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Inefficient Maturation of the Rat Luteinizing Hormone Receptor

A PUTATIVE WAY TO REGULATE RECEPTOR NUMBERS AT THE CELL SURFACE*

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Increasing evidence suggests that the folding and maturation of monomeric proteins and assembly of multimeric protein complexes in the endoplasmic reticulum (ER) may be inefficient not only for mutants that carry changes in the primary structure but also for wild type proteins. In the present study, we demonstrate that the rat luteinizing hormone receptor, a G protein-coupled receptor, is one of these proteins that matures inefficiently and appears to be very prone to premature degradation. A substantial portion of the receptors in stably transfected human embryonic kidney 293 cells existed in immature form of M_r 73,000, containing high mannose-type *N*-linked glycans. In metabolic pulse-chase studies, only ~20% of these receptor precursors were found to gain hormone binding ability and matured to a form of M_r 90,000, containing bi- and multiantennary sialylated *N*-linked glycans. The rest had a propensity to form disulfide-bonded complexes with a M_r 120,000 protein in the ER membrane and were eventually targeted for degradation in proteasomes. The number of membrane-bound receptor precursors increased when proteasomal degradation was inhibited, and no cytosolic receptor forms were detected, suggesting that retrotranslocation of the misfolded/incompletely folded receptors is tightly coupled to proteasomal function. Furthermore, a proteasomal blockade was found to increase the number of receptors that were capable of hormone binding. Thus, these results raise the interesting possibility that luteinizing hormone receptor expression at the cell surface may be controlled at the ER level by regulating the number of newly synthesized proteins that will mature and escape the ER quality control and premature degradation.

The luteinizing hormone receptor (LHR)[1] mediates the actions of the large pituitary glycoprotein hormone, LH, and its placentally produced homolog, human chorionic gonadotropin (hCG) (1, 2). It belongs to the large G protein-coupled receptor (GPCR) family that is characterized by seven membrane-spanning hydrophobic domains (3). Its large extracellular domain (341 amino acids) contains several conserved cysteines that are thought to form disulfide bonds and comprises a repetitive leucine-rich motif that is found in other leucine-rich repeat-containing GPCRs, like the other glycoprotein hormone receptors, the follicle-stimulating hormone and thyroid-stimulating hormone receptors (1, 2).

Previous studies have shown that a substantial portion of glycoprotein hormone receptors in heterologous expression systems and natural tissues exists in an immature form (4–9), containing high mannose-type *N*-linked glycans, which are typical for proteins located in the endoplasmic reticulum (ER) (10). This suggests that these receptors might mature inefficiently, a phenomenon that has been found to characterize a few other GPCRs (11–15). For example, ~50% of δ opioid receptors are retained in the ER and are targeted for ER-associated degradation (ERAD) in proteasomes (12, 16), a pathway that has subsequently been shown to dispose also other GPCRs, both wild type and mutant forms (14, 17–20). This elimination of newly synthesized receptors may result, at least to some extent, from limitations in the folding kinetics, because a proteasomal blockade was found to lead to enhanced ER export and maturation of wild type δ opioid (16) and olfactory receptors (21) and mutant rhodopsin (17).

These data led us to hypothesize that inefficient processing and maturation might be a more common property among GPCRs than has been thought before and may provide a means to control the number of receptors at the cell surface. In an attempt to test this notion, we set out to examine processing and maturation of the rat LHR in stably transfected human embryonic kidney (HEK)-293 cells in more detail and determine, whether the newly synthesized receptors are prone to premature ERAD. The results demonstrate that the LHRs that gain hormone binding ability are exported out of the ER and reach the cell surface. However, a substantial portion of the newly synthesized receptors does not appear to fold correctly and tends to form disulfide-bonded heterocomplexes and are eventually targeted for degradation in proteasomes. Furthermore, proteasomal blockade was found to lead to enhanced maturation of the receptor. Thus, proteasomal degradation appears to modulate the efficiency of LHR maturation and in doing so may control the expression of mature receptors at the cell surface.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—EXPRE^{35}S^{35}S protein-labeling mix (1,175 Ci/mmol) was obtained from PerkinElmer Life Sciences, and Na[^{125}I]iodide (100 mCi/ml) was from Amersham Biosciences. Human CG (10,000 units/mg, 3,000 units/mg), *N*-ethylmaleimide (NEM), anti-FLAG M2 antibody affinity resin, and FLAG peptide were purchased from Sigma, and SDS-PAGE reagents were from Bio-Rad. Peptide-*N*-

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[1] The abbreviations used are: LHR, luteinizing hormone receptor; Endo, Endo-β-*N*-acetylglucosaminidase; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GPCR, G protein-coupled receptor; hCG, human chorionic gonadotropin; HEK, human embryonic kidney; NEM, *N*-ethylmaleimide; PNGase F, peptide-*N*-glycosidase F.

glycosidase F (PNGase F, EC 3.2.2.52), endoglycosidase H (Endo H, EC 3.2.1.96), and endo-β-galactosidase (EC 3.2.1.103) were purchased from Roche Molecular Biochemicals. Endoglycosidase F_2 (Endo F_2, EC 3.2.1.96) and *O*-glycosidase (EC 3.2.1.97) were from Genzyme, and neuraminidase (EC 3.2.1.18) and proteasomal inhibitors were from Calbiochem. Pregnant mare serum gonadotropin (2,820 units/mg) was from Diosynth. The cell culture reagents were obtained from Invitrogen or Bioclear. The rabbit polyclonal antisera directed against the C-terminal domain of the rat LHR (Gly^{646}-His^{700}), and hCG have been characterized previously (22, 23).

Rat Tissue Collection—Adult Sprague-Dawley male rats (90 days) and immature female rats (25–27-day old) rendered pseudopregnant (24) were killed by inhalation of carbon dioxide. Tissue samples were removed immediately, frozen in liquid nitrogen, and stored at −70 °C. The use of animals was approved by the University of Oulu Committee for the Care of Experimental Animals.

DNA Constructs—The full-length rat LHR cDNA (GenBank™ accession M26199) was subcloned into the EcoRI restriction site of the pcDNA3 expression vector (Invitrogen). The DNA construct encoding the rat LHR with a cleavable influenza hemagglutinin signal peptide (KTIIALSYIFCLVFA), N-terminal Myc tag (EQKLISEEDL) and C-terminal FLAG tag (DYKDDDDK) was created by amplifying the cDNA by polymerase chain reaction using oligonucleotides 5′-CAGCTGATCGA-TCGAGAGCTGTCAGGGTCG-3′ and 5′-AATTCCTAGGCTAGTGAGT-TAACGCTCTCG-3′, digested with ClaI/BlnI, ligated into the modified pcDNA5/FRT/TO© vector (Invitrogen) (called pFT-SMMF) and transformed into *Escherichia coli* JM109. An accidentally included Stop-codon was removed by digesting with HpaI/BlnI and inserting an adapter without TAG-codon. The pFT-SMMF vector was created by ligating the HindIII/KpnI-digested pcDNA5/FRT/TO© vector, inserting the DNA sequence 5′-AAGCTTGCCACCATGAAGACGATCATCGCCC-TGAGCTACATCTTCTGCCTGGTATTCGCCGAGCAAAAGCTCATTT-CTGAAGAGGACTTGGCTAGCATCGATCCCGGGATGCATCCTAGG-GACTACAAGGACGACGATGACAAGTGAGGTACC-3′, and transforming into *E. coli* JM110.

Cell Culture and Transfection—Cells were grown and maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, 100 units of penicillin/ml, 100 μg of streptomycin/ml (complete Dulbecco's modified Eagle's medium), and the appropriate selection antibiotics in a humidified atmosphere of 5% (v/v) CO_2 at 37 °C. HEK-293S cells were transfected with the full-length rat LHR cDNA in pcDNA3 using FuGENE 6 (Roche Applied Science), and clones of stably transfected cells were selected using 400 μg/ml of G-418 (Calbiochem). HEK-293 cells stably expressing the epitope-tagged rat LHR under tetracycline induction were prepared as described elsewhere.[2] For a few experiments, a stably transfected HEK-293 cell line obtained from Dr. Rolf Sprengel (Max Planck Institute for Medical Research, Heidelberg, Germany) was used (see Figs. 1–3). For these cells, Dulbecco's modified Eagle's medium was replaced with minimum essential medium with Earle's salts. The level of receptor expression was estimated by Western blotting. Cells were subcultured in 25-cm^2 or 75-cm^2 culture flasks or 60-mm culture plates, grown to 70–90% confluency prior to experiments, harvested in phosphate-buffered saline containing 20 mM NEM, quick-frozen in liquid nitrogen, and stored at −70 °C.

Metabolic Pulse-Chase Labeling with [^{35}S]Methionine/Cysteine—For pulse-chase labeling, cells were first preincubated in methionine and cysteine-free Dulbecco's modified Eagle's medium for 60 min at 37 °C and then labeled in fresh medium containing 150 μCi/ml [^{35}S]methionine/cysteine. After an incubation of 60 min at 37 °C, the pulse was terminated by washing the cells twice with the chase medium (complete Dulbecco's modified Eagle's medium supplemented with 5 mM methionine) and chased for different periods of time as specified in the figures. When labeling was performed in the presence of proteasome inhibitors, the reagents were added 2 h before the pulse labeling at a final concentration of 10 μM.

Isolation of Cell Surface Receptors—For steady state labeling, [^{35}S]methionine/cysteine (150 μCi/ml) was added to the culture medium for 16 h. Cells were then cooled on ice and incubated for 60 min in the presence of hCG (1 mg/ml) before harvesting. The cell surface receptors were immunoprecipitated using a polyclonal anti-hCG antibody, as described below.

Preparation and Solubilization of Crude Membrane Particles and Cellular Lysates—Ovarian (1.7 g) and testicular samples (8 mg) and cell pellets were suspended in buffer A (phosphate-buffered saline, pH 7.4, 5 mM EDTA, 5 mM NEM, 0.2 mM phenylmethylsulfonyl fluoride) and homogenized with a Polytron homogenizer (Ultra-Turrax T-25, Janke and Kunkel) or by squeezing several times through a 26-gauge needle. Cells expressing the epitope-tagged LHR were homogenized in buffer B (25 mM Tris-HCl, pH 7.4, 20 mM NEM, 2 mM EDTA, 2 μg/ml aprotinin, 0.5 mM PMSF, 2 mM 1,10-phenanthroline, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine). Homogenates were centrifuged at 180 × *g* for 5 min, and the pellets were rehomogenized and recentrifuged. The combined supernatants were further centrifuged at 45,000 × *g* for 30 min, and after washing once with buffer A or B, the crude membrane pellets were used either immediately or quick-frozen in liquid nitrogen and stored at −70 °C. Alternatively, the metabolically labeled cells were sonicated in buffer C (buffer A containing 5 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine). Protein was measured using a Bio-Rad DC assay kit with bovine serum albumin as a standard.

The membrane particles were solubilized in SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromphenol blue) at 22 °C for 30 min and subjected to SDS-PAGE after pelleting the unsolubilized fraction at 16,000 × *g* for 30 min (see Fig. 1*A*). Alternatively, the membranes were solubilized by stirring on ice for 60 min in buffer D (buffer C containing 0.5% (v/v) Triton X-100 and 20% (v/v) glycerol) or buffer E (buffer B containing 0.5% (w/v) *n*-dodecyl-β-D-maltoside and 140 mM NaCl) and centrifuging at 100,000 × *g* for 60 min. The cellular lysates were prepared by solubilizing the thawed cells directly in buffer D. After centrifugation, the soluble fractions were supplemented with 0.1% (w/v) bovine serum albumin and subjected to immunoprecipitation or ligand affinity chromatography.

Immunoprecipitation and Ligand Affinity Chromatography—Immunoprecipitation was performed using a receptor-specific polyclonal antibody directed against the C-terminal domain of the receptor (purified antibody 5 μg/ml, antiserum 1:50) or a polyclonal anti-hCG antiserum (1:100). Samples were first precleared for 60 min using 20 μl of protein G-Sepharose (Amersham Biosciences), and after adding the antibody and protein G-Sepharose they were incubated overnight at 4 °C with gentle agitation. The resin was washed twice with buffer D, twice with buffer F (buffer A containing 0.1% (v/v) Triton X-100, 20% (v/v) glycerol, and 0.36 M NaCl) and twice with buffer G (buffer A containing 0.1% (v/v) Triton X-100 and 20% (v/v) glycerol). The receptors were eluted by incubating the resin for 5 min at 95 °C in SDS-sample buffer. The epitope-tagged LHR was immunoprecipitated as described previously (12). Ligand affinity chromatography was performed using hCG-Affi-Gel 10, which was prepared as described previously (24). The solubilized receptors were incubated with 20 μl of the resin overnight at 4 °C, washed, and eluted as described above.

Enzymatic Digestions of Crude Membrane Particles and Purified LHRs—For glycosidase treatments, the crude membrane particles were solubilized in 1% (w/v) SDS, 50 mM sodium phosphate, pH 5.0 (Endo H) or pH 7.0 (PNGase F), 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride at 22 °C for 60 min. Before centrifugation at 16,000 × *g* for 30 min, the samples were diluted 10-fold by adding 0.5% (v/v) Triton X-100, 50 mM sodium phosphate (pH 5.0 or 7.0), 1% (v/v) β-mercaptoethanol, 50 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride. For PNGase F digestion, the buffer also contained 2 mM 1,10-phenanthroline. After centrifugation, aliquots of the supernatants were heated for 5 min at 95 °C and subjected to digestion with Endo H (100 milliunits/ml) or PNGase F (5 units/ml) at 30 °C for 16 h. The reaction was stopped by adding 4× SDS-sample buffer. The hCG-Affi-Gel 10-purified ^{125}I-labeled receptor was treated with glycosidases as described earlier (25).

Subcellular Fractionation—Cells were thawed and homogenized in buffer H (buffer C containing 2 mM 1,10-phenanthroline and 2 μg/ml aprotinin) using a Dounce homogenizer (30 strokes with the loose pestle and 30 with the tight one). The homogenates were first centrifuged at 1,000 × *g* for 10 min to pellet unbroken cells and nuclei. The supernatant was then centrifuged at 10,000 × *g* for 30 min to pellet the crude membranes, and the supernatant was further centrifuged at 100,000 × *g* for 60 min to clarify the cytosolic fraction. The pellets were washed once with buffer H, centrifuged as above, and solubilized in buffer H containing 0.5% (v/v) Triton X-100. After mixing the suspension on a magnetic stirrer for 60 min, the samples were clarified by centrifugation at 100,000 × *g* for 60 min. Immunoprecipitations from the soluble fraction (supplemented with 0.5% (v/v) Triton X-100), and the solubilized membranes were carried out using the receptor-specific polyclonal antibody in two sequential steps. The bound antigens were first eluted with 1% (w/v) SDS, phosphate-buffered saline, pH 7.4, by incubating at 95 °C for 5 min, and the eluates were diluted 10-fold with buffer H containing 0.5% (v/v) Triton X-100 before the next immunoprecipitation step. The second elution was performed by incubating in SDS-sample

[2] T. T. Leskelä, P. M. H. Markkanen, E. M. Pietilä, J. T. Tuusa, and U. E. Petäjä-Repo, manuscript in preparation.

buffer at 95 °C for 5 min. Denaturation of the samples and the second immunoprecipitation step were necessary because of a nonspecific protein in the cytosolic fraction that co-migrated with the receptor precursor on SDS-PAGE.

SDS-PAGE, Ligand, and Immunoblotting—For SDS-PAGE (7.5% separating gels), samples were denatured by heating at 95 °C for 2 min in the absence or presence of 50 mM dithiothreitol. Molecular mass markers (Bio-Rad) detected by staining with Coomassie Brilliant Blue (Bio-Rad) were used to calibrate the gels. For two-dimensional SDS-PAGE, samples were first run under non-reducing conditions, and a 4-mm strip of the slab gel was cut out and dehydrated by incubating in CH_3CN at 22 °C for 15 min and drying in a SpeedVac concentrator (Savant Instruments). The gel strip was rehydrated in 2% (w/v) SDS, 62.5 mM Tris-HCl, pH 6.8, and 50 mM dithiothreitol at 70 °C for 20 min and after incubation in 100 mM iodoacetamide, 62.5 mM Tris-HCl, pH 6.8, at 22 °C for 60 min, it was placed onto a preparative well on another slab gel. For detection of radioactivity, the gels were treated with EN^{3}HANCE® (PerkinElmer Life Sciences) according to the manufacturer's instructions, dried, and exposed at −70 °C for 1–20 days, using Biomax MR (Eastman Kodak Co.) or Fuji x-ray film (Fuji Photo Film Co.) and intensifying screens. The relative intensities of the bands on the autoradiograms were analyzed by densitometric scanning with Agfa Duoscan HiD laser scanner and quantified using the Scion Image 4.02 software, subtracting the local background from each lane. The data were analyzed using the GraphPad Prism 4.02 software.

For immuno- and ligand blotting, the proteins resolved in SDS-PAGE were transferred electrophoretically to either nitrocellulose (Schleicher & Schuell) or Immobilon P sheets (Millipore). The bound proteins were probed using the polyclonal LHR antibody, followed by horseradish peroxidase-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch). Enhanced chemiluminescence Western blotting detection reagents from Amersham Biosciences were used to reveal the blotted proteins. Ligand blotting was performed by incubating the nitrocellulose sheets with [^{125}I]hCG in the absence or presence on an excess of unlabeled hCG, as described (24).

RESULTS

HEK-293 Cells Express Two LHR Forms of M_r 73,000 and 90,000—To identify the LHRs expressed in HEK-293 cells, solubilized membranes from stably transfected cells were subjected to SDS-PAGE and immunoblotting (Fig. 1*A*). A receptor-specific antibody directed against the C-terminal domain of the receptor recognized two bands with apparent molecular weights of 90,000 and 73,000 (Fig. 1*A*, *lane 2*), the relative intensities of which varied from one experiment to another. These two bands were not present in non-transfected cells (Fig. 1*A*, *lane 1*), indicating that they specifically represent the expressed receptor.

Ligand blotting was then applied to investigate whether the two receptor species detected on Western blots are able to bind hormone. The solubilized membrane proteins were resolved by SDS-PAGE under non-reducing conditions and transferred onto nitrocellulose sheets (Fig. 1*A*). Incubation of the sheets with [^{125}I]hCG revealed specific binding to the M_r 90,000 and 73,000 receptor species (Fig. 1*A*, compare *lane 4* with *6*). The M_r 73,000 receptor band was less intense on ligand blots than on immunoblots, whereas the reverse was true for the M_r 90,000 receptor band (Fig. 1*A*, compare *lane 2* with *4*). The most likely explanation for this observation is that a large fraction of the smaller receptor species is misfolded/incompletely folded and thus is unable to bind ligand (see below). When the samples were reduced prior to SDS-PAGE, no radiolabeled bands were detected (data not shown), indicating that correct conformation for ligand binding requires intact disulfide bonds.

The identities of the two LHR species were studied further by treating the receptors with glycosidases (Fig. 1*B*). Endo H selectively removes unprocessed high mannose-type *N*-linked oligosaccharides from glycoproteins but does not cleave complex type, fully processed glycans (26). Treatment of the membrane-bound proteins with this enzyme did not change the molecular weight of the M_r 90,000 receptor species but reduced that of the M_r 73,000 one to M_r ~62,000 (Fig. 1*B*, *lane 2*). This

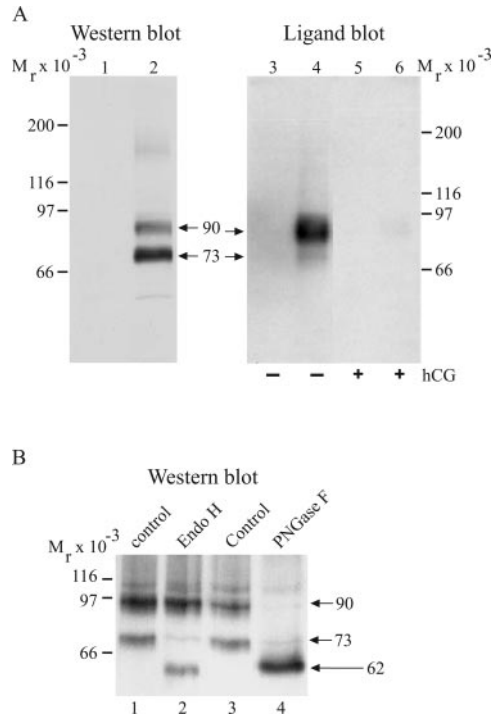

FIG. 1. **Identification of the LHR species expressed in HEK-293 cells.** *A*, crude membrane particles from non-transfected (*lanes 1*, *3*, and *5*) and stably transfected HEK-293 cells expressing the rat LHR (*lanes 2*, *4*, and *6*) were solubilized in SDS sample buffer, subjected to SDS-PAGE under reducing (*lanes 1* and *2*) and non-reducing conditions (*lanes 3–6*), and transferred onto nitrocellulose sheets. The sheets were probed with a polyclonal antibody raised against the C-terminal domain of the rat LHR (*lanes 1* and *2*) or with [^{125}I]hCG in the absence (*lanes 3* and *4*) or presence (*lanes 5* and *6*) of an excess of unlabeled hCG. *B*, SDS/Triton X-100-solubilized crude membrane particles were incubated for 16 h at 30 °C with Endo H (100 milliunits/ml) or PNGase F (5 units/ml). The enzyme-free controls contained buffer only. Reactions were stopped by adding SDS sample buffer, and samples were analyzed by SDS-PAGE and immunoblotting. Molecular weight markers used to calibrate the gels are indicated.

demonstrates that the M_r 73,000 receptor species does not represent a degradation product but rather an intact receptor polypeptide bearing high mannose-type oligosaccharides, whereas the M_r 90,000 receptor species appears to contain complex-type *N*-linked oligosaccharides. This was further supported by the observation that PNGase F, an enzyme that can remove all types of *N*-linked oligosaccharides from glycoproteins (26), was able to digest this receptor species (Fig. 1*B*, *lane 4*). As anticipated, PNGase F was also able to increase the electrophoretic mobility of the M_r 73,000 receptor form (Fig. 1*B*, *lane 4*).

The oligosaccharides of the M_r 90,000 LHR form were studied in more detail by subjecting this species to various exo- and endoglycosidase treatments (Fig. 2). The M_r 90,000 receptor was purified by affinity chromatography on hCG-Affi-Gel 10, labeled with ^{125}I, and separated from the M_r 73,000 species by electroelution from the SDS gel prior to glycosidase treatments. The *N*-linked oligosaccharides were first characterized by treating the receptor with Endo F_2, an enzyme that is able to remove biantennary *N*-linked glycans (26). As seen in Fig. 2, *lane 3*, this enzyme decreased the apparent M_r of the receptor to ~87,000. As complete removal of *N*-linked glycans with PNGase F decreases the M_r to ~62,000 (Fig. 2, *lane 2*), the M_r 90,000 receptor appears to contain some biantennary but also tri- or tetra-antennary *N*-linked oligosaccharides. The receptor was also treated with exoglycosidases, endo-β-galactosidase, and neuraminidase, which decreased the electrophoretic mobility of the M_r 90,000 receptor to about 87,000 and 82,000,

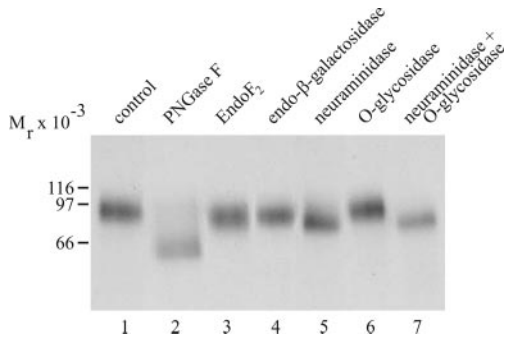

FIG. 2. **Characterization of oligosaccharides of the M_r 90,000 LHR.** Receptors from stably transfected HEK-293 cells were purified by hCG-affinity chromatography, radioiodinated, and subjected to SDS-PAGE. The M_r 90,000 LHR was eluted from the SDS gel and treated with exo- and endoglycosidases at 30 °C. *Lane 1*, untreated control; *lane 2*, PNGase F (2 units/ml, 2 h); *lane 3*, Endo F_2 (4 milliunits/ml, 16 h); *lane 4*, endo-β-galactosidase (20 milliunits/ml, 4 h); *lane 5*, neuraminidase (10 milliunits/ml, 1 h); *lane 6*, *O*-glycosidase (25 milliunits/ml, 16 h); *lane 7*, neuraminidase (10 milliunits/ml, 1 h) and *O*-glycosidase (25 milliunits/ml, 16 h). All reactions were stopped by adding SDS sample buffer, and samples were analyzed by SDS-PAGE and fluorography. The dried gel was exposed to x-ray film to obtain the fluorograph shown.

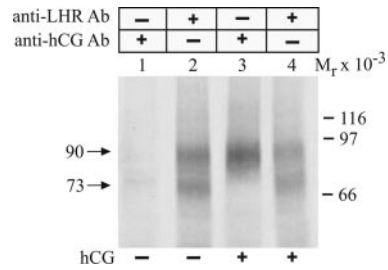

FIG. 3. **Isolation of the LHR species expressed at the cell surface.** Stably transfected HEK-293 cells were labeled for 16 h with 150 μCi of [^{35}S]methionine/cysteine and incubated with or without hCG (1 mg/ml) for 60 min on ice, as indicated. Crude cellular membranes were solubilized and subjected to immunoprecipitation using either a polyclonal receptor-specific antibody or anti-hCG antibody. Immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography.

respectively (Fig. 2, *lanes 4* and *5*). This suggests that the *N*-linked glycans contain poly-*N*-acetyllactosamine sequences (27), and sialic acids linked either α2-3 or α2-3 to galactose (28). Finally, the presence of *O*-linked oligosaccharides was evaluated by subjecting the ^{125}I-labeled receptor to *O*-glycosidase treatment before or after sialic acid removal with neuraminidase. Because this enzyme had no effect on the electrophoretic mobility of the receptor (Fig. 2, *lanes 6* and *7*), it is unlikely that it contains *O*-linked glycans. Thus, these data indicate that the M_r 90,000 receptor species has been processed in the Golgi and therefore represents the fully mature receptor at the cell surface. Furthermore, processing of the *N*-glycans in the stably transfected HEK-293 cells appears to occur in a similar manner than in natural target tissues, rat ovaries (25).

To further investigate the cellular location of the M_r 90,000 and 73,000 receptor species, we used a more direct approach (Fig. 3). Cells were incubated with 150 μCi/ml [^{35}S]methionine/cysteine for 16 h to metabolically label the two receptor species, and after placing the cell culture plates on ice, unlabeled hCG (1 mg/ml) was added for 60 min. This allowed us to label the cell surface receptors, because the hormone is unable to cross the cellular membranes. Membranes were then isolated and solubilized receptors and receptor-hormone complexes were immunoprecipitated using anti-receptor or anti-hCG antibodies, respectively, and analyzed by SDS-PAGE and fluorography. As shown in Fig. 3, *lane 3*, only the M_r 90,000 receptor species was immunoprecipitated with the anti-hCG antibody, confirming that this receptor form is expressed at the cell surface. In contrast, the M_r 73,000 receptor was immunoprecipitated only with the receptor antibody (Fig. 3, *lane 4*), suggesting that it is confined to an intracellular compartment.

Taken together, our observations indicate that the M_r 90,000 LHR is likely to represent fully mature cell surface receptors containing complex-type *N*-linked glycans, whereas the M_r 73,000 species is a biosynthetic intermediate that is located in an intracellular compartment, most likely in the ER.

Maturation of Newly Synthesized LHRs Is Inefficient, and Only Receptors Gaining Hormone Binding Ability Are Exported Out of the ER—The relatively low ratio of the M_r 90,000 LHR form to the M_r 73,000 one detected at steady state (see Fig. 1*A*) could be due to either inefficient maturation of the M_r 73,000 species or to the instability of the M_r 90,000 receptor form at the plasma membrane. To resolve this issue, pulse-chase experiments were performed using stably transfected HEK-293S cells (Fig. 4). Cells were labeled for 60 min in a methionine/cysteine-free medium containing 150 μCi/ml [^{35}S]methionine/cysteine and subsequently chased for periods of up to 20 h in a medium containing an excess of unlabeled methionine. At each time point, cells were harvested, and membranes were prepared. The solubilized receptors were immunoprecipitated and subjected to SDS-PAGE followed by fluorography. As shown in Fig. 4*A*, the M_r 73,000 band was the first component to be labeled during the pulse, and during the subsequent chase, label incorporated into this band decreased in parallel with the appearance of the M_r 90,000 form, confirming the precursor-product relationship between the M_r 73,000 and 90,000 LHR species and the fact that the latter receptor species corresponds to the fully mature form of the receptor.

The kinetic relationships between the two LHR species were assessed by densitometric scanning of the autoradiograms (Fig. 4*C*). The half-life for the M_r 90,000 mature receptor species was about 23 h (23 ± 8.0 h, mean ± S.E.), whereas the turnover rate for the smaller receptor form was substantially faster (137 ± 12 min). The relatively long halftime for conversion of the precursor to the mature form (129 ± 5 min) suggests that export from the ER is a limiting step in the overall maturation process of the receptor. Furthermore, the maturation efficiency was quite low, because the fractional conversion of the precursor to the mature form was ~20% (19.8 ± 1.9%) of the maximum label incorporated into the receptor precursor. Thus, the low ratio of the M_r 90,000 LHR species to the M_r 73,000 ones detected at steady state (see Fig. 1*A*) is likely to result from an inefficient maturation of the M_r 73,000 species.

As only a small fraction of newly synthesized LHRs appears to be processed to fully mature cell surface receptors, they may have an intrinsic tendency to fail in achieving the correct conformation. This was supported by the observation that only a small fraction of receptor precursors was detected by ligand blotting (see Fig. 1*A*), despite the fact that intracellular LHRs have been shown to be able to bind hormone with high affinity (29). Thus, we set out to determine whether only those receptor precursors that gain hormone binding ability are exported from the ER. To this end, the metabolically labeled receptors were subjected to hCG-affinity purification. As shown in Fig. 4, *B* and *D*, all of the M_r 73,000 receptor precursors that were able to bind hormone at the end of the pulse were converted to the mature M_r 90,000 species. This suggests that the precursors that are not converted to the mature form (see Fig. 4*A*) most likely represent misfolded or incompletely folded receptors.

Inefficient Maturation of LHRs Is Not because of Overexpression in a Heterologous Expression System—It could be argued that the inefficient maturation of LHRs in HEK-293S cells might be because of overexpression of the receptor in a heterologous expression system. These cells might lack a factor that

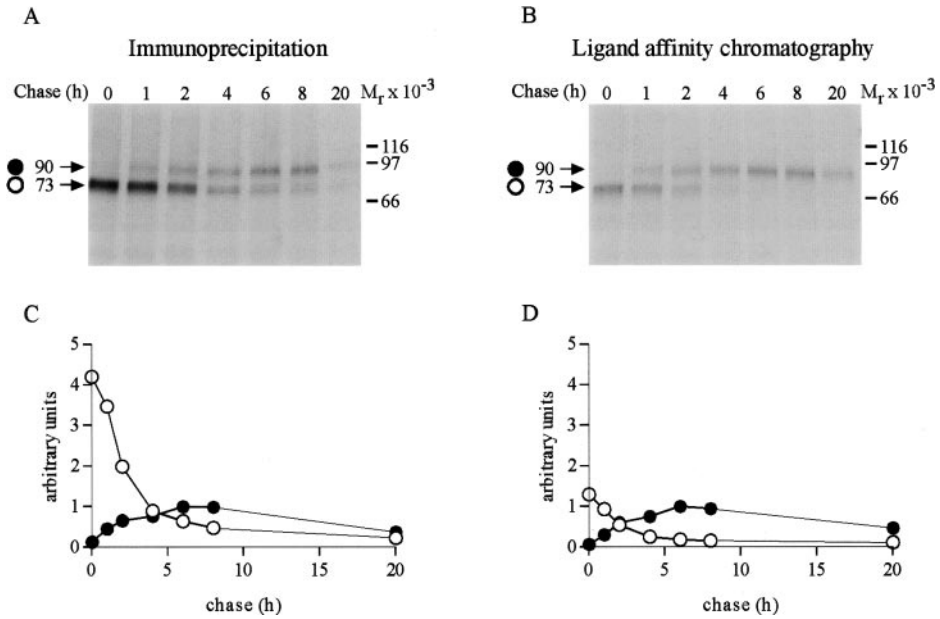

FIG. 4. **Pulse-chase analysis of the LHR synthesis and processing.** Stably transfected HEK-293S cells were pulse-labeled for 60 min with 150 μCi of [^{35}S]methionine/cysteine and chased with complete medium supplemented with 5 mM methionine for the indicated times. Crude cellular membranes were isolated, and the solubilized receptors were divided into two aliquots and subjected either to immunoprecipitation using a receptor-specific polyclonal antibody (*A*) or hCG-affinity chromatography (*B*). The purified samples were analyzed by SDS-PAGE and fluorography. *Arrows* indicate the different receptor forms (●, M_r 90,000; ○, M_r 73,000). *C* and *D* describe the time course of appearance and disappearance of the two labeled receptor species. The symbols refer to those used to identify the different receptor forms. The intensities of the immature and mature LHR species were obtained by densitometric scanning, and the values were normalized to the maximum labeling of the mature receptor at 8 h of chase.

is required for normal receptor processing and trafficking or such a factor may become limiting if the expression level is high. To test directly the possibility that the low maturation efficiency could be because of high expression level attained in stably transfected HEK-293S cells, we expressed the LHR containing an N-terminal Myc epitope tag and a C-terminal FLAG epitope tag under tetracycline induction. This allowed us precise control of the expression level. The cells were treated with an increasing concentration of tetracycline for 24 h, and the receptors were purified by immobilized anti-FLAG M2 antibody and analyzed by immunoblotting. As shown in Fig. 5*A*, the ratio of immature and mature receptors remained unaltered irrespective of the expression level that was attained in these cells. Importantly, no increase in expression of BiP was detected in these cells (data not shown), indicating that the high LHR expression did not induce an unfolded protein response that is a common consequence of accumulation of misfolded proteins in the ER (30). A similar low efficiency of maturation was observed when the LHR was expressed in Chinese hamster ovary cells (data not shown). Taken together, these results indicate that the inefficient conversion of LHR precursors to the mature form is not peculiar to HEK-293S cells nor it is likely to be a consequence of overexpression or result from a differential ability of the receptor-specific antibody to recognize immature and mature receptor forms. Furthermore, the low intrinsic maturation efficiency appears to characterize the LHR also in natural LH target tissues, as both the precursor and mature receptor forms were detected in rat ovaries and testes (Fig. 5*B*, *lanes 1* and *2*, respectively).

Degradation of Misfolded/Incompletely Folded Newly Synthesized LHRs Is Mediated by Proteasomes—Because a large fraction of newly synthesized LHRs is not exported from the ER, we investigated whether proteasomes are involved in degradation of these receptor forms. The stably transfected HEK-293S cells were labeled with [^{35}S]methionine/cysteine for 60 min in the presence or absence of lactacystin, a specific proteasomal inhibitor (31) and harvested immediately or chased for

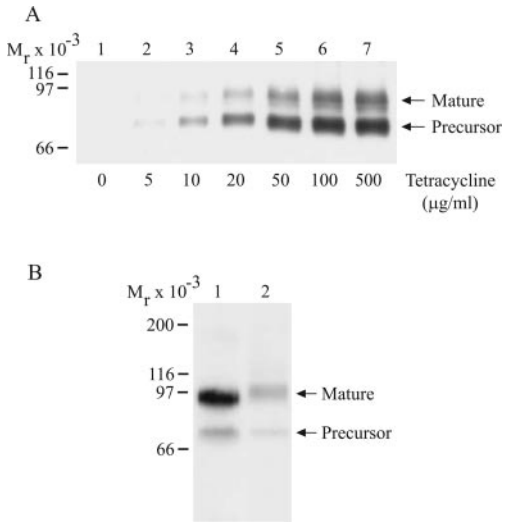

FIG. 5. **LHR expression in an inducible expression system and natural tissues.** *A*, expression of the N-terminally c-Myc-tagged and C-terminally FLAG-tagged LHR in HEK-293 cells was induced by incubating the cells for 24 h at 37 °C in the presence of an increasing concentration of tetracycline, as indicated. Total cellular membranes were isolated, and the solubilized receptors were purified by immunoprecipitation using an immobilized anti-FLAG M2 antibody. Aliquots of the eluates were analyzed by immunoblotting using the receptor-specific polyclonal antibody for detection. *B*, LHRs of pseudopregnant rat ovaries (*lane 1*) and testes (*lane 2*) were purified by hCG-affinity chromatography and analyzed by immunoblotting using the receptor-specific polyclonal antibody.

2 h. Cell lysates were prepared, and receptors were isolated by immunoprecipitation or hCG-affinity chromatography and analyzed by SDS-PAGE and fluorography (Fig. 6*A*). When the cells were treated with lactacystin, a clear increase in the labeling of the immunoprecipitated M_r 73,000 receptor species was detected either at 0 or 2 h of chase (Fig. 6*A*, compare *lanes 1* and *2* with *lanes 3* and *4*). Similar results were obtained using

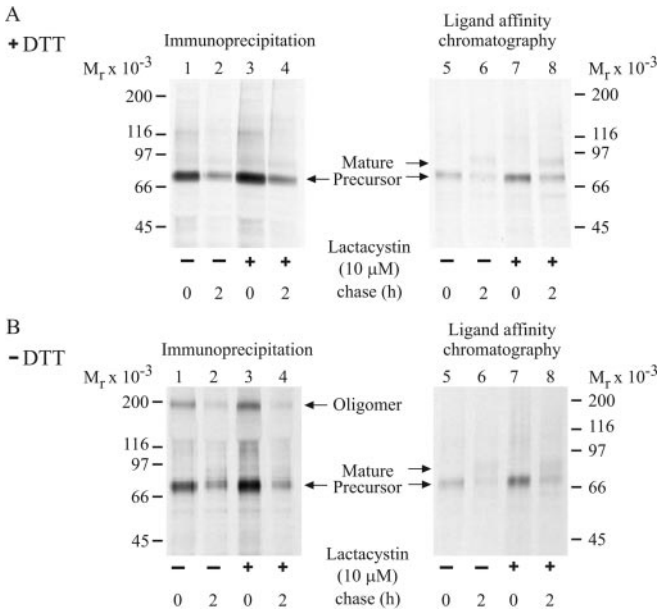

FIG. 6. **Blocking of proteasomal degradation with lactacystin.** Stably transfected HEK-293S cells were pulse-labeled for 60 min with [^{35}S]methionine/cysteine in the presence or absence of lactacystin and either harvested immediately or chased for 2 h in the continued presence of the proteasome inhibitor. Cellular lysates were prepared, and the solubilized receptors were subjected to immunoprecipitation or hCG-affinity purification. The samples were analyzed by SDS-PAGE under reducing (*A*) or non-reducing (*B*) conditions. *DTT*, dithiothreitol.

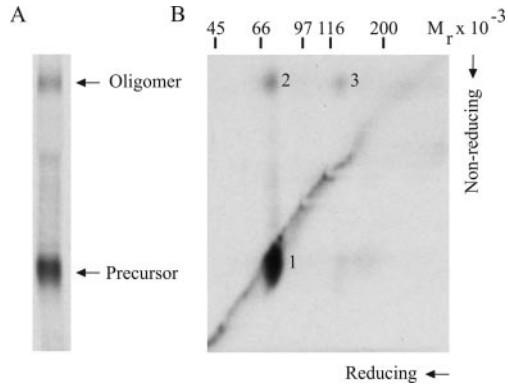

FIG. 7. **Two-dimensional SDS-PAGE analysis of the M_r 180,000 LHR complex.** Stably transfected HEK-293S cells were pulse-labeled for 60 min with [^{35}S]methionine/cysteine in the presence of lactacystin. Cellular lysates were prepared, and the solubilized receptors were subjected to immunoprecipitation using a receptor-specific polyclonal antibody. The samples were divided into two aliquots and analyzed either by one-dimensional SDS-PAGE under non-reducing conditions (*A*) or by two-dimensional SDS-PAGE, first under non-reducing and then under reducing conditions (*B*). *Spot 1* indicates the monomeric LHR precursor in different oxidation states (as evidenced by its elongated shape below the diagonal), and *spot 2* the LHR precursor in the disulfide-bonded complex. *Spot 3* represents an unknown protein interacting with the receptor precursor.

two other proteasomal inhibitors, epoxomicin and MG-132 (data not shown). These results suggested an active role of proteasomes in the elimination of the misfolded/incompletely folded newly synthesized LHRs. Importantly, the inhibition of proteasomal degradation led also to a 1.7-fold increase in the amount of immature and mature receptors that were purified by hCG-affinity chromatography (Fig. 6*A*, compare *lanes 5* and 6 with *lanes 7* and *8*), suggesting that some receptor precursors apparently are targeted for disposal prematurely, most likely because of their slow folding kinetics.

Misfolded/Incompletely Folded Newly Synthesized LHRs Form Disulfide-bonded Complexes in the ER and Do Not Accumulate in the Cytosol—Next we tested the possibility that the newly synthesized misfolded or incompletely folded LHRs might form high molecular weight complexes in the ER. This was suggested by the observation that high molecular weight receptor forms were occasionally observed on Western blots (see Fig. 1*A*, *lane 2*). We reasoned that these complexes might result from inappropriate formation of intermolecular disulfide bonds, because the extracellular domain of the receptor contains several conserved cysteines. It is thought that these cysteines form disulfide bonds, which are required for the correct conformation of the extracellular domain (1). Indeed, when metabolically labeled immunoprecipitated receptors were analyzed under non-reducing conditions, a high molecular mass species of M_r ~180,000 was detected on autoradiograms (Fig. 6*B*, *lane 1*), and the amount of this species was increased in lactacystin-treated cells (Fig. 6*B*, compare *lane 1* with *3*). The high molecular weight receptor form was not due to inappropriate disulfide bond formation during sample preparation, because the free sulfhydryl groups of the receptor were blocked with an alkylating agent, NEM. Furthermore, it was not purified if the receptor-specific antibody was replaced with a non-immune serum (data not shown). Importantly, the high molecular weight species was not detected in samples purified by ligand affinity chromatography (Fig. 6*B*, *lanes 5* and *7*), indicating that it characterizes misfolded or incompletely folded receptor precursors rather than receptors that have acquired native conformation.

To find out whether the high molecular weight complex contains other protein component(s) in addition to the receptor precursor, the [^{35}S]methionine/cysteine-labeled samples treated with lactacystin were analyzed by two-dimensional SDS-PAGE, first under non-reducing and then under reducing conditions (Fig. 7). This technique allows the identification of intermolecular disulfide-bonded complexes, the components of which run above the gel diagonal (32). The M_r 180,000 form detected by one-dimensional non-reducing SDS-PAGE (Fig. 7*A*), run as two labeled spots above the diagonal in the two-dimensional SDS-PAGE (Fig. 7*B*). The smaller species (*spot 2*) ran at precisely the same location as the receptor precursor but the larger one (*spot 3*) had an apparent molecular weight of ~120,000. This indicates that the M_r 180,000 form most likely represents a heterocomplex, containing an unknown protein in addition to the M_r 73,000 precursor.

When proteasomal degradation is inhibited, misfolded membrane-bound glycoproteins have been shown to accumulate in the cytosol in a deglycosylated form (16, 33–36). To test the possibility that the misfolded/incompletely folded LHRs might also accumulate in the cytosol, we fractionated cellular homogenates after metabolic labeling (Fig. 8). Receptors were immunoprecipitated from three fractions: the 1,000 × *g* pellet containing cellular debris, nuclei, and trapped soluble proteins, the 10,000 × *g* pellet containing crude membranes, and the 100,000 × *g* supernatant containing cytosolic proteins. As can be seen in Fig. 8, the M_r 73,000 receptor was apparent in the particulate fractions (*lanes 1*, *2*, *4*, *5*, *7*, *8*, *10*, and *11*), but no receptor species were recovered from the cytosolic fraction (*lanes 3*, *6*, *9*, *12*). The same observation was made whether the samples were reduced or not prior to SDS-PAGE.

Taken together, these data indicate that only a fraction of newly synthesized LHRs is able to fold correctly, gains hormone binding ability, and is transported to the cell surface. The rest appear to be prone to aggregation in the ER as disulfide-bonded complexes and are eventually degraded by proteasomes.

DISCUSSION

The folding and maturation of proteins and the assembly of multimeric protein complexes were thought to be an efficient

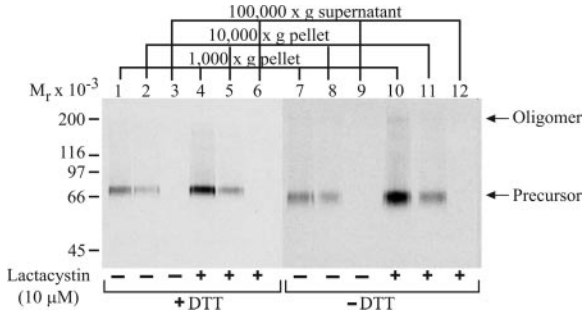

FIG. 8. **Subcellular fractionation of metabolically labeled HEK-293S cells.** Stably transfected HEK-293S cells were pulse-labeled for 60 min with [^{35}S]methionine/cysteine in the presence or absence of lactacystin. Total cellular homogenates were fractionated by differential centrifugation, and the receptors were purified by two-step immunoprecipitation using the receptor-specific polyclonal antibody. The immunoprecipitates were analyzed by SDS-PAGE under reducing or non-reducing conditions. *DTT*, dithiothreitol.

process in the past, and misfolding, misassembly, and degradation were believed to result from changes in the primary structure or errors in translation. However, an increasing body of direct and indirect evidence suggests that this may not always be the case and that even wild type proteins may be processed inefficiently in a regulated manner (34, 37–45). The results of the present study demonstrate that the rat LHR is one of these proteins that has a tendency to fail in acquiring the correct conformation within the time limit allowed by the ER quality control. A majority of the newly synthesized receptors was targeted for degradation, and only a fraction was able to gain hormone binding ability and was transported to the cell surface. Inefficient maturation appears to apply also to some other GPCRs (11–15) and may thus be a more common phenomenon among this group of proteins than has been previously anticipated.

The maturation of the LHR was found to be inefficient irrespective of the expression level or the cell line used, suggesting that this feature may be an intrinsic property of the receptor. As inhibition of proteasomal degradation increased the maturation efficiency, this property is likely to result from slow folding rate of the newly synthesized receptors. This notion is in line with the finding that a substantial portion of LHRs exists in the immature form in natural tissues, as was shown in the present and previously published studies (4, 5, 8, 9). Furthermore, as it has been demonstrated that the relative number of endogenous mature LHR forms to immature ones varies depending on the tissue or the developmental stage of the animal (8, 9), it can be hypothesized that targeting of incompletely folded receptors to ERAD may be a regulated phenomenon providing a means to control the number of cell surface receptors at a post-translational level. Whether this notion could be extended to other GPCRs in general, needs to be tested in future studies. Nevertheless, similar phenomenon has been demonstrated previously for a few other proteins. For example, cellular needs for sterol and non-sterol metabolites were found to control the level of 3-hydroxy-3-methylglutaryl-CoA reductase partially via structural destabilization and targeting of the enzyme to the ERAD pathway (46). Similarly, quantitative differences in T cell antigen receptor expression in immature and mature T cells appears to depend on developmentally regulated differences in the stability of the receptor α-subunit in the ER (38, 47).

The results of the present study revealed that those LHR precursors that had gained hormone binding ability existed as monomers, as only monomeric forms were purified by ligand affinity chromatography. In contrast, a substantial portion of receptor precursors purified by immunoprecipitation migrated as disulfide-bonded complexes in SDS-PAGE under non-reducing conditions, suggesting that they are likely to represent misfolded or incompletely folded receptors destined for degradation. This was further supported by the observation that their amount increased following proteasomal blockade. An inappropriate disulfide bond formation during the sample preparation, a phenomenon that has been shown to apply for the LHR (48), was ruled out by blocking the free cysteine residues with an alkylating agent, NEM. A two-dimensional electrophoresis indicated that in addition to the receptor precursor the complexes contain an unknown protein of M_r 120,000. The identity of this protein remains to be determined in the future, but it can be hypothesized that it might be involved in the unfruitful attempts to fold the receptor or in targeting the misfolded/incompletely folded receptors for degradation. In addition, whether the M_r 180,000 species is part of larger complexes within the ER needs to be tested. In any case, the disulfide-bonded complexes appear to relate to misfolding rather than the normal folding and maturation process of the receptor. The extracellular domain of the receptor contains several conserved cysteines that are thought to participate in intramolecular disulfide bond formation (1), and therefore it is possible that receptor misfolding leads to the formation of inappropriate intermolecular disulfide bonds. Such aberrant disulfide bond formation has been described previously for several other proteins that do not fold correctly or assemble to multisubunit complexes (*e.g.* Refs. 49–52).

In contrast to the present findings, a few other GPCRs have been shown to require dimerization/oligomerization to be able to exit the ER. For example, the metabotropic γ-aminobutyric acid b receptor (GABAbR) is composed of two subunits, GABAbR1 and GABAbR2, the latter of which is needed to mask the C-terminal ER retention signal of the former before the complex is transported to the plasma membrane (53, 54). This is exceptional, however, because most other GPCRs have been found to dimerize via their transmembrane domains (55–59). Recently, disruption of a transmembrane dimerization motif of the β_2-adrenergic receptor was found to prevent normal trafficking of the receptor to the cell surface, suggesting that dimerization of this receptor may be important for normal ER export as well (60). Similarly, several other family A GPCRs have been found to form dimers in the ER in studies using cellular fractionation and fluorescence or bioluminescence resonance energy transfer (56, 61–63). Whether these receptors represent misfolded receptors or correctly folded receptors on their way to the plasma membrane is not known at present.

The misfolded/incompletely folded LHR precursors accumulated in the ER membrane when proteasomal degradation was inhibited, and no cytosolic degradation intermediates were detected. These findings suggest that retrotranslocation of the LHR might be tightly coupled to the function of the proteasome, as has been described for several other proteins (45, 64–68). This is a reasonable hypothesis, because the LHR is a very hydrophobic protein, containing seven transmembrane domains as well as a leucine-rich N-terminal domain, and its exposure to the cytosolic aqueous environment would make it very prone to aggregation. In line with this notion, we were unable to immunopurify receptor forms from Triton X-100-insoluble, SDS-soluble cellular lysates (data not shown), in contrast to what has been reported for mutant forms of the cystic fibrosis transmembrane conductance regulator (69) and rhodopsin (17, 18). The tight coupling of retrotranslocation and degradation is further supported by the finding that the C-terminal domain of the LHR precursor has been found to interact with the p38^{JAB1}, a subunit of the COP9 signalosome (70). This complex has been implicated in the ubiquitin-dependent degradation (71).

Taken together, the results of the present study demonstrate that the rat LHR is a GPCR that appears to be very prone to misfolding and thus joins the increasing number of proteins that are targeted to degradation prematurely by the stringent ER quality control. Whereas the newly synthesized receptors that gained hormone binding ability were able to exit the ER, the rest were prone to form disulfide-bonded complexes and became substrates for ERAD. Thus, in addition to providing important insight into the posttranslational mechanisms that regulate folding and ER export of GPCRs, the present study suggests that inefficient folding and maturation of the receptors in the early secretory pathway may provide a means for controlling their number at the plasma membrane.

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Inefficient Maturation of the Rat Luteinizing Hormone Receptor: A PUTATIVE WAY TO REGULATE RECEPTOR NUMBERS AT THE CELL SURFACE

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