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Homodimerization of the β2-Adrenergic Receptor as a Prerequisite for Cell Surface Targeting*

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Although homodimerization has been demonstrated for a large number of G protein-coupled receptors (GPCRs), no general role has been attributed to this process. Because it is known that oligomerization plays a key role in the quality control and endoplasmic reticulum (ER) export of many proteins, we sought to determine if homodimerization could play such a role in GPCR biogenesis. Using the β2-adrenergic receptor (β2AR) as a model, cell fractionation studies revealed that receptor homodimerization is an event occurring as early as the ER. Supporting the hypothesis that receptor homodimerization is involved in ER processing, β2AR mutants lacking an ER-export motif or harboring a heterologous ER-retention signal dimerized with the wild-type receptor and inhibited its trafficking to the cell surface. Finally, in addition to inhibiting receptor dimerization, disruption of the putative dimerization motif, ^{276}G*XXX*G*XXX*L^{284}, prevented normal trafficking of the receptor to the plasma membrane. Taken together, these data indicate that β2AR homodimerization plays an important role in ER export and cell surface targeting.

Recent studies have established that G protein-coupled receptors (GPCRs),[1] much like other membrane receptors, form both homodimers and heterodimers (1, 2). Various functions have been attributed to the dimerization between different receptor subtypes. For instance, heterodimerization has been proposed to promote the formation of receptors with unique pharmacological properties, contributing to the pharmacological diversity of GPCRs (3–5). Changes in G protein coupling specificity (6) as well as altered receptor endocytosis (3, 4, 6–9) have also been proposed to result from receptor heterodimerization. For the metabotropic GABAb receptor, heterodimerization between GABAb-R1 and GABAb-R2 is an obligatory step for cell surface expression of a functional receptor (10, 11). This was shown to result from the masking of an endoplasmic reticulum (ER) retention signal found in the C terminus of GABAb-R1, presumably through the formation of a coiled-coil domain between the C termini of the two receptor subtypes (12). The obligatory nature of this heterodimerization for cell surface trafficking and signaling was considered for a long time as a particularity of the GABAb receptor, which belongs to a GPCR subfamily (family 3) containing only a few members (13). However, a recent study indicated that the heterodimerization between two closely related members of family 1 GPCRs, the adrenergic α_{1D} and α_{1B} receptors, may be a prerequisite for the proper cell surface expression of the α_{1D} subtype in both experimental models and real tissues (14).

So far, despite the numerous examples of receptor homodimerization reported in the literature (15, 16), the biological function of this phenomenon has remained elusive, probably because it is extremely difficult to experimentally differentiate the dimer properties of identical receptor subtypes from those of monomers. However, a growing number of observations indicates that GPCR homodimerization is constitutive and that it occurs in the ER, suggesting that it might play a role in the quality control and ER export of receptors (17–19). The formation of either homodimers or heterodimers could thus represent a common prerequisite for GPCRs to be exported to the plasma membrane (PM). This hypothesis is supported by the fact that several other membrane proteins have been shown to require oligomerization for their proper maturation and cell surface targeting (20–22). In this study, we sought to determine if homodimerization could indeed play a role in GPCR biogenesis and trafficking.

Because β2AR homodimerization has been well characterized and tools have recently been developed to quantitatively investigate its dimerization in living cells (23–25), we chose to study this receptor as a model. To examine the potential role of β2AR dimerization in receptor trafficking, mutants targeting a putative dimerization interface (23) as well as mutants that are retained within intracellular compartments were used. Here, we report that inhibiting dimerization leads to ER retention of the β2AR. In addition, we show that mutants that are not targeted to the PM act as dominant negative mutants for cell surface expression of the wild-type (WT) receptor through dimerization. Taken with the observation that β2AR dimerization could occur as early as the ER, our data provide direct evidence linking GPCR dimerization to their export to the plasma membrane.

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; β_2AR, β_2 adrenergic receptor; BRET, bioluminescence resonance energy transfer: ER, endoplasmic reticulum; GFP, green fluorescent protein; PM, plasma membrane; *R*luc, *Renilla* luciferase; HA, hemagglutinin; CMV, cytomegalovirus; ELISA, enzyme-linked immunosorbent assay; TM, transmembrane domain; WT, wild type; GABAb, γ-aminobutyric acid type B.

MATERIALS AND METHODS

Cell Culture and Transfection—HEK293T cells were maintained under standard conditions in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and streptomycin, and 2 mM L-glutamine. Transfections were performed using a standard calcium phosphate precipitation protocol (26). Cells were harvested and used 48 h post-transfection.

DNA Constructs and Plasmids—The pcDNA3-HA-β2AR-*R*luc and pcDNA3-myc-β2AR-*R*luc were generated by subcloning the coding sequence of *Renilla* luciferase (*R*luc) from p*R*L-CMV-β2AR-*R*luc (24) into the pcDNA3-His-HA-β2AR (27) and pcDNA3-myc-β2AR (24) construct, respectively. Receptor constructs harboring different mutations in the putative dimerization motif Gly^{276}-X_3-Gly^{280}-X_3-Leu^{284}-X_3 (GGL) present in the 6th transmembrane (TM) domain of the β2AR (β2AR-Leu^{276}, β2AR-Leu^{280}, β2AR-Tyr^{284}, β2AR-Gly^{284}, β2AR-Leu^{276}-Leu^{280}, β2AR-Leu^{280}-Tyr^{284}, and β2AR-Leu^{276}-Leu^{280}-Tyr^{284}) were obtained by PCR-based mutagenesis using pcDNA3-HA-β2AR-*R*luc as template. To generate the GGL mutant receptor-GFP fusion plasmids, a DNA fragment containing the mutation(s) (amino acids 200–344) was subcloned in pcDNA3-His-β2AR-GFP (26). The pcDNA3-myc-β2AR-Ala^{332}-Ala^{336}-*R*luc was generated by PCR based substitution of the Phe^{332} and Phe^{336} by alanine residues using pcDNA3-myc-β2AR-*R*luc as template. The pcDNA3-myc-β2AR-Ala^{332}-Ala^{336}-GFP was then generated by subcloning the DNA fragment containing the F332A and F336A mutation (amino acids 200–344) into pcDNA3-myc-β2AR-GFP. The His-β2AR-*R*luc and His-β2AR-GFP plasmids were described in Mercier *et al.* (25), whereas myc-GABAb-R1-Rluc, myc-GABAb-R1-GFP, and HA-GABAb-R2-GFP were reported in Perroy *et al.* (28).

To replace the region encoding the C-terminal tail of the β2AR with that of the GABAb-R1b, a truncated coding region of the myc-β2AR (including the arginine residue at position 344) was amplified and subcloned into the pcDNA3.1 vector to generate pcDNA3.1-myc-β2AR-trunc. The C-terminal tail of the GABAb-R1b (starting at position Leu^{860}) fused to *R*luc was amplified from the pcDNA3.1-GABAb-R1b-*R*luc plasmid (19) and subcloned in-frame into the pcDNA3.1-myc-β2AR-trunc to generate the pcDNA3-myc-β2AR-$GBR1_{CT}$-*R*luc chimera.

The green fluorescent protein (GFP) chimera proteins were all made using the variant GFP^2 from PerkinElmer Life Sciences, and all PCR-amplified stretches of DNA were verified by automated sequencing. Plasmids and sequences are available upon request.

Luciferase Activity—Luminescence generated by the *R*luc constructs was determined using a LumiCount™ (Packard) following the addition of 5 μM of the luciferase substrate coelanterazine H (Molecular Probes).

Fluorescence Measurement—The total fluorescence was measured using a Packard FluoroCount™ with an excitation and emission filters set at 400 nm and 510 nm, respectively.

BRET Assay—The BRET assay used to detect GPCR dimerization was described previously (24, 25). Briefly, cells resuspended in phosphate-buffered saline/glucose 0.1% or fractionated cell lysates were distributed in 96-well microplates (white Optiplate from Packard Bioscience). The luciferase substrate, DeepBlueC™ (PerkinElmer Life Sciences), was added at a final concentration of 5 μM, and readings were performed using a TopCount NXT™ (Packard Bioscience), which allows the sequential integration of signals detected at 370–450 nm (luciferase) and 500–530 nm (GFP). BRET signals were calculated as the ratio of the light emitted at 500–530 nm by BRET acceptors (receptors fused to GFP), over the light emitted at 370–450 nm by BRET donors (receptors fused to *R*luc). In each experiment, BRET values were corrected by subtracting the background ratios obtained upon expression of the BRET donor constructs alone.

Subcellular Fractionation—Cells were lysed in 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) using a Dounce homogenizer. Cellular debris and unlysed cells were removed by centrifugation at 1,000 × *g* for 5 min at 4 °C. Sucrose was then added to the supernatants to obtain a final concentration of 0.2 M, and lysates were layered on top of a discontinuous sucrose step gradient (0.5, 0.9, 1.2, 1.35, 1.5, and 2.0 M). Samples were centrifuged for 16 h at 27,000 rpm in a Beckman SW28 rotor. Thirty-one fractions of 1 ml were collected from the top of the tubes. BRET was measured on 100-μl aliquots of each fraction. *R*luc activity was also measured in each fraction to monitor cellular distribution of *R*luc-fused receptors. Samples containing PM and ER were identified by immunoblotting using anti-Na/K-ATPase (A-276, Sigma) and anti-calnexin (SPA-860, Biotechnologies) antibodies, respectively.

Immunofluorescence Microscopy—Cell surface receptors were labeled with polyclonal anti-myc (A14, 1:100, Santa Cruz Biotechnology) and/or a monoclonal rat anti-HA (3F10, 1:100, Roche Applied Science) antibodies and appropriate secondary antibodies (anti-rabbit Alexa-596 and anti-rat Oregon green, Molecular Probes). For intracellular staining, cells were fixed and permeabilized with 0.15% Triton X-100 in phosphate-buffered saline supplemented with 3% bovine serum albumin. Samples were processed for epifluorescence microscopy on a Nikon inverted microscope Eclipse TE2000-U using a ×100 planapochromat immersion CFi60 objective and a Roper CoolSnap HQ™ 16 bits charge-coupled device camera for acquisitions. The overlay were generated with the Metamorph™ software. Co-localization experiments were carried out with a Leica confocal TCS-SP2 microscope using a PL APO ×63 1.4 immersion objective. The TGN46 polyclonal antibody (*trans*-Golgi marker) was from Serotec, and the polyclonal anti-calnexin antibody (C-20) was from Santa Cruz Biotechnology.

Quantitative Measurement of Cell Surface Receptors—Cell surface expression of HA-GPCR-*R*luc constructs was determined by ELISA using the monoclonal 12CA5 anti-HA antibody and the horseradish peroxidase-conjugated secondary anti-mouse antibody (Amersham Biosciences). Total receptor level was assessed in the same sample by measuring the *R*luc activity. The relative efficiency of cell surface targeting was deduced from the slope of the correlation between total receptor expression (*R*luc) and the corresponding cell surface signal (ELISA) obtained for various expression levels.

RESULTS

Subcellular Distribution of β2AR Dimers—Recent studies, based on co-immunoprecipitation and resonance energy transfer approaches, have revealed that many GPCRs form homodimers independently of receptor activation (15). To gain insight into the site of formation of the dimers and thus their potential role in receptor biology, the subcellular distribution of β2AR dimers, detected by bioluminescence resonance energy transfer (BRET), was studied after cell fractionation. For this purpose, His-β2AR-*R*luc was expressed alone or in combination with His-β2AR-GFP, and PM was resolved from ER by ultracentrifugation on a sucrose gradient (18, 19). The Na/K-ATPase and calnexin were used as markers for PM (fractions 7–15) and ER (fractions 19–27), respectively (data not shown). Furthermore, the ER-retained myc-GABAb-R1-*R*luc (12) was used as a specific marker of GPCR distribution in the ER fractions (Fig. 1). His-β2AR-*R*luc was mostly associated with the PM (fractions 9–13) but a small proportion also co-sedimented with the ER (fractions 19–25). A significant BRET between His-β2AR-*R*luc and His-β2AR-GFP was observed in both PM and ER fractions, indicating that dimers exist in both compartments. Because BRET is a ratiometric value, the comparable BRET observed in ER and PM indicates that the proportion of His-β2AR-*R*luc interacting with His-β2AR-GFP is similar in the two compartments. Receptor dimerization was also found to be specific and not a mere results of receptor overexpression, because no BRET was detected in any fraction when His-β2AR-*R*luc was co-expressed with the PM-exported isoform of the GABAb receptor (12) fused to GFP (HA-GABAb-R2-GFP). Our observations therefore suggest that specific β2AR dimerization can occur as early as the ER. However, one cannot exclude the possibility that, as a result of overexpression, the high ER concentration of receptors could favor specific dimerization in this compartment rather than later in the maturation pathway.

Mutants of β2AR, Which Are Retained Within Intracellular Compartments, Have a Dominant Negative Effect on WT β2AR Targeting to the Cell Surface—The potential biological role of early receptor dimerization was further investigated by studying the effect of two receptor mutants, expected to be retained inside the cell, on WT β2AR trafficking. The first mutant (myc-β2AR-$GBR1_{CT}$-Rluc) was generated by replacing the carboxyl tail of the β2AR with that of the GABAb-R1 that contains a well characterized arginine-based ER retention signal (12). In the second mutant, the ^{332}F*XXX*F*XXX*L^{340} motif, previously re-

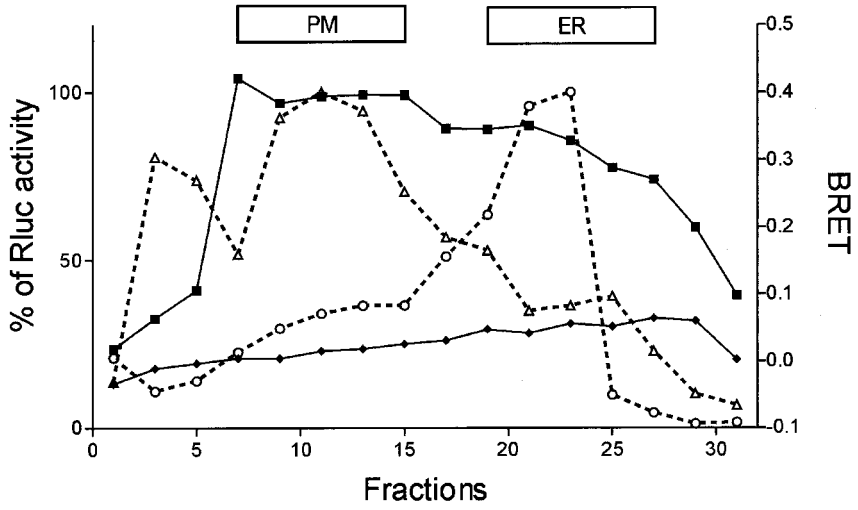

FIG. 1. **Subcellular distribution of β2AR dimers.** HEK293T cells expressing receptor-*R*luc and receptor-GFP fusions were lysed, and the material was fractionated on a discontinuous sucrose gradient. PM-associated Na/K-ATPase and ER calnexin were detected in *fractions 7–15* and *19–27*, respectively (see *bars above the curves*). The distribution of each receptor-*R*luc construct was determined by measuring the luciferase activity in each fraction. The His-β2AR-*R*luc (△) was found mainly in the PM and to a lesser extent in the ER fractions, whereas, as expected, the ER-resident myc-GABAb-R1-*R*luc (○) was primarily associated with the ER fractions. The presence of β2AR dimers in cells co-expressing His-β2AR-*R*luc and His-β2AR-GFP was assessed by measuring BRET in every fraction (■). The specificity of the interaction was shown by the lack of significant BRET in cells co-expressing His-β2AR-*R*luc and HA-GABAb-R2-GFP (◆). Curves for Rluc activity are represented by *dotted lines*, whereas the BRET curves are show as *solid lines*.

ported as a binding site for DRIP-78, a protein involved in the ER export of the D1-dopamine receptor (29), was mutated to ^{332}A*XXX*A*XXX*L^{340} (myc-β2AR-Ala^{332}-Ala^{336}-Rluc). As predicted, both constructs were completely retained inside the cells (Fig. 2*A*, *panels c–f*) as shown by the lack of membrane receptor staining in non-permeabilized cells. This contrasts with the predominant PM expression of the WT β2AR (Fig. 2*A*, *panel a*). The subcellular distribution of the mutant receptors was further documented in confocal fluorescence microscopy experiments using anti-TGN46 and anti-calnexin antibodies to label the Golgi apparatus and the ER, respectively. As shown in Fig. 3, the myc-β2AR-GBR1$_{CT}$-Rluc was found to co-localize strongly with the ER marker and only marginally with the Golgi marker, a labeling pattern that is identical to that of the WT myc-GABAb-R1-*R*luc and most likely reflects the ER retention of the constructs. In contrast, myc-β2AR-Ala^{332}-Ala^{336}-Rluc was found to co-localize with both calnexin and TGN46, indicating that the block in PM trafficking most likely occurred at the level of the *trans*-Golgi network for this mutant.

When co-expressed with either myc-β2AR-GBR1$_{CT}$-*R*luc or myc-β2AR-Ala^{332}-Ala^{336}-*R*luc, the WT HA-β2AR was retained inside the cells and co-localized with these receptor mutants (Fig. 2*B*, *panels a–f*). The dominant negative effect of mutant receptors on WT receptor export to the PM was specific to constructs bearing the β2AR sequence, because cell surface expression of the WT β2AR was not affected by the co-expression of the ER-retained myc-GABAb-R1-*R*luc (Fig. 2*B*, *panels g–i*).

To determine more quantitatively the effect of dominant negative mutants on WT receptor cell surface expression, a PM receptor targeting assay was designed. For this purpose, a β2AR bearing an HA epitope at the N terminus and *R*luc at the C terminus was used. In each experiment, total WT receptor expression was monitored by measuring the luciferase activity with a cell permeable substrate (coelenterazine h) that can access receptor constructs both at the PM and in intracellular compartments. HA immunoreactivity of surface targeted receptors was measured, in parallel, by ELISA on non-permeabilized cells. The relative cell surface expression of the WT β2AR, determined as the ELISA/luminescence ratio, was assessed in the presence or absence of one of the retained mutants. As shown in Fig. 4*A*, co-expression of myc-β2AR-Ala^{332}-Ala^{336}-GFP led to a 60% reduction of WT HA-β2AR-*R*luc cell surface expression, an effect likely resulting from a dimerization between the WT HA-β2AR-*R*luc and the intracellularly retained myc-β2AR-Ala^{332}-Ala^{336}-GFP, as indicated by the robust BRET signal measured between these two proteins (Fig. 4*B*). The impaired transport of the β2AR to the PM did not result from a nonspecific effect that could be attributed to any intracellularly retained receptors. Indeed, the ER-retained myc-GABAb-R1-GFP construct that cannot dimerize with the HA-β2AR-Rluc, as shown by the lack of BRET between the two constructs (Fig. 4*B*), had no effect on β2AR cell surface targeting (Fig. 4*A*).

β2AR Dimerization-deficient Mutants Are Retained in the ER—The data described above are consistent with a model whereby dimerization is a process involved in the normal receptor trafficking to the PM. Accordingly, inhibiting dimerization should impact on this process. To directly test this hypothesis, a previously predicted putative dimerization motif present in the 6th TM domain of the β2AR, Gly^{276}-*X*$_3$-Gly^{280}-*X*$_3$-Leu^{284}-*X*$_3$ (GGL) (23) was targeted by site-directed mutagenesis. Each of the three amino acids within this motif was mutated individually or in combination, and receptors tagged with *R*luc or GFP were generated for each mutant. The effect of the mutations on receptor dimerization was then assessed by quantitative BRET. Mutation of glycine residues at positions 276 (β2AR-Leu^{276}), 280 (β2AR-Leu^{280}), or 276 and 280 (β2AR-Leu^{276}-Leu^{280}) did not significantly affect receptor dimerization (Fig. 5*A*). In contrast, all constructs harboring a mutation at position L284 (β2AR-Tyr^{284}, β2AR-Gly^{284}, β2AR-Leu^{280}-Tyr^{284}, and β2AR-Leu^{276}-Leu^{280}-Tyr^{284}) showed a reduction of ~40–50% in BRET, indicating impaired dimerization. BRET titration curves (25) were then performed at a constant level of BRET donor and increasing concentrations of BRET acceptor. As shown in Fig. 5*B*, the titration curve of the β2AR-Leu^{280}-Leu^{280}-Tyr^{284} mutant was significantly right-shifted when compared with that of the WT receptor, indicating a decreased propensity of receptor mutants to self assemble as compared with WT β2AR.

Next, the efficiency of WT and dimerization-deficient mutant receptors to reach the cell surface was compared, using the PM receptor targeting assay described above. For each receptor,

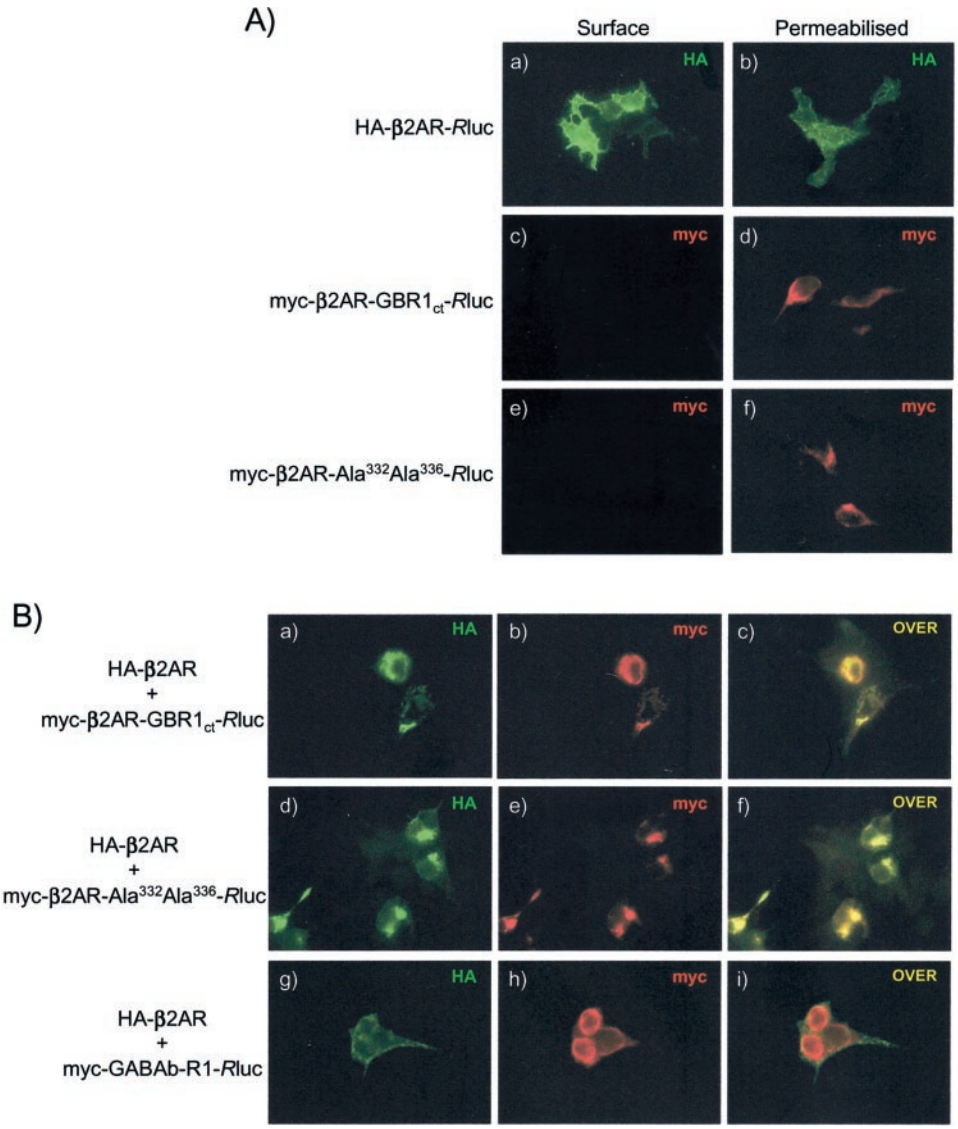

FIG. 2. **Export-deficient β2AR mutants act as dominant negative mutants for the surface expression of the WT receptor.** *A*, immunofluorescence experiments were performed on intact (*panels a*, *c*, and *e*) or permeabilized (*panels b*, *d*, and *f*) HEK293T cells expressing WT HA-β2AR-*R*luc (*panels a* and *b*), myc-β2AR-GBR1$_{CT}$-*R*luc (*panels c* and *d*) or myc-β2AR-Ala^{332}-Ala^{336}-*R*luc (*panels e* and *f*). Following a first incubation with the anti-myc or anti-HA antibodies, Oregon green-conjugated anti-rat and Alexa-594-conjugated anti-rabbit secondary antibodies were used to detect the HA and myc epitopes, respectively. *B*, double labeling immunofluorescence experiments were performed on permeabilized HEK293T cells co-expressing the HA-β2AR and either the myc-β2AR-GBR1$_{CT}$-*R*luc (*panels a–c*), the myc-β2AR-Ala^{332}-Ala^{336}-*R*luc (*panels d–f*), or the myc-GABAb-R1-*R*luc (*panels g–i*). The images shown are representative of three independent experiments.

cell surface expression was determined as a function of total receptor expression. As shown in Fig. 6*A*, the slope of the correlation plot between total and cell surface receptors for the HA-β2AR-Leu^{276}-Leu^{280}-Tyr^{284}-*R*luc and HA-β2AR-Tyr^{284}-*R*luc was significantly lower than that observed for the WT or the HA-β2AR-Leu^{276}-Leu^{280}-*R*luc mutant. Similar blunted slopes, reflecting altered cell surface targeting, were also observed for the other receptors mutated at position L284 (HA-β2AR-Gly^{284}-*R*luc, and HA-β2AR-Leu^{280}-Tyr^{284}-*R*luc) but not for the HA-β2AR-Leu^{276}-*R*luc and HA-β2AR-Leu^{280}-*R*luc mutants (data not shown). By measuring the ratio between the slope values calculated for the mutants and those determined for the WT receptor, cell surface targeting of HA-β2AR-Leu^{276}-Leu^{280}-Tyr^{284}-*R*luc and HA-β2AR-Tyr^{284}-*R*luc mutants was estimated to be impaired by >50% as compared with WT (Fig. 6*B*). Consistent with its lack of effect on dimerization measured by BRET, substitution of Gly^{276} and Gly^{280} to leucine residues did not affect cell surface targeting of the HA-β2AR-Leu^{276}-Leu^{280}-*R*luc. These data are consistent with dimerization being necessary for normal receptor trafficking to the PM. Subcellular fractionation experiments, comparing the relative distribution pattern of WT and dimerization-deficient mutants, revealed that the reduction of receptor expression in PM-enriched fractions was correlated with an increased association with ER enriched fractions (Fig. 6*C*), indicative of a block in the biosynthetic pathway.

To further confirm the intracellular compartments in which the dimerization-deficient mutants were retained, confocal microscopy experiments were carried out using the HA-β2AR-Leu^{276}-Leu^{280}-Tyr^{284}-*R*luc triple mutant. As can be seen in Fig. 7, the intracellularly retained receptor showed a high degree of co-localization with calnexin and no detectable Golgi labeling, consistent with the notion that the export block occurs in the ER.

DISCUSSION

In this study, two lines of evidence support the hypothesis that homodimerization may be a prerequisite for the targeting

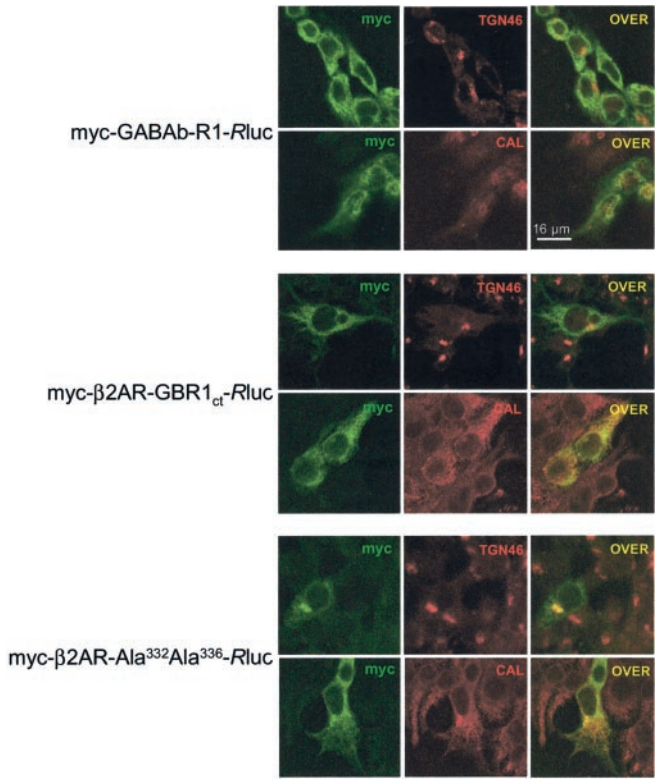

FIG. 3. **Subcellular localization of export-deficient β2AR mutants.** Confocal immunofluorescence experiments were performed on permeabilized HEK293T cells expressing myc-GABAb-R1-Rluc, myc-β2AR-GBR1$_{CT}$-*R*luc, or myc-β2AR-Ala^{332}-Ala^{336}-*R*luc. Receptors (*green*), *trans*-Golgi and ER (*red*) were immunostained with anti-myc (*myc*), anti-TGN46 (*TGN46*), and anti-calnexin (*CAL*) antibodies, respectively. Preparations were analyzed by confocal microscopy. Co-localization appears in *yellow* in overlays (*OVER*).

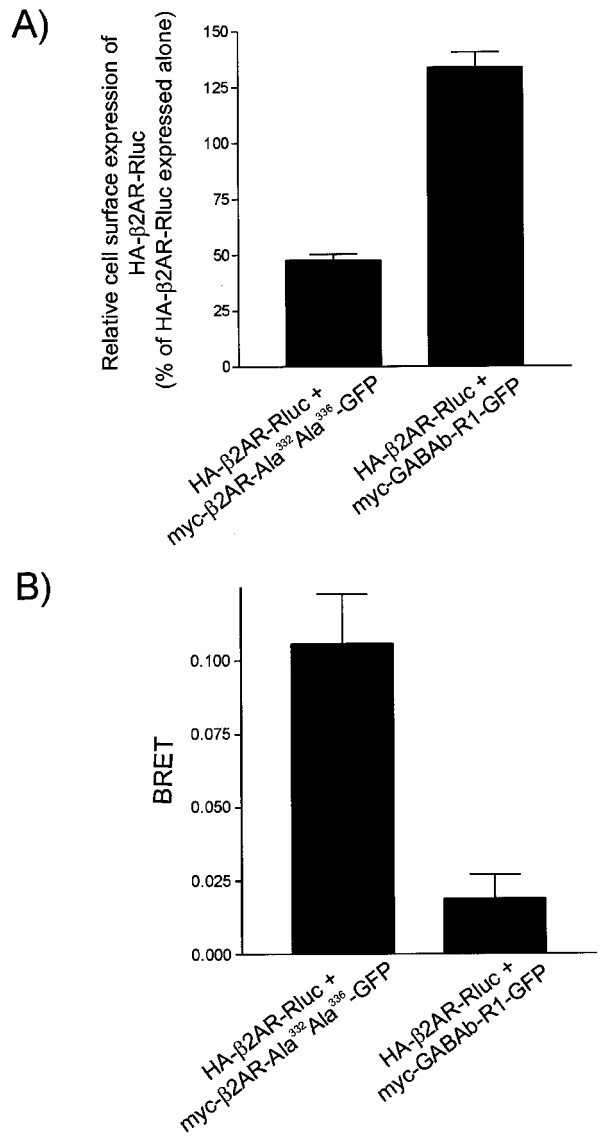

FIG. 4. **Quantitative analysis of the dominant negative effect of myc-β2AR-Ala^{332}-Ala^{336}-GFP on the cell surface expression of WT HA-β2AR-*R*luc.** *A*, the relative cell surface expression of HA-β2AR-*R*luc was determined in HEK293T cells by measuring cell surface HA immunoreactivity (ELISA) and luciferase activity. The surface localization of this construct was then determined in cells co-expressing either myc-β2AR-Ala^{332}-Ala^{336}-GFP or the myc-GABAb-R1-GFP. The ELISA/*R*luc signals are expressed as the percentage of the ratio observed in cells expressing the HA-β2AR-*R*luc construct alone. *B*, BRET measurements in cells co-expressing WT HA-β2AR-*R*luc and either myc-β2AR-Ala^{332}-Ala^{336}-GFP or myc-GABAb-R1-GFP.

of the β2AR from the ER to the PM. First, homodimerization of β2AR can occur as early as the ER, and mutations, which impair cell surface targeting without affecting dimerization, confer a dominant negative effect on the WT β2AR export. Second, mutations that inhibit β2ARs dimerization perturb receptor cell surface targeting.

Our data are consistent with a recent report indicating that homodimerization of the yeast α-mating factor receptor is important for its normal PM targeting (30). This conclusion was based on the observation that, as reported here, mutations of a putative dimerization interface significantly reduced both dimerization and cell surface targeting. Interestingly, however, the identified glycophorin-like dimerization motif was located in TM1 rather than TM6, as in our study. These observations indicate that multiple interfaces may contribute to receptor dimerization or that the dimerization interface could be receptor-specific.

Although these two studies are the only ones establishing a direct link between the homodimerization of a GPCR and its PM targeting, other data available for various receptor subtypes are consistent with such a model (15). These include reports of physiopathologically relevant, naturally occurring mutations, which cause receptor retention within intracellular compartments and behave as dominant negative species blocking WT receptor surface expression (31–34). Even though this dominant negative effect has often been used as an indication that association between receptors occurs early in the biosynthetic pathway, little direct experimental evidence was available to support this mechanism. The present study directly demonstrates that β2AR mutants retained in the biosynthetic pathway can interact with the WT receptor and provides both qualitative and quantitative assessment of the intracellular retention. Our data also exclude the possibility that the retention of the WT receptors comes from a general inhibition of the export resulting from a congestion of the secretory pathway, because accumulation of an ER-retained receptor, the GABAb-R1 that cannot interact with β2AR, had no effect on its transport.

A few reports are in apparent contradiction with the growing body of evidences supporting the concept that early dimerization is involved in GPCR processing and export to the PM. For instance, mutations in the N-terminal and carboxyl tail, which were found to affect the dimerization of the B2-bradykinin (35) and δ-opioid (36) receptors respectively, were without apparent effect on the binding and signaling activity of the receptors. These data could be interpreted as an indication that dimerization is not required for receptor cell surface targeting. However, it should be noted that this parameter was not quantified

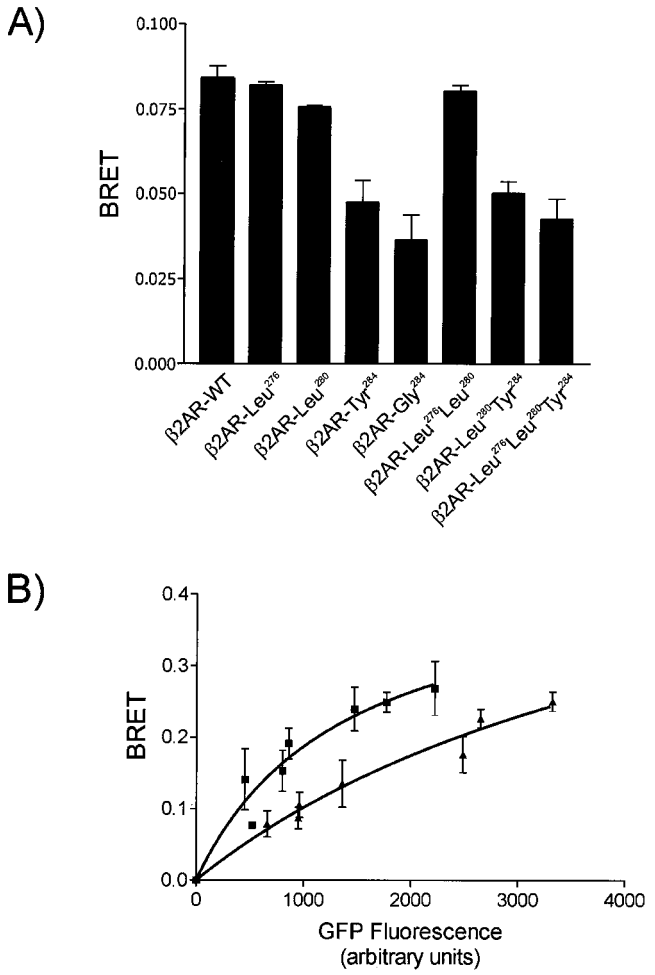

FIG. 5. **Quantitative BRET analysis of WT and dimerization-defective β2AR mutants.** *A*, BRET analyses were carried out in HEK293T cells expressing equimolar amounts of HA-β2AR-*R*luc and β2AR-GFP or the indicated pair of mutant receptors. BRET values are expressed as the mean ± S.E. of three to five independent experiments. *B*, BRET titration curves for WT-β2AR (■) and the β2AR-Leu^{276}-Leu^{280}-Tyr^{284} (▲) mutant receptor. HEK293T cells were co-transfected with a constant DNA concentration of either HA-β2AR-*R*luc or HA-β2AR-Leu^{276}-Leu^{280}-Tyr^{284}-*R*luc and increasing DNA concentrations of the corresponding construct fused to GFP. Forty-eight hours after transfection, the BRET values and total fluorescence were measured in parallel. BRET levels are plotted as a function of the total fluorescence signal used as an index for the concentration of receptor-GFP constructs expressed. The results are expressed as the mean ± S.E. of three independent experiments. The curves were fitted using a non-linear regression equation assuming a single binding site (GraphPad Prism).

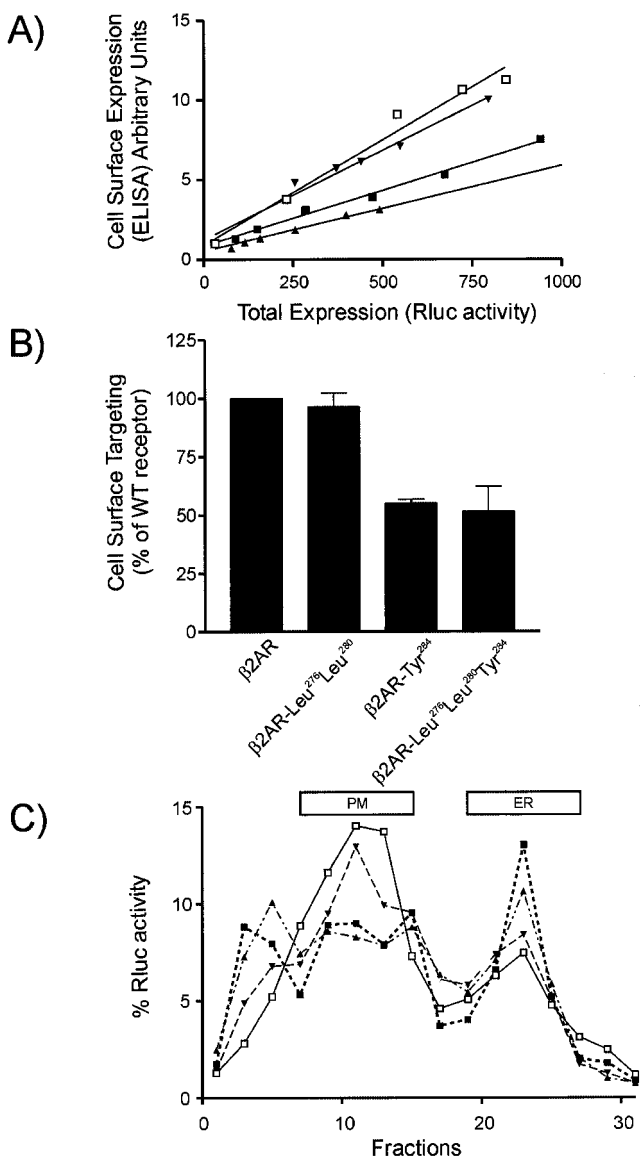

FIG. 6. **Correlation between receptor expression and surface targeting for WT and dimerization-deficient β2AR.** *A*, increasing concentrations of DNA encoding WT HA-β2AR-*R*luc (□), HA-β2AR-Leu^{276}-Leu^{280}-*R*luc (▼), HA-β2AR-Gly^{284}-*R*luc (■), or HA-β2AR-Leu^{276}-Leu^{280}-Tyr^{284}-*R*luc (▲) were transfected in HEK293T cells. The total amount of receptor expression and receptor localized at the cell surface were determined as in Fig. 4*A*. Values for cell surface receptor expression (ELISA) were plotted as a function of corresponding amounts of total receptor (luciferase activity). The data were fitted using a linear regression equation (GraphPad Prism). *B*, indices of cell surface targeting, expressed as the percentage of WT, were obtained by dividing the values of the slopes determined in *A* for the mutant receptors by that of the WT receptor. *C*, cell lysates prepared from cells expressing WT HA-β2AR-*R*luc (□), HA-β2ARTyr^{284}-*R*luc (■), HA-β2AR-Leu^{276}-Leu^{280}-*R*luc (▼), and HA-β2AR-Leu^{276}-Leu^{280}-Tyr^{284}-*R*luc (▲) were resolved on discontinuous sucrose gradients (as in Fig. 1). Receptor distribution was determined by measuring the luciferase activity in each fraction and expressed as the percentage of the total amount of luciferase activity recovered within all the fractions. The *bars above the curves* indicate the fractions enriched in PM and ER markers.

in these early studies. Thus, receptor function could have been maintained despite a decrease of cell surface receptors. This possibility is fully supported by the observation that a reduction in cell surface targeting of the α-mating factor dimerization-deficient mutant did not result in a loss of function (30).

More recently, substitution of two residues in TM1 and 4 of the chemokine CCR5 receptor led to a loss of function, even if the mutant receptor was normally expressed at the cell surface (37). This mutation also resulted in a loss of fluorescence resonance energy transfer, a result that was interpreted as a loss of homodimerization. Although consistent with the proposed interpretation, the loss of fluorescence resonance energy transfer could alternatively result from conformational changes within dimers and, as such, does not prove lack of dimerization. Distinguishing between these two possibilities would necessitate more quantitative analysis, such as the titration assays reported in the present study, allowing the assessment of the relative propensity of receptors to self-assemble. Further studies will therefore be required to determine if CCR5 export to the cell surface requires homodimerization, as in the cases of the β2AR and α-mating factor receptor, or if alternative mechanisms need to be invoked.

Various other oligomerization-based mechanisms have been shown to be involved in the maturation and export of specific

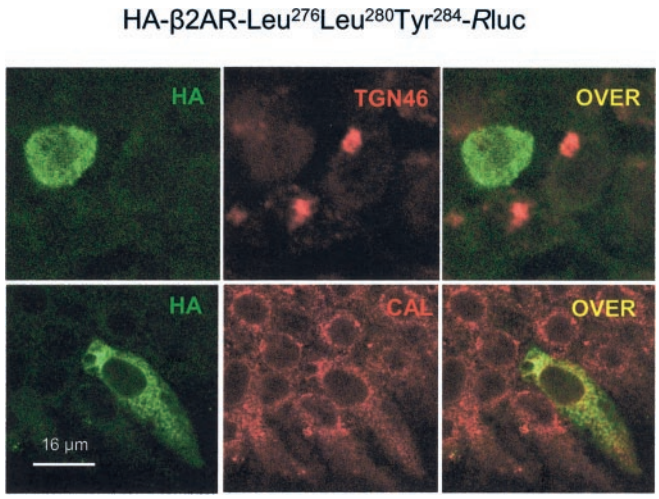

FIG. 7. **Subcellular localization of dimerization-deficient β2AR mutant.** Confocal immunofluorescence experiments were performed on permeabilized HEK293T cells expressing HA-β2AR-Leu^{276}-Leu^{280}-Tyr^{284}-*R*luc. Receptors (*green*), *trans*-Golgi and ER (*red*) were immunostained with anti-myc, anti-TGN46 (*TGN46*), and anti-calnexin (*CAL*) antibodies, respectively. Preparations were analyzed by confocal microscopy. Co-localization appears in *yellow* in overlays (*OVER*).

GPCRs. In addition to the examples of GABAb-R1/GABAb-R2 and α_{1D}/α_{1B}AR heterodimerization already discussed in the introduction (10, 14), the observation that co-expression of the taste receptors T1R2 and T1R3 or T1R1 and T1R3 are required to generate functional sweet and unami taste receptors (38, 39) strongly suggest an obligatory heterodimerization requirement for the biogenesis of these sensory receptors. In the case of the calcitonin receptor-like receptor, heterodimerization with a single TM domain protein (the receptor activity-modifying protein) rather than with another GPCR is required for its surface expression (40). In *Caenorhabditis elegans*, the single TM ODR-4 also acts as a factor involved in the exit from the ER (41), an event that leads to the proper localization of odorant receptors in the sensory organs (42). More recently, it was reported that the pheromone receptors, V1R and V2R, need to interact with the M10 major histocompatibility complex class Ib molecules for their proper transport to the cell surface (43).

A role for oligomerization in ER processing and cell surface trafficking is not restricted to GPCR and seems to be a general theme for a number of membrane proteins, including the shaker potassium channels (44), the alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid calcium channel (45), the transforming growth factor β receptor (20), as well as the insulin receptor (21). Although the mechanistic basis of this general need for oligomerization is incompletely understood, recent studies suggest that the oligomerization of proteins ready to be exported to the PM increases the avidity for dimeric molecular chaperones, such as those of the 14-3-3 family, which mediate the release of maturating proteins from the ER (46).

An increasing number of molecular chaperones have been proposed to play a role in maturation and ER export of GPCRs. For rhodopsin, NinaA (47) and HSJ1B (48) have been shown to facilitate trafficking to the PM by acting as chaperone and co-chaperone in *Drosophila* and mammals, respectively. Another ER export factor, DRIP-78, was found to play an important role in cell surface export of the mammalian D1-dopamine receptor (29). It will be interesting to examine whether GPCR dimerization is required for the interaction with these chaperones.

In the case of the GABAb receptor, it has been shown that the interaction of GABAb-R1 with the GABAb-R2 is necessary to mask an ER retention signal present on the GABAb-R1, thus allowing the targeting of the heterodimer to the PM (12). In contrast, no known canonical ER retention signal is present in the β2AR. Whether unknown specific motifs or exposed hydrophobic patches, shown to be involved in the ER retention of other proteins (49, 50), are implicated in the retention of dimerization-deficient β2AR mutants remains to be investigated. Although our study in heterologous systems clearly points to the ER as the initial site of dimer formation, additional studies will be required to demonstrate that this is also the case for native systems. Indeed, crowding of the ER quality control system, which can accompany heterologous expression, could slow down export, allowing enough time for specific dimerization to occur earlier than it would normally do. However, the observation that the ER exit represents the rate-limiting step for GPCR processing and PM targeting, even at low expression levels (51), is consistent with the ER as a privileged site for oligomeric assembly.

In conclusion, our study indicates that homodimerization of the β2AR is intimately linked to its export and cell surface trafficking. Although, heterodimerization among GPCR subtypes or between GPCR and escort proteins had been previously shown to play an important role in the biogenesis of a few receptors, these were generally considered as exceptions. Our study supports the notion that, in addition to heterodimerization, homodimerization between identical receptor subtypes can also play an important role in receptor biosynthesis and transport. Because a large number of GPCR has now been shown to form constitutive homodimers, our results suggest that homodimerization may be a general mechanism contributing to the processing and quality control of this important class of signaling molecules.

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