking toward the cell surface. Studies of mammalian olfactory receptors suggest that there are multiple trafficking checkpoints at the ER/Golgi boundary where signaling partners and chaperones could conceivably interact with receptors (3). The ER export step is probably the most important rate-limiting trafficking step in receptor biosynthesis, where early folding occurs (4). It is known that some receptor oligomers such as the GABAβ1, CCR5, and β2AR are assembled in the ER (5–8).

Individual Rab GT Pases govern discrete transport steps between organelles and along both endocytic and exocytic pathways (9–11). ER-to-Golgi transport is regulated by Rab1 and Rab2 (12–15); intra-Golgi transport requires Rab6 (16), and trans-Golgi-to-plasma membrane transport proceeds via Rab8 or Rab11 (17–19). Recent studies have demonstrated that the trafficking itinerary of angiotensin II receptor subtype 1 and β2-adrenergic receptors from the ER to the Golgi is regulated by a Rab1-dependent pathway (12, 13). The putative role of the ER-Golgi transport in G protein ontogeny remains controversial (20, 21). The Gβγ subunits of the heterotrimeric G protein localize to the ER when expressed alone, whereas co-expression with Ga leads to plasma membrane targeting of the heterotrimer (21–24). These results have suggested a model whereby Ga and βγ interact to form heterotrimers before trafficking to the cell surface, with the ER being critical for formation and processing of Gβγ. However, little is known about the role of trafficking in the assembly of 7TM-R signaling complexes and whether or not trafficking of these complexes depends on assembly as well. We have demonstrated recently that agonist-sensitive effector-G protein complexes are formed in the ER (25), suggesting that decisions regarding signaling specificity and complex formation are made early during biosynthesis.

In this study, we examined the reciprocal relationship between localization and interaction of the β2AR and its cognate heterotrimeric G protein partners. Using a novel combination of BRET and BiFC (26, 27), we demonstrate that co-expression of core G protein partners independently resulted in a different trafficking itinerary compared with when the members of the 7TM-R-G protein complex were expressed together.

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# The abbreviations used are: 7TM-R, heptahelical receptor; β2AR, β2-adrenergic receptor; ER, endoplasmic reticulum; BRET, bioluminescence resonance energy transfer; BiFC, bimolecular fluorescence complementation; PBS, phosphate-buffered saline; GFP, green fluorescent protein; HA, hemagglutinin; YFP, yellow fluorescent protein; CYP, 12R-cyclopindolol; WT, wild type; DN, dominant negative; GABAβ, γ-aminobutyric acid, type B; FWD, forward; RVS, reverse; VSV, vesicular stomatitis virus.
EXPERIMENTAL PROCEDURES

Reagents were obtained from the following sources: Dulbecco’s modified Eagle’s medium high glucose and Lipofectamine 2000 transfection reagent were from Invitrogen; fetal bovine serum, anti-FLAG M2 monoclonal antibody, and protein A-Sepharose were from Sigma; Alexa-Fluor 555 goat anti-mouse IgG and Alexa-Fluor 647 goat anti-rabbit IgG were from Molecular Probes (Eugene, OR); anti-VSV-g polyclonal antibody, monoclonal anti-HA raw ascites, and anti-Myc 9E10 were from Bio/Can Scientific (Etobicoke, Ontario, Canada); rabbit polyclonal anti-GFP was from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal anti-Refilina luciferase antibody, coelenterazine H, and coelenterazine 400a were from Cedarlane (Hornby, Ontario, Canada).

**Constructs**—Constructs encoding β2AR, β2AR-RLuc, β2AR-GFP10, Ga11-GFP10, Ga12-GFP10, GFP-G2, RLuc-G2, Ga2, and Ga3 were used as described previously (28, 29). For rat Ga11-RLuc, the RLuc moiety was introduced between Leu-91 and Lys-92 in the loop connecting helices A and B analogous to a YFP fusion protein that has been described previously (30). This construct was a generous gift from Dr. Céline Galés (Université de Montréal). All constructs used for BRET were shown to be functional (25, 28, 29). The Ga11-EE, FLAG-Gβ1, HA-Gγ2, Rab1α, -2α, -6α, and -11α clones were obtained from the UMR cDNA Resource Center.

**Rab Constructs**—Rab1 S25N, Rab2 S20N, and Rab11 S25N were amplified by PCR with the following: BHG RVS primer (5’-TAGAAGGCACAGTCGAGG-3’); Rab1 S25NFWD (5’-AGCTCGGATCCACCATGTCCAGCATGAATCCCGAATATGATGAT-3’); and Rab2 S20NFWD (5’-AGCTCGGATCCACCATGTCCAGCATGAATCCCGAATATGATGAT-3’). Rab11 S25NFWD (5’-AGCTCGGATCCACCATGTCCAGCATGAATCCCGAATATGATGAT-3’); Rab2 S20NFWD (5’-AGCTCGGATCCACCATGTCCAGCATGAATCCCGAATATGATGAT-3’); and Rab11 S25NFWD (5’-AGCTCGGATCCACCATGTCCAGCATGAATCCCGAATATGATGAT-3’). The different Rab constructs (WT and mutant) were subcloned into a pcMV Myc vector using BamHI-Xhol. All constructs were confirmed by bidirectional sequencing.

**Cell Culture and Transfection**—HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium high glucose supplemented with 10% fetal bovine serum and transfected using Lipofectamine 2000 as per the manufacturer’s instructions. Cells were plated at a density of 3 × 10⁴ cells/well in 6-well plates. Experiments were carried out 24 h after transfection with the exception of co-immunoprecipitation experiments which were conducted 48 h after transfection.

**BRET**—HEK 293 cells were co-transfected with vectors expressing the GFP and RLuc fusion proteins. Twenty-four hours after transfection, cells were harvested and washed once with phosphate-buffered saline (PBS). The cells were then suspended in PBS+ (PBS + 0.1% glucose) and distributed into 96-well microplates (white Optiplate; PerkinElmer Life Sciences). Most experiments were conducted using the BRET² technology, using Coelenterazine 400a at a final concentration of 5 μM. Signals were collected on a Packard fusion instrument (PerkinElmer Life Sciences) using either 410/80- (luciferase) and 515/30-nm (RLuc) band pass filters for GFP10 constructs. BRET² was also used for constructs incorporating YFP. These experiments allowed us to combine a split YFP (BiFC (26, 27)) strategy to produce BRET with a third luciferase-tagged partner. The BRET¹-BiFC signal was determined by the ratio of the light emitted by the 450/58- (luciferase) and 535/25-nm band pass filters (YFP) using coelenterazine H as a substrate. Whether or not BRET occurred was determined by calculating the ratio of the light passed by the 515/30 filter to that passed by the 410/80 or 535/25 to the 450/58 filter. To avoid possible variations in the BRET signal resulting from fluctuation in the relative expression levels of the energy donor and acceptor, we designed transfection conditions to maintain constant GFP/RLuc expression ratios in each experimental set.

**Radioligand Binding Assay**—Twenty-four hours post-transfection, cells were assayed as described previously (31) to determine the number of β2-adrenergic receptors. Binding of the hydrophobic ligand [125I]-cyanoindol (CYP) and the hydrophilic ligand (–)-[3H]CGP12177 was used to determine the total number and cell surface receptors, respectively. Non-specific binding was determined by including 10 μM (–)-propranolol. These experiments (except for washing steps) were conducted at 37 °C. For cell surface binding, cells were incubated with the hydrosoluble ligand CGP12177 for 30 min, and for total binding cells were incubated with the membrane-permeant ligand CYP for 60 min and then filtered with a Brandel cell harvester. Raw data in the presence of each WT GTPase (mean ± S.E.) for three independent experiments conducted in triplicate for specific CYP binding (dpm) were as follows: Rab1, 93,095 ± 8375; Rab2, 95,356 ± 9144; Rab6, 96,406 ± 10125; Rab8, 93,790 ± 8694; Rab11, 92,806 ± 8592; and Sar1, 93,352 ± 7823. Data were analyzed by normalizing specific binding to 100% for each individual GTPase WT isoform for both surface and total receptor measurements. As an independent measure of total receptor levels, we also measured total luminescence or fluorescence of RLuc- and GFP-tagged receptor between WT and DN GTPase groups, and these were consistently similar as well (see supplemental Fig. 2). Statistical analyses of normalized data between WT and DN GTPase groups were performed using a one-tailed Student’s t test.

**Immunoprecipitation**—48 h after transfection, cells were washed with PBS and harvested. Samples were lysed in 0.8 ml of RIPA buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 2 μg/ml aprotonin, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 100 μM iodoacetamide and DNase I). Expression levels of various partners as well as the different WT and DN GTPase constructs were also verified by Western blotting using total cell lysates.

The lysate was solubilized by incubation at 4 °C for 30 min, pre-cleared with 50 μl of protein A-Sepharose beads at 4 °C for 1 h, and clarified by centrifugation at 14,000 rpm for 10 min.
The pre-cleared lysate was incubated with an anti-HA antibody for 30 min, and 50 μl of protein A-Sepharose beads was then added and the mixture incubated for 1 h. After extensive washing with RIPA buffer, the immunoprecipitated proteins were eluted from beads with 50 μl of SDS sample buffer and resolved by SDS-PAGE, and Western blots were performed either with anti-GFP (Invitrogen) or with anti-HA (Covance) antibodies. For analytical purposes, we compared the ratio of Goαs-GFP or β2AR-GFP10 co-precipitated with HA-tagged receptor normalizing the amount pulled down in the presence of WT Sar1 or H79G Sar1.

Confocal Microscopy—Twenty four hours post-transfection, HEK 293 cells were harvested and seeded on laminin-coated coverslips for 4 h at 37 °C. The cells were then fixed for 20 min in PBS, pH 7.4, containing 3% (w/v) paraformaldehyde, then washed three times in PBS, and incubated for 1 h at room temperature in PBS containing 2% normal goat serum plus 0.2% (v/v) Triton X-100. Excess serum was removed, and the cells were incubated overnight at 4 °C with primary antibody diluted in PBS containing 1% normal goat serum and 0.04% (v/v) Triton X-100. The coverslips were then washed with PBS, drained, and incubated for 1 h at room temperature with the appropriate secondary antibody. The coverslips were washed with PBS, drained, and mounted onto glass slides using a drop of 0.4% 1,4-diazabicyclo{2.2.2}octane/glycerol medium. Coverslips were fixed to the slides with nail polish.

Confocal microscopy was performed with an unmodified Zeiss LSM-510 system (with PMT detection) using a 63/1.4 oil Plan-Apochromat objective. GFPs (green) were excited at 488 nm with an argon laser, emitting fluorescence at either 510 or 525 nm depending on the GFP variant. Secondary antibodies were goat anti-mouse Alexa-Fluor 555-conjugated or goat anti-rabbit Alexa-Fluor 647-conjugated IgG. Alexa-Fluor 555 was excited with an HeNe1 laser at 543 nm, emitted fluorescence at 570 nm, and is represented in red. Alexa-Fluor 647 was excited with an HeNe2 laser at 633 nm, emitted fluorescence at 668 nm, and is represented in blue. For deconvolution, the three-dimensional image data were transferred to an AZ-10 work station (Azunis Technologies), and images were processed with Huygens 2 software with a theoretical point spread function run-ning on LINUX RedHat. Deconvoluted Z-stacks were transferred back into LSM-510 software for three-dimensional reconstruction. Control experiments omitting primary antibodies revealed absent or very low level background staining. Experiments with primary antibodies on nontransfected mammalian cells indicated no nonspecific staining.

Subcellular Fractionation—HEK 293 cells were transiently transfected with β2AR-RLuc. 48 h after transfection, the cells were washed three times with ice-cold PBS, scraped off flasks, and lysed with 300 μl of ice-cold hypotonic lysis buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 2 mM EGTA, 6 mM magnesium chloride, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM aprotinin, 1 mM benzamidine). The cell lysate was homogenized with 50 strokes of a Dounce homogenizer. Cellular debris and unlysed cells were removed by centrifuging at 1,000 × g for 5 min at 4 °C. The supernatant was collected, and sucrose was added to obtain a final concentration of 0.2 M. The discontinuous sucrose step gradient was made using the above hypotonic lysis buffer with the addition of sucrose at the following final molar concentrations: 0.5, 0.9, 1.2, 1.35, and 1.5. Each step in the gradient had a total volume of 400 μl. The samples were centrifuged for 2 h at 35,000 rpm in a TLS55 rotor using an OptimaMAX tabletop ultracentrifuge (Beckman). A total of six fractions of 400 μl each were collected from the top of the tube. To monitor the cellular distribution of the β2AR-AR-Luc, Renilla luciferase activity was measured in 100 μl of each fraction using 5 μM coelenterazine H as a substrate. The efficiency of the sucrose gradient fractionation to resolve the plasma membrane from ER fractions was verified by immunoblotting with anti-calnexin antibodies (StressGen Biotechnologies).

RESULTS

Characterization of Receptor/G Protein Interactions Using BRET—Before undertaking an analysis of the role of receptor trafficking in the formation of receptor signaling complexes, we first characterized the interactions between the β2AR and the Gi heterotrimer using BRET. As shown in Fig. 1c (see also supplemental Fig. 1), we detected interactions described previously between receptors in a homodimer (32), receptor, and Goαs (28, 33) and between receptor and Gβγ subunits (28, 33). It is clear that co-expression of Gβγ improves the interaction between receptor and Goαs (Fig. 1, a and b) consistent with previous results (28). As a negative control, CD4-RLuc was also tested for interaction with the β2AR-GFP10 to demonstrate the specificity of the receptor/receptor and subsequent receptor/G protein interactions. To more clearly see the effects on these and other interactions, we present the data as net BRET (with the negative controls subtracted from the total BRET signal). However, we also present raw BRET values to demonstrate how net BRET values are calculated (see supplemental Fig. 1 and in the text where applicable). For BRET experiments to accurately reflect a given protein/protein interaction in a living cell, the interaction must be as follows: 1) saturable in the sense that increasing the amount of the acceptor (GFP-tagged partner) will eventually result in transfer of all available energy from the donor (luciferase-tagged partner) and 2) be competed by untagged versions of the partners. All three interactions are saturable (Fig. 1b), and increasing amounts of “cold” Goαs can compete for the BRET interaction between β2AR-RLuc and Goαs-GFP but not β2AR-AR-Luc/β2AR-GFP (Fig. 1c). In contrast, nonspecific interactions such as we show between CD4-RLuc and either β2AR-GFP, Goαs-GFP, and Gβγ-GFP or between soluble RLuc alone and these GFP-tagged constructs possess neither of these qualities even when the proteins have been shown to co-localize (i.e. using CD4-RLuc for membrane localized events and RLuc for cytosolic events), which makes them useful as negative controls.

Trafficking Itinerary of β2AR Determined Using Dominant Negative Rab1 and Sar1 Isoforms—Confocal microscopy was performed on HEK 293 cells expressing the β2AR labeled with GFP (β2AR-GFP10), and the receptor trafficking itinerary was visualized in the presence of either wild type (WT) or dominant negative (DN) Rab1/Sar1 GTPases. The receptor itinerary appears to be dependent on both Sar1 (ER-localized) and Rab1 (ER/ER-Golgi intermediate compartment), because DN isoforms of these proteins were able to reduce export of the recep-
tor to the cell surface, as demonstrated by the intracellular colocalization of the receptor with particular dominant negative GTPase isoforms (Fig. 2, a–f). These experiments also served to demonstrate that expression levels of the various wild type and dominant negative GTPases were more or less constant from experiment to experiment.

FIGURE 1. Initial characterization of receptor-Goβγ interactions. a, HEK 293 cells were transfected with recombinant plasmids to express β2AR-RLuc and β2AR-GFP and GFP-Gγ2 or Goxs-GFP. In addition, cells expressed untagged Gβ1 and/or Gγ2 as indicated. CD4-RLuc/β2AR-GFP interactions were used as a negative control in all cases and subtracted off as described under “Experimental Procedures” and shown in supplemental Fig. 1. b, representative BRET saturation experiments using a constant background of β2AR-RLuc with increasing amounts of cDNAs coding (leading to the ratios shown on the x axis of each curve) for β2AR-GFP (left panel), GFP-Gγ2 (with co-transfected Gβ1, middle panel), and Goxs-GFP (with co-transfected Gβ1γ2, right panel). c, competition of BRET between β2AR-RLuc and β2AR-GFP or β2AR-RLuc and Goxs-GFP with increasing amounts of untagged Gαs diminishes receptor/G protein interaction as measured using BRET. Data in a and c are expressed as mean ± S.E. of at least three different experiments. * indicates p < 0.05 compared with controls using a one-tailed Student’s t test.
To confirm results obtained using confocal microscopy, we also evaluated the effects of the different WT and DN GTPase isoforms on cell surface and total β2AR binding. The results show that the DN Rab1 (S25N and N124I) reduced cell surface binding by 63 ± 4% (p = 0.0001) and 61 ± 6% (p = 0.0013) compared with cells expressing WT Rab1 (Fig. 2g). Sar1 T39N and Sar1 H79G DN GTPases also inhibited binding of the hydrophilic ligand (125I)cyanopindolol. A membrane-permeable ligand (125I-cyanopindolol) assessed the total cellular pool of β2AR. Fig. 2h shows that the co-expression of the DN Rab1 or Sar1 did not affect total receptor number. Thus, our results demonstrate that co-expression of DN Rab1/Sar1 affects the relative distribution of the β2AR rather than the level of expression per se. The fact that total receptor levels remain unaltered also indicated that folding of the receptors into functional conformations is not affected by the DN GTPases. These results were further supported by sucrose density gradient centrifugation experiments used to enrich plasma membrane and ER fractions. ER fractions were enriched in the chaperone, calnexin (Fig. 2i, inset). Cells expressing β2AR-AR-Luc with either Rab1 or Sar1 WT or DN versions were fractionated and luciferase levels measured by an enzymatic assay. In the presence of either WT Rab1 or Sar1, there were significant amounts of β2AR-AR-Luc in the calnexin-free fractions, although this proportion decreased dramatically in the presence of either Rab1 N124I or Sar1 H79G (Fig. 2, i and j).

Interactions among 7TM-R Signaling Partners Are Differentially Affected by Alterations in Receptor Trafficking Itinerary—The precise localization of receptor signaling complex formation remains obscure, but the existence of constitutive and stable receptor-G protein complexes suggests that it may occur before plasma membrane targeting. HeterotrimERIC G proteins mediate a large part (but not all) of receptor-dependent signal- ing. We assessed the interaction of the heterotrimeric G protein with the receptor using BRET, after altering receptor distribution in different intracellular compartments using the DN GTPases. First, to establish the validity of our approach, we confirm as described previously (8) that β2AR homodimers are assembled intracellularly, more specifically in the ER. Our results demonstrate that none of the GTPases regulating export from ER to Golgi affect formation of homodimers between β2AR-AR-Luc and β2AR-AR-GFP10, even if they inhibit receptor trafficking to the cell surface (Fig. 3a). We next verified the relatively stable interaction between Gβ and Gγ subunits. We measured BRET between GFP10-Gβ1 and RLuc-Gγ2. Our results demonstrate that the interaction between Gβ1 and Gγ2 was also unaffected by the different GTPases mediating export from ER to Golgi (Fig. 3a), which is consistent with published reports (20). We were also interested in determining whether the interaction between the receptor and Gβγ (28) was an early event in the maturation of receptor signaling complexes. Using the same tools, we measured BRET between β2AR-AR-Luc and GFP10-Gγ2, in the presence of stoichiometric amounts of co-expressed Gβ1. Again, the DN isoforms of Sar1 and Rab1 GTPases had no effect on the interaction between the receptor and Gβγ (Fig. 3a) indicating that this interaction occurs initially in the ER as well.

A functional receptor-G protein complex could not be complete without the presence of a Ga subunit. Therefore, we next measured interactions between the β2AR and the two Ga subunits to which it is primarily coupled Ga11 and Ga12. Fig. 3b shows that in the absence of co-expressed Gβγ subunits, the weak interactions between receptor and Go were insensitive to the DN GTPases. Co-expression of Gβγ subunits considerably augments the BRET between the receptor and Gaαo, and the interaction was now quite sensitive to the presence of Sar1 H79G and Rab1 N124I DN GTPases. The interaction was almost completely reduced to ratios seen in the absence of co-expressed Gβγ subunits, respectively, compared with the presence of WT Rab1 or Sar1. Curiously, Rab1 S25N and Sar1 T39N DN GTPases had no effect on the interaction between the receptor and Ga subunit despite their effects on β2AR trafficking (Fig. 3b). This suggests that the interaction between receptor and Ga takes place after the formation of the ER export domain but before final fusion with the Golgi apparatus (34), which is to say that they are differentially affected by GTPase mutants that mimic either the GTP-bound state (i.e. Sar1 H79G and Rab1 N124I) or the GDP-bound state (Rab1 S25N and Sar1 T39N). The GDP- and GTP-bound GTPases play distinct roles in vesicle formation, budding, and targeting. Our data also suggests that both Sar1 and Rab1 (in addition to the role of the latter in vesicle targeting) play a role in vesicle budding, which is blocked by their respective DN isoforms that render them incapable of hydrolyzing GTP (35, 36).

As for Gaα, the interaction between the receptor and Gaα11 (to which it is also coupled (37)) was also increased upon co-expression of Gβγ subunits (Fig. 3c). The effect of DN GTPases on the formation of the receptor-Gα11 complex was also seen only in the presence of Gβγ subunits. When the β2AR-AR-Luc and Gaα11-GFP10 were co-expressed with Gβγ subunits, the interaction was diminished in the presence of Sar1 H79G or Rab1 N124I DN GTPases, in comparison to that seen with WT GTPase isoforms. Once again, the effects of either Sar1 T39N or Rab1 S25N were not significantly different from their respective WT GTPases. Also, similar results were obtained when the BRET tags were reversed to obtain Gaα11-RLuc and β2AR-GFP10, confirming the validity and specificity of the interaction (i.e. their independence of the relative position of BRET donor and acceptor tags; see Fig. 3c). Once again, increasing amounts of cold Gaα can compete for the BRET interaction between β2AR-AR-Luc and Goai-GFP but not β2AR-AR-Luc/β2AR-GFP (Fig. 3d), indicating the specificity of the BRET signal.

To confirm that reduced BRET signals were not a result of changes in expression levels of the two partners in different experiments, we also quantified total GFP fluorescence and luciferase activity independently (see supplemental Fig. 2, a and b). No significant differences in expression levels were detected for any of the relevant BRET pairs in any of the experiments we describe. To confirm this independently, we also demonstrate that total receptor levels remain unaltered in the presence of various WT and DN Rabs and Sar1 as measured by radioligand binding (Fig. 4, Fig. 6, and Fig. 7) and that Gaα-GFP and Gaα-GFP levels as determined by Western blotting were unaltered (supplemental Fig. 2c). Furthermore, we confirmed equivalent
Biosynthetic Events Important for 7TM-R Complex Assembly

Receptor  GTPase  Merge

a  
Sar1 WT

b  
Sar1 T39N

c  
Sar1 H79G

d  
Rab1 WT

e  
Rab1 N124I

f  
Rab1 S25N

g  
% of $^3$H-CP12177 surface binding

h  
% of $^{125}$I-CYP total cell binding

i  
Calnexin

j  
Relative Luminescence Units

- Sar1 WT
- Sar1 H79G

- Rab1 WT
- Rab1 N124I
amounts of other co-expressed proteins by Western blot or confocal microscopy (data not shown).

To provide independent verification of the interactions and their sensitivity to DN GTPase constructs, we used a co-immunoprecipitation approach. Fig. 4, a and c, show the co-immunoprecipitation of Gαi, in presence of Sar1 WT or DN H79G. The DN construct partially blocked the interaction between the β2AR and Gαi, although it had no effect on the β2AR-β2AR interaction (Fig. 4, b and d). Note also that comparable levels of receptor were immunoprecipitated in the presence of WT or DN Sar1, suggesting that levels of expression per se were not altered by DN Sar1 (Fig. 4, a and b).

Receptor/G Protein Interaction Regulates Their Individual Subcellular Trafficking Itineraries—By using a technique we recently developed (25) combining BRET and BiFC (26, 27), we further evaluated the trafficking itinerary of the G protein in the

**FIGURE 3.** Interactions among 7TM-R signaling partners are not affected by alterations in receptor trafficking itinerary. We assessed the interaction of various receptor signaling partners using BRET, after blocking anterograde transport at different steps using dominant negative Sar1 and Rab1 GTPases. α, β2AR-GFP/β2AR-RLuc; Gβi, GFP10/Gγ1, RLuc, and β2AR-RLuc/Gβ1, GFP. Note that BRET values are smaller in some cases than those presented in Fig. 1a but still fall on the curves presented in Fig. 1b suggesting that co-expression of additional proteins (i.e. the RabS) may reduce overall protein expression. b, the localization of the initial interaction between Gα and β2AR was assessed by using different WT and dominant negative GTPase constructs. In absence of Gβγ, little interaction is detected using BRET. Co-expression of Gβi, γ1 significantly augments the interaction between Gαi and β2AR and renders their interactions sensitive to DN Rab1 and Sar1. c, co-expression of Gβi, γ2 also significantly favors the interaction between Gαi and β2AR and renders their interactions sensitive to DN Rab1 and Sar1. d, similar competition experiments as shown in Fig. 1c with increasing amounts of untagged Gαi, diminished receptor/G protein interaction. CD4-RLuc/β2AR-GFP interactions were used as a negative control in all cases and subtracted off as described under "Experimental Procedures" and shown in supplemental Fig. 1. Data were expressed as mean ± S.E. of at least three independent experiments.

**FIGURE 2.** Trafficking itinerary of β2-adrenergic receptor determined using dominant negative Rab isoforms. a–f, deconvolved confocal microscopy images were taken from cells after co-expression of the β2AR-GFP and various GTPase constructs (WT or dominant negative) to assess their effects on the trafficking itinerary of the receptor. The left panel (green) represents the receptor-GFP fluorescence; the middle panel (red) represents the labeling of individual GTPase isoforms with an Alexa-Fluor 555 goat anti-mouse antibody, recognizing the monoclonal FLAG, VSV-g or Myc antibodies on the particular tagged GTPase constructs (FLAG-tagged Rab1 N124I, VSV-g-tagged Sar1 constructs, and various other Myc-tagged Rab1 constructs). Right panel (yellow) superimposes the two images that are representative of multiple experiments. Yellow arrows highlight the plasma membrane. When yellow and cyan arrows were used it was to highlight two cells (specifically for the Rab S25N and Sar T39N constructs) that expressed the receptor and the depicted Rab construct (yellow arrow) or not (cyan arrow). Similar levels of expression for WT and DN GTPases (here and those shown in Fig. 7) were also confirmed by Western blotting against c-Myc (mouse monoclonal) for Rabs 1, 2, 6, and 11, VSV-g (rabbit polyclonal) for Sar1 and FLAG (mouse monoclonal) for Rab8. Antibodies were used at 1:1000 dilutions (gel lanes were loaded with 50 μg of protein from cell lysates; data not shown). g, surface, and h, total cellular expression of β2AR in the presence of various WT or dominant negative constructs of Rab and Sar and GTPases. Cell surface receptor number was assessed using (−)3H)-Propranolol (125I-cyanopindolol binding, although total receptor number was measured using 125I-cyanopindolol binding. (−)-Propranolol was used to determine nonspecific binding in all cases. Data are expressed as means ± S.E. of at least three different experiments. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 compared with controls using a one-tailed Student’s t-test. i, subcellular fractionation of β2AR-RLuc in the presence of WT or N124I Rab1 using sucrose density gradient centrifugation. Enrichment of the ER marker, calnexin in heavier fractions, measured by Western blotting is shown in the inset. j, subcellular fractionation of β2AR-RLuc in the presence of WT or H79G Sar 1 using sucrose density gradient centrifugation. Data in i and j are representative of two separate experiments.
presence or absence of the β₂AR. BiFC/BRET permits measures of the interaction between three partners, where one BRET pair is formed by two proteins each containing half of GFP. We used constructs encoding the Gα₁ subunit, tagged with the first 158 amino acids of YFP, and Gα₂ tagged with the remaining C terminus of YFP (26) in combination with Gβ₁-RLuc. To obtain a BRET signal, the split YFP partners must associate and recreate functional YFP with which the RLuc construct will generate a BRET signal. We co-expressed the β₂AR with the G protein subunits along with the different GTPase constructs to determine the effect of receptor on the assembly of the G protein. Co-expression of the receptor (Fig. 5a, left panel) alters GTPase-insensitive assembly of the G protein (Fig. 5, a, right panel, and c) to a Sar1/Rab1-sensitive pathway, as for the β₂AR/Gα₁ interaction. We also evaluated using BRET whether the possible interaction between Gα and Gβγ subunits was altered by the presence of the receptor. Fig. 5b shows the effects of Sar1 and Rab1 on intra-G protein subunit interactions Gγ₂-RLuc and Gα₁-GFP₁₀ (with Gβ₂) in absence of the receptor. Neither this interaction nor Gγ₂-RLuc and Gβ₁-GFP₁₀ (Fig. 3a) were sensitive to the DN Sar1 or Rab1. These results suggest that G proteins can follow different trafficking itineraries resulting in the formation of heterotrimers in the presence (Rab1/Sar1-dependent) or absence (Rab1/Sar1-independent) of a co-expressed receptor. Although co-expression of the receptor has an effect on the assembly of the G protein heterotrimer, the modification of receptor trafficking does not alter the final localization of all Gα subunits. A certain proportion of Gα subunits (compared with the more severe impairment of receptor trafficking) is still found at the plasma membrane with either co-expression of WT Sar1 (Fig. 5c, top panel) or DN Sar1 H79G (Fig. 5c, bottom panel).

Of course, it is possible that the effects we have seen are generalized trafficking defects because of the DN GTPase constructs. HEK 293 cells used in our experiments remained similar in viability when transfected with either wild type or mutant Rabs (data not shown), and receptors were expressed to similar levels (see “Experimental Procedures”) arguing against this notion. However, to provide a second, independent strategy to result in mistargeted receptor-G protein complexes, we used a
number of G protein trafficking and interaction mutants. Using modified G protein subunits that are retained in the ER/Golgi compartment or mistargeted to mitochondria, we evaluated the role of the receptor-G protein interactions for plasma membrane targeting and localization (Fig. 6a). We first made use of G\(\alpha\)s and G\(\gamma\)2 subunits mistargeted to the mitochondria (22, 38). Co-expression with the \(\beta\)2AR diminished receptor cell surface binding as measured again with \(^{3}\text{H}\)CGP12177 binding by 21 \pm 9 (\(p = 0.003\)) and 25 \pm 11\% (\(p = 0.004\)), respectively. G\(\beta\)_\(\gamma\)18E (39), a mutant that disrupts (but does not entirely prevent entirely) the interaction between G\(\beta\) and G\(\gamma\), also partially blocked surface expression of the \(\beta\)2AR (30 \pm 18\% \(p = 0.049\)). Interestingly, another G\(\alpha\)\(\delta\) mutant, C3S, which cannot be palmitoylated and is subsequently retained intracellularly (20), also reduced cell surface receptor expression by 42 \pm 20\% (\(p = 0.035\)). No effect was seen for any of these treatments on total receptor levels (Fig. 6b). Although each of these constructs had an effect on receptor plasma membrane localization, there was an obligate requirement for expression of the heterotrimeric G protein to see maximal effects on receptor targeting. Expression of the various mistargeted or mutant G protein subunits was confirmed by Western blotting (Fig. 6c). These data indicate that mistargeting the G protein can lead to a mistargeting of the receptor, something that in principle should not occur if receptor and G protein traffic independently to the cell surface before interacting in response to agonist.

**Post-ER Effects on Receptor Trafficking and G Protein Interactions**—We next assessed the role of several other GTPases important for post-ER protein trafficking. These GTPases were selected for their particular subcellular localization and function to assess post-ER events and an alternative (Rab2) ER trafficking pathway to the Golgi apparatus. The receptor itinerary appears to be dependent on Sar1 (ER-localized) and Rab1 (ER/ER-Golgi intermediate compartment or ER/Golgi intermediate compartment-localized, as shown in Fig. 1) (13) but also on Rab6 (Golgi-localized), Rab8, and Rab11 (Golgi/plasma membrane-localized), because DN isoforms of these proteins were able to reduce export of the receptor to the cell surface, as demonstrated by the intracellular co-localization of the receptor with particular dominant negative GTPase isoforms (Fig. 7, a–h). The alternative ER/Golgi trafficking pathway involving Rab2 does not seem to be involved in anterograde \(\beta\)2AR trafficking, as no effect of DN Rab2 S20N GTPase was observed (Fig. 7, a and b). The data are again supported by comparing cell surface binding with total levels of receptor (Fig. 7, i and j). We also tested the interactions between different combinations of partners after blocking either the alternative ER to Golgi pathway dependent on Rab2 (Fig. 8a) or subsequent intra-Golgi traffic dependent on Rab6 (Fig. 8b) and Golgi to plasma membrane traffic dependent on Rab11 (Fig. 8c). Here, we show that none of these DN GTPases had any effect on the interactions we measured.

Taken together, our results suggest multiple steps in the assembly of the core receptor signaling complex, with G\(\alpha\) subunit association with receptor occurring last, perhaps to maintain the complex inactive until it reaches its final target destination.

**DISCUSSION**

We show here that the interaction of different components of \(\beta\)2AR signaling complexes, including receptor homodimers and \(\beta\)2AR/G\(\beta\)_\(\gamma\), occurs in or before targeting to the ER, whereas the subsequent \(\beta\)2AR/G\(\alpha\) interaction requires the presence of G\(\beta\)_\(\gamma\) and appears to occur later in the trafficking itinerary. We also demonstrate that independent expression of the core partners leads to distinct trafficking itineraries compared to when they are expressed together. Confirming previ-
Biosynthetic Events Important for 7TM-R Complex Assembly

![Graph](image)

**FIGURE 6.** Mistargeted G protein subunits lead to intracellular retention of receptor. *a,* trafficking-altered signaling components such as ER resident or mistargeted G protein subunits reduce surface receptor localization as measured by $[^{125}]$I-cyanopindolol binding. Data are expressed as means ± S.E. of at least three different experiments. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared with controls using a one-tailed Student's $t$ test. *b,* trafficking-altered signaling components do not affect total receptor levels as measured by $[^{125}]$I-cyanopindolol binding. Data are expressed as means ± S.E. of at least three different experiments. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared with controls using a one-tailed Student's $t$ test. *c,* expression of constructs used in a shown by Western blotting (IB). Images are representative of three independent experiments. *mito,* mitochondria.

**TABLE**

| Condition                  | % of $[^{125}]$I-CYP Total cell binding |
|----------------------------|------------------------------------------|
| No G protein               | 100                                      |
| G protein WT               | 75                                       |
| G protein mito             | 50                                       |
| G protein mito + G protein | 25                                       |

Although 7TM-R biosynthesis and transport toward the cell surface remains poorly characterized in general, ER exit was defined as a crucial step controlling their plasma membrane expression (4). Incompletely folded or misfolded proteins are retained in the ER before ultimately being targeted for degradation, although only correctly folded proteins transit out of the ER. The formation of oligomeric complexes represents an important step in ER quality control because it may mask retention sequences or hydrophobic patches that would otherwise result in protein retention (46). Although a general role for heterodimerization and/or homodimerization in 7TM-R quality control and ER export has not yet been established, several studies have demonstrated that 7TM-R dimerization occurs in the ER (8, 47–49). Dimerization of the $\beta{\gamma}$ complex is essentially blocked completely by modulating anterograde protein transport or by mistargeting the G protein. Our data suggest that the receptor makes a portion of heterotrimer formation sensitive to dominant negative Sar1/Rab1 because the receptor acts as a site for assembly of a specific receptor-G protein-effector complex, i.e., the receptor acts as a scaffold for a particular signaling system to form, and the formation and trafficking of these complexes are now sensitive to blockers of specific transport steps as well. Thus assembly and trafficking pathways exert mutual regulatory effects on one another.

Recently, it has been demonstrated that some 7TM-Rs traffic from the ER to the Golgi via a Rab1-dependent pathway (13), as we confirm here. However, the trafficking itinerary of 7TM-Rs after ER exit remain unclear. Our results suggest that Sar1,
Rab6, Rab8, and/or Rab11 are also important for correct plasma membrane localization of the βAR. Previous studies established these exocytic pathways as typical for a number of other proteins as well (16–18).

The interaction between the receptor and the Gα subunit appears to be dependent on co-assembly of receptor with Gβγ subunits. Gβ and Gγ subunits exist as a tightly bound complex, which can only be dissociated under denaturing conditions and which functions as a single entity throughout the signaling cycle. Gβ subunits are unstable in the absence of Gγ subunits (50). As for the Gα subunits, the Gβγ complex also requires heterotrimer formation, together with isoprenylation of the Gγ for efficient plasma membrane targeting (22). Much less well understood is the cellular trafficking pathway by which the G protein subunits reach the plasma membrane after synthesis and entry into the ER-Golgi complex. Gβγ remains ER-localized when expressed without Gα again corroborating the notion that heterotrimer formation is also a key event in trafficking of these complexes. Co-expression of Gα leads to strong plasma membrane localization of the heterotrimer (21–24). These results and others highlight G protein assembly events that occur before trafficking to the plasma membrane. A recent study has demonstrated that a protein that interacts with Gα and Gα at ER/Golgi exit sites, called GIV, may play a role in vesicle trafficking (51). We would suggest that it (or an analogous molecule) may act as a chaperone between Gα subunits and the nascent receptor-Gβγ complex. Here we demonstrate that the interaction of each subunit of the G protein is necessary for its assembly with the receptor but that trafficking of the G protein can be either dependent or independent of the receptor. Our results show that a fraction of G proteins can still traffic to the plasma membrane, even when the receptor is retained intracellularly, but that DN GTPases such as Rab1 N124I and Sar1 H79G block the interaction between the receptor and the Gγ subunit at ER export sites (34). Our results thus help reconcile certain discrepancies observed with respect to G protein trafficking (20, 21). G protein assembly and association with 7TM-Rs can be modulated by GTPases usually mediating trafficking of the receptor to the Golgi, but the trafficking itinerary of the G protein can also be totally independent of Sar1/Rab1/Rab2.

The idea that signal transduction occurs as the result of random collisions between receptors, G proteins, and effectors in a
cell containing multiple G protein signaling components is difficult to reconcile with the observed specificity and efficacy of signal transduction (52). These problems are eliminated in an alternative view where receptors with their cognate G proteins and effectors are organized into complexes, and there is a significant amount of both historical and more recent data to support this notion. Kinetic and biochemical data suggest that G proteins and their effectors form stable complexes (reviewed in Refs. 53 and 54). Most early evidence for existence of stable protein/protein interactions between G proteins and effectors came from in vitro biochemical studies (for example see Ref. 55 and for review see Ref. 56). The first proposal that G proteins and effectors existed as a complex in cell membranes was based on data indicating that signal transduction displays first order kinetics (53, 54, 57–62). It has become clear that 7TM-Rs interact constitutively and stably with their G protein partners (28, 52, 63) and that signaling can even proceed when Go and Gβ subunits were fused genetically in yeast (64). However, a recent study showing little basal FRET but a large agonist-stimulated signal between $\gamma_1$ and its cognate G protein challenges this view (65). We would contend that interpretation of the absence of a basal signal is difficult because the FRET/BRET pair may be in a nonpermissive orientation. There is also evidence for Gβγ-dependent interactions between receptors and effector molecules such as the Kir3.1 potassium channel, which by itself cannot exit from the ER (66). It is likely that other components of 7TM-R signaling pathways are also assembled before ultimate targeting events, including G protein/effecter interactions (29). Taken together, these findings modify the current view of 7TM-R signaling that posits mainly transient interactions at the cell surface between signaling partners in response to agonist stimulation. We can suggest that stable, pre-assembled complexes might be a way to ensure both specificity and efficiency of signaling. Our data argue that both spatial and temporal considerations determine the formation of specific signaling complexes in that trafficking decisions to some extent depend on assembly and vice versa.

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