Constitutive and Agonist-dependent Self-association of the Cell Surface Human Lutropin Receptor*

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The human lutropin receptor (hLHR) is a G proteincoupled receptor (GPCR) that plays an essential role in reproductive physiology. The present studies were undertaken to determine whether the hLHR self-associates. We show that high molecular weight complexes of the hLHR can be co-immunoprecipitated from 293 cells transfected with differentially tagged hLHRs. These complexes are detected only in extracts from cells that have been co-transfected and not in extracts combined from cells expressing only one form of tagged hLHR, confirming the in vivo self-association of the receptor. In transiently transfected cells, in which a small percentage of cells overexpress hLHR and most of the hLHR is located intracellularly in the ER, the self-associated hLHR is composed predominantly of immature hLHR. When cells were transiently co-transfected with wildtype hLHR and a misfolded mutant of the hLHR, a physical association of the ER-localized misfolded mutant with the immature hLHR was observed, resulting in a decreased cell surface expression of the wild-type receptor. In contrast, in stably transfected cells, where the majority of cells express receptor and there is much less intracellular accumulation of hLHR, the self-associated forms of the hLHR are composed predominantly of cell surface receptor. The abundance of cell surface hLHR dimers and oligomers, as detected on SDS gels, is increased further upon human choriogonadotropin treatment of the stably transfected cells. In addition to documenting the self-association of cell surface hLHR, our results underscore the importance of the cellular distribution of recombinant GPCR as it relates to the nature of the GPCR dimerization and oligomerization.

Although dimerization has long been recognized to be involved in the signal transduction of integral membrane receptors such as receptors for growth factors and cytokines (1), G protein-coupled receptors (GPCRs)¹ were believed to act as monomers interacting with a single G protein. However, in recent years, biochemical, biophysical, and functional studies have increasingly suggested that GPCRs exist in cells as dimers or higher ordered oligomers (for reviews, see Refs. 2–4). In addition to homodimers of a given GPCR, specific heterodimerization between distinct GPCRs has also been documented. That GPCR heterodimerization is of functional consequence is best documented by those instances where the pharmacological or trafficking properties of the heterodimer are distinct from the individual GPCRs (4–6). In contrast, the functional role of GPCR homodimerization is still quite unclear.

The lutropin receptor (LHR) is a GPCR that plays an essential role in reproductive physiology in both males and females. It is a member of the rhodopsin-like or Group A family of GPCRs and couples primarily to Gs (see Ref. 7 for a recent review on the LHR). The LHR and structurally related follitropin receptor and thyrotropin receptor, collectively referred to as the glycoprotein hormone receptors, in turn form a subset of rhodopsin-like GPCRs characterized by a large N-terminal extracellular domain that is responsible for the high affinity binding of the respective glycoprotein hormone. Prior to the cloning of the LHR cDNA, studies examining the equilibrium sedimentation of detergent-solubilized LHR and radiation inactivation of LHRs on gonadal cells had indicated that the LHR might exist as dimers or oligomers (8, 9). A more recent study using cells stably expressing recombinant rat LHR demonstrated fluorescent resonance energy transfer between derivatized hormone bound to LHR-expressing cells consistent with the self-association of cell surface LHR (10). Furthermore, the desensitized rat LHR exhibits an increase in fluorescent resonance energy transfer, suggestive of larger LHR complexes formed upon LHR desensitization (11). Using a functional complementation approach, recent studies have also shown that the co-expression of an hLHR defective in hormone binding with an hLHR defective in signaling restores hormonestimulated signaling (12, 13), further suggesting that the cell surface LHR self-associates.

The present studies were undertaken to determine whether self-associated complexes of the hLHR could be detected biochemically. By co-immunoprecipitation of differentially tagged hLHRs, we show that the hLHR does indeed self-associate. However, our data demonstrate a profound difference in the nature of the self-association of the hLHR depending upon whether cells express most of the receptor at the cell surface or if there is a significant accumulation of receptor in the ER.

MATERIALS AND METHODS

Hormones and Supplies—Highly purified hCG was purchased from the NIDDK, National Institutes of Health, National Hormone and Pituitary Program and Dr. A. F. Parlow. hCG was iodinated following the procedure described for the iodination of hCG (14). Cell culture medium was obtained from the Media and Cell Production Core of the Diabetes and Endocrinology Research Center of the University of Iowa. Tissue culture reagents were purchased from Invitrogen, and Corning plasticware was obtained from Fisher.

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¹ The abbreviations used are: GPCR, G protein-coupled receptor; LHR, lutropin receptor; hLHR, human lutropin receptor; ER, endoplasmic reticulum; GFP, green fluorescent protein; Endo H, endoglycosidase H; BSA, bovine serum albumin; hCG, human choriogonadotropin.

Preparation and Characterization of Tagged Receptor Constructs— The wild-type hLHR cDNA was kindly provided by Ares Advanced Technology (Ares-Serono Group, Randolph, MA) and was subcloned into pcDNA3.1 (Invitrogen). Using the PCR overlap extension method (15, 16), a FLAG or c-Myc epitope tag was inserted after the signal peptide such that the tag would be on the N terminus of the mature protein (referred to herein as FLAG-hLHR or Myc-hLHR). For confocal microscopy, the hLHR was tagged at the C terminus with enhanced green fluorescent protein by subcloning the coding sequence of the hLHR cDNA into pEGFP-N1 (BD Biosciences Clontech). This latter construct is referred to herein as GFP-hLHR.

The entire coding region of each construct was sequenced to ensure the fidelity of the epitope tagged cDNA. Automated DNA sequencing was performed by the DNA Core of the Diabetes and Endocrinology Research Center of the University of Iowa. Control experiments showed that the placement of c-Myc or FLAG epitope tags at the N terminus of the hLHR does not adversely affect the cell surface expression, ligand binding properties, or signaling properties of the receptor. Although the placement of Myc or FLAG tags at the C terminus decreases cell surface expression of the hLHR, the placement of the larger GFP sequence at the C terminus of the hLHR did not decrease cell surface expression of the hLHR, and it did not affect signaling as measured by hormonestimulated cAMP production (data not shown).

Cells and Transfections-HEK293 cells were maintained at 5% CO₂ in a culture medium consisting of Dulbecco's modified Eagle's medium containing 50 µg/ml gentamicin, 10 mM Hepes, and 10% newborn calf serum. Cells for experiments were plated on gelatin-coated plasticware. Transfections were performed using the calcium precipitation method (17), using 4 μ g of plasmid in 2.0 ml per 35-mm dish or 20 μ g of plasmid in 10 ml per 100-mm dish. For transient transfections, cells were used for experiments 48 h after transfection. To establish cell lines stably expressing the hLHR, 293 cells were plated into 100-mm gelatin-coated dishes and transfected with either Myc-hLHR in pcDNA3.1(neo), FLAG-hLHR in pcDNA3.1(zeo), or both constructs. Cells were selected in medium containing 700 µg/ml G418 (for Myc-hLHR-transfected cells), 600 µg/ml zeocin (for FLAG-hLHR-transfected cells), or both G418 and zeocin (for co-transfected cells). Stable cell lines were maintained in the medium containing the same antibiotics as described above.

Western Blotting of Detergent-solubilized Cell Extracts-HEK293 cells expressing tagged hLHRs were analyzed for receptor protein by Western blotting. Cells were washed, and detergent-solubilized cell extracts were prepared using lysis buffer (0.5% Nonidet P-40 in 150 mM NaCl, 20 mM Hepes, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride and 1 mm EDTA as protease inhibitors) containing 100 mm iodoacetate. The extract was incubated in an ice bath for 30 min with intermittent vortexing. The lysate was then cleared by centrifuging 15 min at 4 °C in a microcentrifuge at maximal speed. Protein concentrations in the supernatants were measured using the Bradford assay (18). Samples were diluted 1:6 in a 6-fold concentrated Laemmli sample buffer containing reducing agents (12% (w/v) SDS, 40% glycerol, 109 mM EDTA, 1.5 M Tris/HCl, 98 mg/ml dithiothreitol, and 6% (v/v) β-mercaptoethanol), incubated for 1 h at room temperature, fractionated by SDS-PAGE on a 7.5% gel, and transferred to a polyvinylidene difluoride membrane. The membrane was probed with anti-Myc monoclonal antibody (9E10; 1:500 dilution) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad). The immunoreactive bands were visualized using an ECL detection system (Amersham Biosciences).

Co-immunoprecipitation of Differentially Tagged hLHRs-HEK293 cells were transiently or stably co-transfected with Myc-hLHR and FLAG-hLHR, and detergent-solubilized lysates were prepared as described above. One day prior to the experiment, 5 μ l of rabbit polyclonal antibody against FLAG (catalog no. F-7425; Sigma) was conjugated to 50 μ l of protein G-Sepharose (Santa Cruz Biotechnology) by rotating overnight at 4 °C. On the day of the experiment, the conjugated anti-FLAG resin was washed with lysis buffer and added to 10 mg of cell extract. After rotating for 90 min at 4 °C, the beads were washed four times with lysis buffer, and the immunoprecipitate was eluted with 50 μ l of Laemmli sample buffer containing reducing agents. Twenty-five microliters of each immunoprecipitate was resolved by SDS-PAGE under reducing conditions and transferred to a polyvinylidene difluoride membrane as described above. The membrane was probed with anti-Myc monoclonal antibody (9E10; 1:500 dilution) (Santa Cruz Biotechnology), and horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad). The immunoreactive bands were visualized using an ECL detection system (Amersham Biosciences).

Treatment of Solubilized Extracts with Glycosidases-Detergent-sol-

ubilized extracts were incubated for 24 h at 37 °C with either no additions or with 300 milliunits/ml endoglycosidase H (Endo H) or 300 milliunits/ml neuraminidase (both enzymes were purchased from Roche Applied Science).

Proteolysis of Intact Cells Expressing the hLHR—Proteolysis of the cell surface hLHR was performed as previously described (19). Cells were cooled on ice for 15 min, washed twice with cold Hanks' balanced salt solution containing 1 mg/ml bovine serum albumin and twice with Hanks' balanced salt solution, and then fresh Hanks' balanced salt solution was added. Protease XIV (Sigma) was added to give a final concentration of 250 μ g/ml, and the cells were incubated on ice for 30 min. To terminate the reaction, Waymouth's medium containing 10% newborn calf serum and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM pepstatin, and 100 μ g/ml leupeptin) was added. The cells were then collected and used for cell surface binding assays (performing the assay in plastic tubes rather than culture dishes) or for the preparation of solubilized extracts. The cell surface binding of cells after treatment with protease was 4.4 ± 0.7% (mean ± S.E.) that of untreated cells (n = 3).

Biotinylation of Intact Cells Expressing the hLHR-Cell surface proteins of 293 cells were biotinylated as described previously (20). Cells were washed four times with ice-cold calcium-free, magnesium-free phosphate-buffered saline, pH 8.0, and then biotinylated during two consecutive 15-min incubations at room temperature with 0.5 mg/ml solutions of sulfosuccinimidyl-6-(biotinamido)hexanoate (Vector Laboratories) in the same buffer. The cells were then incubated with cold Dulbecco's modified Eagle's medium containing 10% fetal calf serum for 15 min to quench the reaction, washed twice with ice-cold PBS, twice with 150 mM NaCl containing 2 mM Hepes, pH 7.4, and lysed on ice for 30 min with lysis buffer containing a Complete Protease Inhibitor tablet from Roche Applied Science used at the concentration suggested by the manufacturer. Solubilized extracts were then either immunoprecipitated with anti-Myc monoclonal antibody 9E10 or purified on an immobilized avidin column (Pierce). For the immunoprecipitation protocol, 10 μ l of anti-Myc monoclonal antibody was combined with 30 μ l of protein G-Sepharose, rotated overnight at 4 °C, and washed. Biotinylated cell extract (100 μ g) was combined with a given batch of conjugated antibody and rotated at 4 °C for 90 min. After extensive washing with lysis buffer, the immunoprecipitates were eluted with Laemmli sample buffer, separated by SDS-PAGE under reducing conditions, transferred to polyvinylidene difluoride membranes, blocked overnight with blocking buffer, and probed with streptavidin conjugated with horseradish peroxidase (0.1 μ g/ml in blocking buffer; Vector Laboratories), and visualized with an ECL detection system. For the affinity purification protocol, 10 mg of biotinylated cell extracts were applied to 1 ml of immobilized avidin (Pierce). After the extract entered the column, the column was incubated at room temperature for 30 min and then washed extensively with PBS until the absorbance at 280 nm reached the value of buffer only. Bound proteins were eluted with 0.1 ${\mbox{\tiny M}}$ Tris buffer (pH 2.8) and immediately neutralized after collection with 0.1 M Tris, pH 9.5. These fractions were concentrated using Centricon YM-30 columns (Millipore Corp.), resolved by SDS-PAGE under reducing conditions, transferred to polyvinylidene difluoride membranes, and blotted with anti-Myc monoclonal antibody as described above.

¹²⁵I-hCG Binding to Intact Cells—The levels of cell surface hLHR were determined by ¹²⁵I-hCG binding assays to intact cells. HEK293 cells were placed on ice and washed two times with cold Waymouth's MB752/1 medium modified to omit the sodium bicarbonate and to contain 50 μ g/ml gentamicin and 1 mg/ml BSA. The cells were then incubated overnight at 4 °C in the same medium containing a saturating concentration of ¹²⁵I-hCG (500 ng/ml final concentration) with or without an excess of unlabeled hCG (50 IU/ml final concentration). The cells were collected into plastic tubes on ice, centrifuged, and washed twice with cold Hanks' balanced salt solution modified to contain 50 μ g/ml gentamicin and 1 mg/ml BSA. The cells were then pelleted and counted in a γ counter.

Confocal Imaging of GFP-hLHR in Stably and Transiently Transfected Cells—The day prior to the experiment, 293 cells expressing GFP-hLHR were plated onto lysine-coated slides (Biocoat cellware from Falcon). All reagents and incubations for the immunohistochemistry were at room temperature. Cells were washed three times with filtered 137 mM NaCl, 2.7 mM KCl, 1.4 mM $\rm KH_2PO_4$, 4.3 mM Na_2HPO_4, pH 7.4 (PBS-IH) and then fixed with 4% paraformaldehyde in PBS-IH for 30 min. Cells were permeabilized with 1% Triton X-100 in PBS-IH for 4 min. After incubating with blocking solution (5% BSA in PBS-IH) for 1 h, the cells were incubated with monoclonal antibody to protein-disulfide isomerase (catalog no. MA3-018; Affinity Bioreagents) diluted 1:75 in PBS-IH containing 1 mg/ml BSA (PBS-IH/BSA). After washing



FIG. 1. Cells transiently transfected with the hLHR exhibit high molecular weight complexes on Western blots in addition to the mature and immature monomeric forms of the receptor. 293 cells were transiently transfected with Myc-hLHR, and lysates were resolved by SDS-PAGE under reducing conditions. Western blots were probed with anti-Myc antibody. A representative gel is shown.

with PBS-IH, cells were incubated for 1 h with Texas Red-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) diluted 1:500 in PBS-IH/BSA. Cells were washed with PBS-IH and allowed to dry, and then Vectashield mounting medium (Vector Laboratories) and a coverslip were placed on top. Images were collected with a Bio-Rad 1024 laser confocal microscope.

Co-localization of Differentially Tagged hLHRs—Cells transiently or stably co-transfected with Myc-hLHR and FLAG-hLHR were plated on coverslips, fixed, and permeabilized as described above. After blocking, the cells were incubated for 1 h with anti-FLAG monoclonal antibody (catalog no. F3165; Sigma) and rabbit anti-Myc antibody (catalog no. sc-789; Santa Cruz Biotechnology) diluted 1:200 and 1:100, respectively, in PBS-IH/BSA. After washing, they were incubated for 1 h with CyTM5-conjugated sheep anti-mouse IgG (catalog no. 515-175-062; Jackson ImmunoResearch Laboratories) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG diluted 1:500 and 1:1000, respectively, in PBS-IH/BSA. Control experiments (not shown) confirmed that there was no cross-reactivity of the anti-rabbit secondary antibody with the monoclonal primary antibody or cross-reactivity of the anti-mouse secondary antibody with the rabbit primary antibody. The cells were examined by confocal microscopy as described above.

RESULTS

Self-association of the hLHR in Transiently Transfected Cells-To facilitate the study of potential hLHR self-association, constructs were made in which a FLAG or Myc epitope was placed at the amino terminus of the mature protein. Our own studies (data not shown) and those of others (19) have shown that the placement of these relatively small epitope tags at the amino terminus of the hLHR does not adversely affect receptor cell surface expression or ligand binding and signaling properties. Experiments were initially performed with 293 cells that were transiently transfected with one or both constructs. Western blots of extracts of cells that had been transiently transfected with Myc-hLHR display four prominent bands (Fig. 1). The 67- and 84-kDa bands have previously been shown to represent immature precursor and mature cell surface forms of the hLHR, respectively (19-24). Based upon the predicted size of the protein translated from the cDNA and the resulting molecular masses calculated from SDS gels, it can be concluded that the 67-kDa immature and 84-kDa mature forms of the receptor represent monomeric forms of the protein. In addition, however, two additional higher molecular mass bands of 166 and ~ 240 kDa are also observed on Western blots. These higher molecular weight complexes may represent self-associated hLHR and/or hLHR complexed with other protein(s). The complexes are not disulfide-bonded, since they are observed in the presence of reducing agents. However, the noncovalent interactions between the proteins in the complexes are strong enough to withstand the effects of SDS.



FIG. 2. Co-immunoprecipitation of differentially tagged hLHRs in transiently transfected cells reveals self-associated hLHR complexes. 293 cells were transiently transfected with the indicated constructs. Lysates were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates (*IP*) were resolved by SDS-PAGE under reducing conditions, and Western blots were probed with anti-Myc antibody. A representative gel is shown.

To determine whether the high molecular weight complexes observed on SDS gels represent self-associated forms of the hLHR, we performed co-immunoprecipitation experiments on cells transiently co-transfected with Myc-hLHR and FLAGhLHR. In the experiment shown in Fig. 2, cells were transfected, lysed, and immunoprecipitated with anti-FLAG, the immunoprecipitates were resolved on a reducing gel, and the gel was immunoblotted with anti-Myc. In this and all subsequent experiments, iodoacetate was included in the lysis buffer to prevent the artifactual reduction and reoxidation of disulfide bonds that can occur during solubilization of the LHR (25). As shown in Fig. 2, Myc-hLHR and FLAG-hLHR can be co-immunoprecipitated with each other as 166- and ~240-kDa complexes. These data clearly demonstrate that the high molecular weight complexes of hLHR detected on Western blots of cell lysates from hLHR-expressing cells result from the physical self-association of the hLHR. As with other self-associated GPCRs, a definitive assessment of the stoichiometry of receptor molecules in each complex is lacking. However, based on the sizes of the complexes and by analogy with other GPCRs shown to self-associate, we designate the 166-kDa complex as a dimer and the \sim 240-kDa complex as an oligomer of the hLHR.

Most of the hLHR Dimers and Oligomers in Transiently Transfected Cells Are Composed of Immature Receptor-To determine whether the high molecular weight complexes of hLHR in transiently transfected cells were composed of immature and/or mature receptor, we utilized several different experimental approaches. In one, we examined the sensitivity of the complexes to endoglycosidases (Fig. 3). Lysates from 293 cells transiently transfected with Myc-hLHR were subjected to treatment with Endo H, which cleaves high mannose-containing immature N-linked carbohydrates from proteins, or neuraminidase, which cleaves sialic acids from mature glycoproteins. It has previously been shown that the 67-kDa monomeric form of the hLHR is sensitive to Endo H, but not neuraminidase, consistent with it being a precursor form of the receptor in the ER, whereas the 84-kDa form of the hLHR has been shown to be sensitive to neuraminidase, but not Endo H, indicative of it containing fully processed carbohydrates and thus having exited the Golgi (21-24). The data in Fig. 3 confirm these earlier studies and further show that neither high molecular weight complex exhibits any sensitivity to neuraminidase, whereas both exhibit a partial sensitivity to Endo H treatment. That the



FIG. 3. Endoglycosidase treatments of hLHR species from transiently transfected cells suggest that the high molecular weight complexes contain immature hLHR. 293 cells were transiently transfected with Myc-hLHR. Cell lysates were incubated 24 h at 37 °C in the absence of endoglycosidases or with Endo H or neuraminidase as described under "Materials and Methods." The extracts were then resolved by SDS-PAGE under reducing conditions, and Western blots were probed with anti-Myc antibody. The relevant portion of a representative gel is shown.

high molecular weight complexes are only partially sensitive to Endo H may be due to steric hindrance of the *N*-linked carbohydrates in the complexes to endoglycosidases. This is supported by the observation that the smaller 166-kDa complex shows more degradation upon Endo H treatment than the \sim 240-kDa complex.

We also examined the susceptibility of the different species of hLHR to be degraded by limited proteolysis of intact cells (19). Cells transiently transfected with Myc-hLHR were subjected to protease treatment (under conditions where parallel binding experiments confirmed that 95% of the cell surface binding activity was lost), the lysates were resolved by SDS-PAGE, and Western blots were probed with anti-Myc antibody (Fig. 4A). As would be expected, protease treatment caused the selective loss of the 84-kDa monomeric form of the mature hLHR on the cell surface but had no effect on the 67-kDa immature, monomeric hLHR. These results also show that protease treatment of the intact cells did not diminish the abundance of either the 166- or \sim 240-kDa complex. To further assess the cellular localization of the hLHR oligomers in transiently transfected cells, we determined if either high molecular weight hLHR complex could be detected in cells transiently transfected with MychLHR and biotinylated to label cell surface proteins. Biotinylated proteins in the lysate were purified on a streptavidin column, resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-Myc antibody. As shown in Fig. 4B, only the mature, monomeric 84-kDa form of the hLHR was detected as a biotinylated species. As would be expected, the 67-kDa immature, precursor form of the receptor was not observed to be biotinylated. The lack of detection of either the 166- or ~240-kDa forms of the hLHR under these conditions suggests that these complexes are not at the cell surface or are otherwise inaccessible to biotinylation.

Taken altogether, the data in Figs. 3 and 4 are consistent with the conclusion that the 166- and \sim 240-kDa complexes in transiently transfected cells represent complexes composed primarily of self-associated immature forms of the hLHR that are located intracellularly, most likely in the ER. If so, the abundance of hLHR dimers and oligomers in transiently transfected cells should not increase upon hCG treatment of the cells. As shown in Fig. 5, hCG does not affect the amounts of hLHR dimers and oligomers in transfected cells. Although these data alone cannot be used to demonstrate the intracellular localization of the hLHR dimers and oligomers in the transfected cells.



FIG. 4. hLHR species detected after protease treatment or biotinylation of transiently transfected intact cells suggest that high molecular weight complexes are composed of immature hLHR. 293 cells were transiently transfected with Myc-hLHR and then subjected to protease treatment (A) or biotinylation treatment (B) as described under "Materials and Methods." Extracts were resolved by SDS-PAGE under reducing conditions, and Western blots were probed with anti-Myc antibody. A and B are from separate representative experiments, where only the relevant portions of the gels are shown.



FIG. 5. hCG treatment of transiently transfected cells does not increase the abundance of hLHR dimers or oligomers. 293 cells were transiently transfected with Myc-hLHR. They were incubated for 30 min at 37 °C with the indicated final concentrations of hCG. Cell lysates were prepared and resolved by SDS-PAGE under reducing conditions, and Western blots were probed with anti-Myc. A representative gel is shown.

siently transfected cells, the observation of hCG-dependent changes in hLHR dimer and oligomer abundance would have argued for the cell surface localization of these complexes. The lack of effect of hCG on the levels of hLHR dimers and oligomers is therefore consistent with the data presented in Figs. 3 and 4 that strongly suggest an intracellular localization of the hLHR dimers and oligomers in transiently transfected cells.

Transient Co-expression of a Misfolded hLHR Mutant with the Wild-type hLHR Decreases Cell Surface Expression of the Wild-type Receptor Due to Heterodimerization / Heterooligomerization in the ER-It has previously been shown that misfolded mutants of the hLHR and other glycoprotein hormone receptors are retained partially or entirely (depending upon the extent of misfolding) in the ER, resulting in a decrease in the expression of the mutant receptor at the cell surface (26-29). It has been shown for other GPCRs that the transient co-expression of a wild-type receptor with a misfolded mutant thereof causes a decrease in cell surface expression of the wild-type receptor (30–35). The data from our present study suggested to us that this phenomenon might be due to the aggregation of misfolded mutant receptor in the ER with immature wild-type receptor in the ER. To examine this, we transiently co-transfected cells with the wild-type hLHR and either of two different misfolded hLHR mutants. The two mutants, A593P and S616Y, are naturally occurring inactivating mutants of the hLHR (36-38). These mutants are observed on Western blots as immature receptor, sensitive to Endo H, where the majority of each receptor is present as high molecular weight dimerized/oligomer-



FIG. 6. Inactivating hLHR mutants A593P and S616Y are present primarily as intracellular high molecular weight complexes in transiently transfected cells. *Top*, 293 cells were transiently transfected with Myc-hLHR(A593P) or Myc-hLHR(S616Y). Cell lysates were incubated 24 h at 37 °C in the absence of endoglycosidases or with Endo H or neuraminidase as described under "Materials and Methods." The extracts were then resolved by SDS-PAGE under reducing conditions, and Western blots were probed with anti-Myc antibody. The relevant portion of a representative gel is shown. *Bottom*, 293 cells were transiently transfected with GFP-hLHR(A593P) or GFP-hLHR(S616Y) and visualized by confocal laser microscopy. *A* and *D*, the localization of the hLHR-GFP; *B* and *E*, staining for protein-disulfide isomerase (*PDI*); *C* and *F*, merged images.

ized forms (Fig. 6, *top*). Confocal laser microscopy of 293 cells transiently transfected with hLHR(A593P) or hLHR(S616Y) labeled with GFP indicates that there is detectable cell surface expression of each (Fig. 6, A and D). However, in both cases, most of the mutant is localized intracellularly. Similar intracellular retention of the mutants was also observed in cells stably transfected with each of the mutants (data not shown). The same cells that were transiently transfected with each of

the mutants were also stained for protein-disulfide isomerase (PDI), a luminal ER protein (Fig. 6, B and E). Surprisingly, we observed only partial co-localization of each mutant with PDI (Fig. 6, C and F). Cells co-transfected with the wild-type hLHR and each of the misfolded hLHRs display reduced cell surface binding as compared with cells expressing only the wild-type receptor (Fig. 7). Co-immunoprecipitation experiments using differentially tagged wild-type and mutant hLHRs confirm the



FIG. 7. Misfolded mutants co-expressed with the wild-type **hLHR** decrease the cell surface expression of the wild-type receptor (*WT*). 293 cells were transiently transfected with the indicated Myc-labeled hLHR constructs. Empty vector was used to maintain the same amount of plasmid transfected into each group of cells. ¹²⁵I-hCG binding was determined on intact cells as described under "Materials and Methods."

formation of heterodimers and oligomers between the intracellularly localized immature wild-type hLHR and the misfolded mutant (Fig. 8). The co-aggregation of wild-type and mutant hLHR in the ER, therefore, probably causes the decrease in cell surface expression of the wild-type hLHR.

Transient, but Not Stable, Expression of the hLHR Results in a Large Percentage of Receptor Localized Intracellularly-The above results showing dimerization/oligomerization of the immature hLHR in the ER of transiently transfected cells, but not dimerization/oligomerization of cell surface hLHR, raised the critical question as to whether this was a peculiarity of transiently transfected cells. To address this issue, we compared hLHR expression, as determined both by ¹²⁵I-hCG binding to intact cells and by confocal microscopy, in cells transiently or stably expressing GFP-hLHR. The stably transfected cell line used for this study bound 6.3 \pm 0.9 ng of $^{125}\mbox{I-hCG}/10^6$ cells, and the transiently transfected cells bound 2.9 \pm 1.1 ng of ¹²⁵I-hCG/ 10⁶ cells. Confocal images of 293 cells transiently transfected with GFP-hLHR show only a few cells expressing the receptor (Fig. 9; compare A, showing hLHR-expressing cells, with B, showing all cells as stained with anti-PDI). In the hLHRexpressing cells arising from transient transfections, there is, in addition to cell surface receptor, a large percentage of the hLHR located intracellularly (Fig. 9A) in a compartment that co-localizes with the ER marker PDI (Fig. 9C). In contrast, in stably transfected cells, the majority of the cells express hLHR, as would be expected (Fig. 9, compare D and E). However, the intensity of cell surface receptor expression is less than that in the transiently transfected cells. Therefore, the binding data (which measures total amount of hormone bound in a given well of cells) for the transiently transfected cells is misleading. since it reflects the average of a small percentage of cells overexpressing receptor plus a larger percentage of cells expressing no receptor.

Self-association of the hLHR in Stably Transfected Cells— These results prompted us to examine the issue of hLHR self-association in stably transfected cells. Western blots of cells stably transfected with the hLHR also exhibit high molecular weight complexes (Fig. 10A). However, in contrast to the transiently transfected cells, a much smaller percent-



FIG. 8. Heterodimerization/oligomerization of misfolded mutant hLHR with immature wild-type hLHR in the ER of transiently transfected cells. 293 cells were transiently transfected with the indicated epitope-tagged hLHR constructs. Lysates were prepared and subjected to immunoprecipitation with anti-FLAG antibody. Immunoprecipitates (*IP*) were resolved by SDS-PAGE under reducing conditions, and Western blots were probed with anti-Myc antibody. The relevant portion of a representative gel is shown.

age of total receptor appears as high molecular weight complexes (Fig. 10A). Furthermore, whereas the transiently transfected cells exhibit very broad bands whose centers correspond to 166 and \sim 240 kDa, the stably transfected cells exhibit sharper bands at 189 and \sim 240 kDa. To determine whether the high molecular weight complexes in stably transfected cells are composed of self-associated hLHR, a stable cell line was created that co-expressed both Myc-hLHR and FLAG-hLHR. This cell line was then used to examine whether the two epitope tagged versions of the hLHR could be co-immunoprecipitated. As shown in Fig. 10B, bands of 189 and \sim 240 kDa are indeed observed as co-immunoprecipitated hLHR species. These high molecular weight complexes were only observed in cells co-transfected with the different epitope-tagged hLHRs and were not observed when extracts from cells transfected with each construct were mixed together and then immunoprecipitated (Fig. 11), confirming that the complexes exist in the cell prior to their solubilization.

Most of the Dimerized hLHR Complexes in Stably Transfected Cells Are Composed of Mature, Cell Surface Receptor-As we had done for the transiently transfected cells, we then performed experiments to determine whether the high molecular weight hLHR complexes in stably transfected cells were composed of immature or mature forms of the receptor. As shown in Fig. 12A, protease treatment of intact cells stably expressing the hLHR led to the disappearance of the 84-kDa monomeric form of the mature hLHR and the 189-kDa dimeric form of the hLHR. Endoglycosidase treatment of extracts from cells stably transfected with hLHR depicted a sensitivity of the 189-kDa complex to neuraminidase but not Endo H (Fig. 12B). A shift in the \sim 240-kDa band was not apparent with either Endo H or neuraminidase treatment. The data from the protease and glycosidase experiments suggest that the 189-kDa complex is composed predominantly of cell surface hLHR. The composition of the ~240-kDa complex cannot, however, be ascertained from these experiments.

Surprisingly, when stably transfected cells were biotinylated and lysed and the biotinylated proteins were isolated by avidin chromatography, only the mature monomeric 84-kDa form of the hLHR was detected, and the high molecular weight hLHR oligomers were not observed as biotinylated (data not shown). We also examined stably transfected cells that were biotinylated and lysed, and the biotinylated hLHR species were immunoprecipitated with anti-Myc and visualized on Western blots Self-association of the hLHR



cells transiently versus stably expressing the hLHR. 293 cells were transiently (A-C) or stably (D-F) transfected with GFP-hLHR and visualized by confocal laser microscopy. A and D, localization of the GFP-hLHR; B and E, staining for protein-disulfide isomerase (PDI); C and F, merged images. Also shown are ¹²⁵I-hCG binding data (saturation binding to intact cells) on the cells transiently versus stably transfected with GFP-hLHR.

FIG. 9. Confocal microscopy of 293

¹²⁵I-hCG Binding (ng / 10⁶ cells)

with streptavidin. Again, only the mature monomeric 84-kDa form of the hLHR was detected (data not shown). We presume that lack of labeling of either high molecular weight hLHR complex by biotin in intact cells may be due to the steric inaccessibility of the reactive lysines to biotin when the receptor is self-associated.

Agonist Increases Dimerization/Oligomerization of the Cell Surface hLHR in Stably Transfected Cells—We then examined the effects of hCG treatment on the abundance of the high molecular weight complexes in cells stably transfected with hLHR. Under basal conditions, the ratios of the 189- and ${\sim}240$ kDa hLHR complexes relative to the 84-kDa monomeric mature form of the hLHR on SDS gels ranged from 0.15-0.52 and 0.34–0.63, respectively. Upon incubation of the cells with hCG, there was a marked increase in the abundance of the 189- and ~240-kDa complexes at the higher concentrations of hCG (Fig. 13A). To compare several experiments quantitatively, we defined the ratios of the 189-kDa hLHR complex relative to the 84-kDa monomeric mature hLHR and the \sim 240-kDa hLHR complex relative to the 84-kDa monomeric mature hLHR in the basal state each to 1. Fig. 13B depicts the -fold increases in each of the two ratios with increasing hCG concentrations and shows an ~4-fold increase in each ratio at the highest concentration of hormone tested. These data clearly show that a portion of the cell surface hLHR in stably transfected cells exists as self-associated

complexes, presumably dimeric and oligomeric forms, under basal conditions and that the relative amounts of these complexes increase further with agonist treatment.

Importantly, the observation of an hCG-induced increase in the 189- and \sim 240-kDa complexes in stably transfected cells is consistent with their being localized to the cell surface, since hCG is not membrane-permeable. This is in contrast to the transiently transfected cells, where no effect of hCG on the abundance of the high molecular weight hLHR complexes was observed (Fig. 5). Immunohistochemistry experiments (Fig. 14) further show a co-localization of Myc-hLHR and FLAG-hLHR on the cell surface of stably transfected cells. Although cell surface co-localization is also observed in transiently co-transfected cells, most of the receptors co-localize in the ER.

DISCUSSION

Using a co-immunoprecipitation technique in 293 cells transfected with differentially tagged hLHRs, we show that the hLHR can be detected as two distinct high molecular weight complexes and that each is composed of self-associated hLHR. The molecular weights of the complexes are consistent with the smaller one being a dimer of the hLHR and the larger one a higher ordered oligomer. However, in the absence of a more definitive determination of the stoichiometry of each complex, the terms dimer and oligomer are used tentatively. One also must consider the possi-



FIG. 10. High molecular weight complexes of self-associated hLHR are also detected in stably transfected 293 cells. 293 cells were transiently or stably transfected with Myc-hLHR (A) or stably transfected with both Myc-hLHR and FLAG-hLHR (B). Lysates were not immunoprecipitated (A) or were immunoprecipitated (IP) with anti-FLAG (B). The samples were then resolved by SDS-PAGE under reducing conditions, and Western blots were probed with anti-Myc. The relevant portion of a representative gel is shown.



FIG. 11. hLHR self-association does not occur after solubilization. 293 cells were stably transfected with Myc-hLHR alone, with FLAG-hLHR alone, or with Myc-hLHR and FLAG-hLHR. Lysates were prepared and subjected to immunoprecipitation (*IP*) with anti-FLAG antibody. In one case (*third lane* from the *left*), the lysates from the Myc-hLHR cells and the FLAG-hLHR cells were first combined, and then the mixed lysate was subjected to immunoprecipitation with anti-FLAG antibody. Immunoprecipitates were resolved by SDS-PAGE under reducing conditions, and Western blots were probed with anti-Myc antibody. The relevant portion of a representative gel is shown.



FIG. 12. Glycosidase treatment and protease treatment of cells stably expressing the hLHR. 293 cells stably transfected with MychLHR were subjected to protease treatment (A), or cell extracts were incubated with endoglycosidases (B) as described under "Materials and Methods." Extracts were resolved by SDS-PAGE under reducing conditions, and Western blots were probed with anti-Myc antibody. The relevant portion of a representative gel is shown.



FIG. 13. Agonist-dependent increase in the abundance of cell surface hLHR dimers and oligomers in stably transfected cells. 293 cells were stably transfected with Myc-hLHR. They were incubated for 30 min at 37 °C with the indicated final concentrations of hCG. Cell lysates were prepared and resolved by SDS-PAGE under reducing conditions, and Western blots were probed with anti-Myc. A, one representative gel depicting the relevant portion of the gel is shown. B, gels from five independent experiments were scanned and quantified. For each gel, the ratio of the 189-kDa dimeric hLHR relative to the 84-kDa monomeric mature hLHR and the ratio of the ~240-kDa oligomeric hLHR relative to the 84-kDa monomeric mature hLHR were determined, and the ratios for the untreated cells were defined as 1. Within a given experiment, the -fold increases of each ratio in the treated samples versus the untreated samples were then determined. The graph depicts the mean \pm S.E. of the -fold increases in the ratios taken from the five experiments. Under basal conditions, the ratios of 189kDa hLHR/84-kDa hLHR ranged from 0.15 to 0.52 with the mean \pm S.E. being 0.34 \pm 0.07, and the ratios of ${\sim}240{\text{-kDa}/\text{84-kDa}}$ hLHR ranged from 0.34 to 0.63 with the mean \pm S.E. being 0.51 \pm 0.08.

bility that one or more other proteins may also be physically associated with the self-associated hLHR complexes.

The self-association of the hLHR was examined in transiently transfected cells and stably transfected cells, where the cellular localization of the hLHR was shown to differ markedly. Under the conditions used herein, the transiently transfected cells showed expression of the hLHR in a small percentage of cells (as would be expected), where receptor was apparent at the cell surface, but was mostly localized to the ER. In contrast, the stably transfected cells expressed hLHR in nearly all of the cells and the localization of receptor was predominantly at the cell surface, with very little in the ER. Importantly, whether the dimeric and oligomeric forms of the hLHR were found to be composed primarily of immature versus mature hLHR differed greatly between the two groups of transfected cells. In the transiently transfected cells, the sensitivities of the complexes to protease treatment and endoglycosidases suggest that they are composed mostly of immature receptor localized in the ER. We cannot rule out the possibility that a portion of hLHR dimer and/or oligomer in transiently transfected cells is composed of mature hLHR. Indeed, this is probably the case. However, its detection is made difficult by the large percentage of immature hLHR in these complexes in the transiently transfected cells. In contrast, in stably transfected cells, protease and endogly-

Co-expressed FLAG-hLHR and Myc-hLHR

Transients

Stables



FIG. 14. **Co-localization of Myc-hLHR and FLAG-hLHR co-expressed transiently** versus stably. 293 cells were transiently versus stably co-transfected with Myc-hLHR and FLAG-hLHR. The top panels show the visualization of the FLAG-hLHR, the middle panels show the Myc-hLHR, and the bottom panels show the merged images.

cosidase treatment readily suggest that the dimeric form of the hLHR in the stably transfected cells is composed mostly of mature cell surface receptor. Unfortunately, the composition of the oligomeric form cannot be ascertained using these methods, possibly due to the steric inaccessibility of the hLHR in the larger complex to enzymes. However, hCG treatment of stably, but not transiently, transfected cells increased the abundance of hLHR dimer and oligomer observed on Western blots. We cannot yet discriminate between whether hCG treatment increases the abundance of cell surface hLHR dimers and oligomers in vivo or whether hCG binding stabilizes constitutively expressed hLHR complexes to detergent solubilization (see below). Either scenario, however, would necessitate hCG binding to a cell surface hLHR, since hCG is not membranepermeable. Therefore, the observed lack of effect of hCG on the abundance of hLHR dimers and oligomers in transiently transfected cells and the hCG-induced increase in hLHR dimers and oligomers in stably transfected cells strongly supports our conclusion that the dimers and oligomers in the hLHR complexes in transiently transfected cells are composed mostly of immature intracellular receptor and that the complexes in stably

transfected cells are composed mostly of mature cell surface receptor. This conclusion is further supported by the observations that differentially tagged hLHRs co-localize to the cell surface and ER in transiently transfected cells, with most being in the intracellular compartment, whereas their co-localization is only observed on the cell surface of stably transfected cells.

The observation of differences in the composition of the hLHR dimers and oligomers in cells expressing most of the receptor on the cell surface and little in the ER (in this case in stably transfected cells) as opposed to cells expressing receptor on the cell surface but with most in the ER (in this case in transiently transfected cells) underscores the importance of ascertaining the cellular localization of a GPCR in cells used for the study of dimerization/oligomerization. For example, if much of the GPCR is localized to the ER, then the results obtained (by co-immunoprecipitation, fluorescent resonance energy transfer, or BRET) could be confounded by interactions between immature GPCRs in the ER as opposed to or in addition to cell surface GPCRs. Along these lines, the observation of apparently promiscuous heterodimerization between different GPCRs in transiently transfected cells might be attributed to

interactions of excess GPCRs in the ER if the receptors were overexpressed (39). It should be pointed out that the conditions used for the transient transfections of cells in this study utilized maximal concentrations of plasmid. It may be possible to prevent the accumulation of excess GPCR in the ER in transiently transfected cells by utilizing lower concentrations of plasmid. Also, although the cells stably transfected with hLHR exhibit little accumulation of receptor in the ER, this may not be true for all GPCRs. Indeed, our studies with cells stably transfected with the human thyrotropin receptor suggest a large accumulation of this receptor in the ER even under these conditions. Therefore, the issue is not necessarily whether cells are transiently *versus* stably transfected but rather that conditions are found where it can be verified by imaging that the GPCR is expressed primarily at the cell surface.

Despite the very different expression patterns of hLHR observed in the transiently *versus* stably transfected cells used in this particular study, receptor expression as quantified by hormone binding assays to intact cells was similar between the two groups of cells (if anything, the maximal binding capacity was somewhat higher in the stably transfected cells). Therefore, binding data on transiently transfected cells is misleading because it reflects the average of a small percentage of cells expressing an excessive amount of receptor with a larger number of cells that do not express any receptor. This can pose a serious caveat to the commonly used approach of utilizing binding data on cells transiently transfected with a GPCR as a means to determine whether or not the expression levels are physiological.

Significantly, dimerization and oligomerization of the cell surface hLHR were determined biochemically by co-immunoprecipitation of differentially tagged receptors in stably co-transfected cells. These complexes were observed under reducing conditions, confirming that the associations between hLHR molecules are not disulfide-linked. The high molecular weight complexes of hLHR observed on Western blots clearly are resistant to dissociation by SDS. We considered the possibility that a portion of each complex may be dissociating during exposure to SDS and that we are observing the residual complexes. If this were the case, then one would expect to observe the 84-kDa mature monomeric hLHR in the co-immunoprecipitation experiments in addition to the 169- and ~240-kDa complexes. However, the monomeric, mature form of the hLHR is not observed, suggesting that the complexes are not dissociating upon SDS treatment. That the self-associated complexes of hLHR are not disrupted by SDS is an intriguing but not unique observation. There have been several reports of other GPCRs that form self-associated complexes that are not disulfide bonded but are stable to SDS (40-43). We cannot, however, rule out the possibility that detergent solubilization of the cells disrupts self-associated complexes of the receptor. If this were occurring, we would not detect the dissociated 89-kDa monomeric mature hLHR in co-immunoprecipitation experiments, because the complex dissociation would occur prior to the immunoprecipitation step. Therefore, we cannot directly determine whether this is occurring. Interestingly, recent studies by Mercier et al. (44) using quantitative bioluminescence resonance energy transfer suggested that the majority of cell surface β -adrenergic receptor exists constitutively in self-associated forms. Similarly, studies by Guo et al. (45) using cysteine crosslinking of the dopamine receptor suggest that most or all cell surface dopamine receptors exist as homodimers in the plasma membrane and that detergent solubilization disrupts these complexes. It is possible, therefore, that the proportion of dimers and oligomers of hLHR detected in our study, both under basal conditions and after hCG treatment, may be an underestimate.

Previous studies have shown that the co-expression of a misfolded GPCR mutant with the wild-type cognate GPCR results in a decrease in the cell surface expression of the wild-type receptor. Dominant negative mutations affecting GPCR trafficking have been reported in, among other proteins, rhodopsin (30), the calcium-sensing receptor (31), platelet-activating factor receptor (32), dopamine receptor (33, 34), and chemokine receptor (35). We similarly show herein that two different hLHR mutants that are poorly expressed on the cell surface due to their retention in the ER, when transiently co-expressed with the wild-type hLHR, decrease the cell surface expression of wild-type hLHR. Our studies show that this is due to the heterodimerization/oligomerization within the ER of the misfolded, immature form of the mutant with the immature form of wild-type hLHR. The aberrant folding of the mutant presumably causes the heterocomplexes of mutant and wild-type hLHR as well as the homocomplexes of mutant hLHR to be retained in the ER and probably then degraded. Our observations raise the question of whether the heterodimerization/oligomerization of the wild-type and misfolded hLHRs is an artifact of the overexpression of receptor in transiently transfected cells (causing an accumulation of immature wild-type hLHR in the ER) or whether it would occur in stably transfected cells expressing physiological levels of receptor (or for that matter in gonadal cells in individuals heterozygous for misfolded inactivating hLHR mutations). Unfortunately, technical issues have thus far prevented us from resolving this question. It should be pointed out that individuals who are heterozygous for inactivating hLHR mutations that result in decreased cell surface expression of the mutant do not have detectable reproductive impairments (46). However, it is also known that gonadal cells have large numbers of spare receptors, and hence hormonal responsiveness does not decrease proportionally with decreased receptor numbers (47, 48). Therefore, it would not be inconsistent for there to be a heterodimerization/aggregation of mutant and immature wild-type hLHR in heterozygotes and a consequent decrease in the cell surface expression of wild-type hLHR in these individuals and yet observe normal reproductive functioning.

Although numerous GPCRs have been shown to form homodimers and oligomers (for reviews, see Refs. 2-4), the functional roles of the self-association of the cell surface GPCRs remain unclear. Since GPCR dimerization has at times been found to increase upon agonist stimulation (see Refs. 2-4) and because a peptide corresponding to the sixth transmembrane region of the β_2 -adrenergic receptor has been shown to inhibit both homodimerization and agonist-dependent stimulation of the receptor (40), it has been suggested that homodimerization may be involved with GPCR activation. There are, however, conflicting data in this area and, therefore, as for other GPCRs, the critical question of the physiological role of the self-association of cell surface hLHRs remains to be determined. The observation that immature hLHR in the ER also forms dimers and oligomers raises additional questions. Importantly, is this phenomenon solely an artifact of the overexpression of receptor in the transiently transfected cells, or is it more readily detected under those conditions but also occurring with more physiological levels of recombinant receptor in stably transfected cells or with the endogenous receptor in gonadal cells? Are the hLHR complexes in the ER a result of misfolded receptors aggregating together as a prelude to their being degraded, and/or do the correctly folded receptors first form dimers and oligomers in the ER and then move on to the plasma membrane? It is anticipated that ongoing and future studies will help to resolve these questions.

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