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Luteinizing Hormone Receptor Ectodomain Splice Variant Misroutes the Full-Length Receptor into a Subcompartment of the Endoplasmic Reticulum[D]

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The luteinizing hormone receptor (LHR) is a G protein–coupled receptor that is expressed in multiple RNA messenger forms. The common rat ectodomain splice variant is expressed concomitantly with the full-length LHR in tissues and is a truncated transcript corresponding to the partial ectodomain with a unique C-terminal end. Here we demonstrate that the variant alters the behavior of the full-length receptor by misrouting it away from the normal secretory pathway in human embryonic kidney 293 cells. The variant was expressed as two soluble forms of M_r 52,000 and M_r 54,000, but although the protein contains a cleavable signal sequence, no secretion to the medium was observed. Only a very small fraction of the protein was able to gain hormone-binding ability, suggesting that it is retained in the endoplasmic reticulum (ER) by its quality control due to misfolding. This was supported by the finding that the variant was found to interact with calnexin and calreticulin and accumulated together with these ER chaperones in a specialized juxtanuclear subcompartment of the ER. Only proteasomal blockade with lactacystin led to accumulation of the variant in the cytosol. Importantly, coexpression of the variant with the full-length LHR resulted in reduction in the number of receptors that were capable of hormone binding and were expressed at the cell surface and in targeting of immature receptors to the juxtanuclear ER subcompartment. Thus, the variant mediated misrouting of the newly synthesized full-length LHRs may provide a way to regulate the number of cell surface receptors.

INTRODUCTION

Newly synthesized proteins destined for the secretory pathway enter the endoplasmic reticulum (ER) and after correct folding and assembly are either secreted from the cell or transported to their site of action in other cellular compartments. Folding of the nascent proteins is constantly monitored by the ER quality control apparatus. It has an important task in maintaining the cellular homeostasis by preventing premature export of incompletely folded and assembled proteins and removing misfolded and unassembled ones via the ER-associated degradation (ERAD) pathway, presumably by recognizing structural signals that are enriched in incompletely folded proteins (Ellgaard and Helenius, 2003; Helenius and Aebi, 2004). Recent evidence suggests that the ER quality control does not only target permanently misfolded proteins to ERAD but may also dispose some folding competent ones, possibly due to their slow folding kinetics. This applies also to several polytopic membrane proteins, including G protein–coupled receptors (GPCRs; Petäjä-Repo *et al.*, 2000; Imai *et al.*, 2001; Lu *et al.*, 2003; Wüller *et al.*, 2004; Pietilä *et al.*, 2005). Thus, export from the ER is the limiting step in their expression and probably provides a mean to regulate the number of functional receptors at the cell surface. This is supported by our recent finding that final maturation of the rat luteinizing hormone receptor (rLHR) was found to be developmentally regulated in target tissues (Apaja *et al.*, 2005). Cell surface expression of GPCRs has also been found to be modulated by their corresponding splice variants and naturally occurring mutant forms. They appear to function in a dominant negative manner and hinder cell surface expression of the corresponding wild-type receptors, most likely through association in the ER (Benkirane *et al.*, 1997; Grosse *et al.*, 1997; Karpa *et al.*, 2000; Brothers *et al.*, 2004; Kaykas *et al.*, 2004; Nakamura *et al.*, 2004; Sarmiento *et al.*, 2004).

The LHR is a GPCR that recognizes glycoprotein hormones LH and chorionic gonadotropin (CG) that are secreted by the pituitary gland and placenta, respectively. These hormones are the major regulators of reproductive-related endocrine functions and steroidogenesis (Pierce and Parsons, 1981). The LHR is comprised of a large N-terminal ectodomain that is responsible for high-affinity hormone binding and a seven-transmembrane region with a C-terminal endodomain that is distinctive to other members of the family A GPCRs (Ascoli *et al.*, 2002;

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Abbreviations used: BSA, bovine serum albumin; CG, chorionic gonadotropin; DDM; *n*-dodecyl-β-D-maltoside; Endo H, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GPCR, G protein–coupled receptor; HA, haemagglutinin; HEK293, human embryonic kidney 293; LH, luteinizing hormone; μOR, μ opioid receptor; PNGase F, peptide-*N*-glycosidase F; R, receptor; STI, soybean trypsin inhibitor; UPR, unfolded protein response.

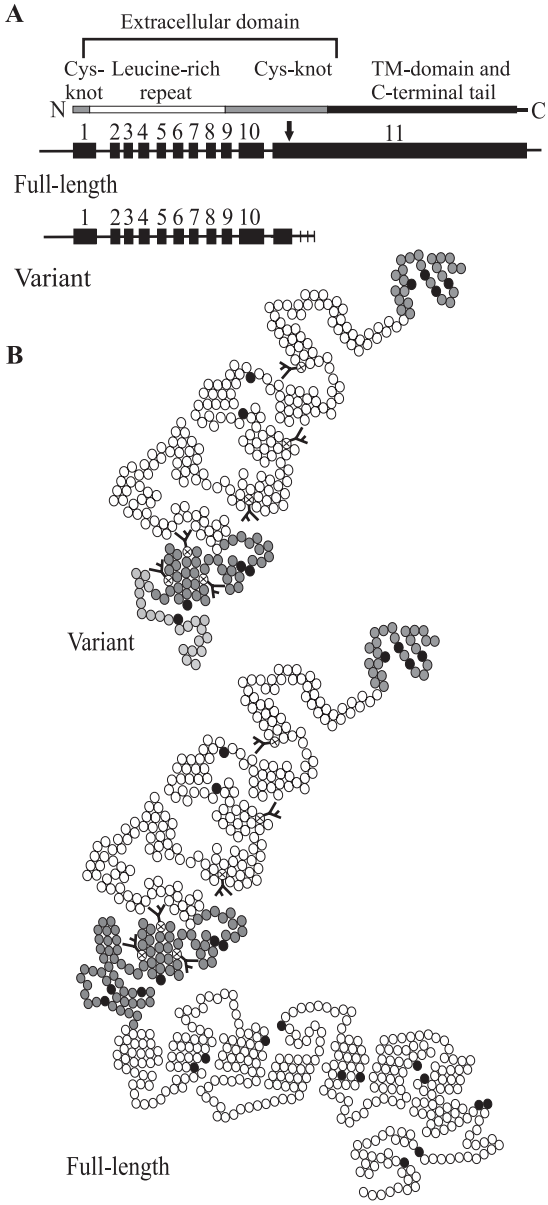

Figure 1. Schematic presentation of the rat LHR gene structure and topography of the full-length receptor and its ectodomain splice variant. (A) Exons 1–10 and the 5′ end of exon 11 code for the extracellular domain, whereas the rest of exon 11 encodes the seven transmembrane sequences and the C-terminal intracellular domain of the receptor. The arrow indicates the alternative splice site within exon 11. Cys-knot, cysteine knot; TM, transmembrane. (B) The truncated variant contains the N-terminal cysteine-knot and part of the C-terminal one (dark gray) with a unique C-terminal tail (light gray). The six potential N-glycosylation sites in the ectodomain and cysteine residues (black circles) are indicated.

Vassart *et al.*, 2004). The rLHR ectodomain splice variant differs from the full-length LHR mRNA by having a deletion of 266 base pairs, resulting from usage of an alternative splice site in exon 11 (Tsai-Morris *et al.*, 1990; Aatsinki *et al.*, 1992; Figure 1). This deletion causes a frame shift in the reading frame and creates a truncated translation product lacking amino acids 295–674 (corresponding to half of the C-terminal cysteine knot in the ectodomain, transmembrane regions and C-terminal tail), and concomitantly creates a unique C-terminus of 22 amino acids. We have previously shown that overexpression of the ectodomain splice variant in transgenic mice leads to alterations in pituitary-gonadal functions, steroidogenesis and morphology of the adrenals and kidneys (Apaja, Poutanen, Aatsinki; Petäjä-Repo, and Rajaniemi, unpublished results). One explanation for this phenotype could be changes in cell surface expression of the full-length LHR in cells that concomitantly express the receptor and the splice variant. On the basis of this hypothesis, we characterized expression of the LHR ectodomain splice variant (here called LHRvariant) in stably transfected human embryonic kidney (HEK) 293 cells and tested the possibility that coexpression with the variant might lead to alterations in the behavior of the full-length receptor. Results presented here provide evidence supporting the hypothesis and show that the LHRvariant may control cell surface expression of the full-length receptor by inducing misrouting of the newly synthesized receptors in the ER.

MATERIALS AND METHODS

Materials

Flp-In-293 cells, Flp-In T-Rex Core Kit, and pcDNA3 plasmid were purchased from Invitrogen (Carlsbad, CA). Human CG (6300 U/mg), protease inhibitors, poly-L-lysine and tunicamycin were obtained from Sigma (St. Louis, MO) and reagents for SDS-PAGE from Bio-Rad (Hercules, CA). Peptide-*N*-glycosidase F (PNGase F) and Endo-β-*N*-acetylglucosaminidase H (Endo H) were from Roche (Indianapolis, IN), brefeldin A and lactacystin from Alexis (San Diego, CA) and protein G-Sepharose from Amersham Pharmacia Biotech (Piscataway, NJ). Cell culture reagents were from BioWhittaker (Rockland, ME), Invitrogen, or Sigma. The anti-hemagglutinin (HA) and anti-cMyc antibody affinity resins, monoclonal mouse antibodies against HA (clone 7), BiP, β-actin, and FLAG M2 were products of Sigma. The rabbit anti-calnexin (SPA-865, SPA-860) and anti-calreticulin (SPA-600) antibodies and the mouse anti-KDEL-antibody (SPA-827) recognizing BiP were from Stressgen (Victoria, British Columbia, Canada) and the rabbit anti-cMyc antibody from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti-cMyc antibody (9E10) was obtained either from Santa Cruz or was produced by the core facility at the Department of Biochemistry, University of Montréal, Canada as ascites fluid. The cMyc peptide (EQKLISEEDL) was either from Sigma or was synthesized by the Biocenter Oulu protein analysis core facility. The Alexa488- and Alexa568-conjugated secondary goat anti-mouse and anti-rabbit antibodies were from Molecular Probes (Eugene, OR) and the phycoerythrin-conjugated rat anti-mouse antibody and mouse anti-GM130 antibody were from Becton-Dickinson (Lincoln Park, NJ). The rabbit antibody directed against the N-terminal domain of the rLHR (L^3-P^{43}) has been described previously (Aatsinki and Rajaniemi, 2005). All the other reagents were of analytical grade and were obtained from various commercial suppliers.

DNA Constructs

The expression vectors for epitope tagged receptor proteins were created by inserting DNA cassettes encoding the cleavable influenza HA signal peptide (MKTIIALSYIFCLVFA; Guan *et al.*, 1992), N-terminal *Myc*- (EQKLISEEDL) or HA- (YPYDVPDYA) epitope tags and C-terminal *Flag*-epitope tag (DYKDDDDK) into plasmids pcDNA5/FRT/TO or pcDNA3 with the same strategy as described earlier (Pietilä *et al.*, 2005). The C-terminal *Flag*-tag was excluded from some constructs. DNA constructs encoding the rLHR (GenBank accession no. M26199), rLHRvariant (Tsai-Morris *et al.*, 1990; Aatsinki *et al.*, 1992), and human μ opioid receptor (hμOR; GenBank accession no. NM000914) were created with a method similar to that described previously (Pietilä *et al.*, 2005). The rLHRvariant cDNA encoding the protein with its endogenous signal peptide and without epitope tags was subcloned into BamHI sites in the expression vector pNeoNUT containing the mouse metallothioneine-1 promoter (a generous gift from Professor Ilpo Huhtaniemi, Department of Physiology, University of Turku, Finland).

Cell Culture

Cells were grown routinely in DMEM containing 10% (vol/vol) fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin with appropriate selection antibiotics in a humidified atmosphere of 5% CO_2 at 37°C. A stable cell line expressing the Tet repressor ($HEK293_i$) was established by introducing the pcDNA6/TR plasmid into the Flp-In-293 cells according to the manufacturer's instructions and selecting and maintaining the cells under Zeozin (100 μg/ml; Invitrogen) and BlasticidinS (4 μg/ml; Invivogen, San Diego, CA). Stable cell lines with inducible expression were established by cotransfecting the pOG44 and the appropriate pFT-SMMF plasmids into the $HEK293_i$ cells with the Lipofectamine 2000 transfection reagent (Invitrogen) under BlasticidinS (4 μg/ml) and hygromycin (400 μg/ml; Invivogen) selec-

tion. They were isolated, expanded and tested for Zeozin sensitivity, β-galactosidase activity and tetracycline (0.5 μg/ml) inducible expression of the appropriate recombinant proteins by Western blotting. Stable cell lines with constitutive expression were prepared as above by transfecting the Flp-In-293 cells ($HEK293_c$). The *HA*-rLHR in pcDNA3 was transfected into random integration sites in the inducible $HEK293_i$-*Myc*-rLHRvariant cells under G-418 (400 μg/ml; Calbiochem, La Jolla, CA) selection to generate stable double expression cell lines. G-418 selection was also used to generate a stable HEK293 cell line expressing the rLHRvariant with its endogenous signal peptide.

For experiments, cells were cultured routinely in 75-cm^2 culture flasks and grown to 80–90% confluency before adding tetracycline for 24 h. Lactacystin (10 μM) to block proteasomal degradation and tunicamycin (5 μg/ml) to induce unfolded protein response (UPR) were added to the culture medium 6 and 24 h before harvesting, respectively. HEK293 cells expressing the LHRvariant with it endogenous signal peptide were treated with $ZnCl_2$ (0.1 mM) for 24 h to induce expression of the variant. Cells were harvested in phosphate-buffered saline (PBS) containing 20 mM *N*-ethylmaleimide. In some cases also medium (with or without serum) was collected and both cell and medium samples were quick frozen in liquid nitrogen and stored at −70°C.

For transient transfections, cells were cultured to 50% confluency on glass coverslips in six-well plates. Twenty-four hours after plating, they were transfected with Lipofectamine 2000 (dilution 1:2) and 200 ng of the appropriate DNA constructs. The medium was replaced 24 h later with one containing selection antibiotics and tetracycline and the cells were incubated further for 24 h.

Preparation and Solubilization of Cellular Membranes and Lysates

Total cellular membranes were prepared by homogenizing cells with a Polytron homogenizer (Ultra-Turrax T-25, IKA, Wilmington, NC) in buffer A (25 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor [STI], 10 μg/ml benzamidine, 2 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], and 2 mM 1,10-phenantroline) and centrifuging at 800 × *g* for 5 min. The membrane particles were then collected by centrifuging the supernatant for 20 min at 45,000 × *g*. After washing the membranes twice with buffer A they were solubilized by stirring on ice for 60 min in 500 μl of buffer B (buffer A containing 0.5% [wt/vol] *n*-dodecyl-β-D-maltoside [DDM] and 140 mM NaCl) and centrifuging at 100,000 × *g* for 60 min. Protein concentration in each sample was determined by the Bio-Rad DC assay kit using bovine serum albumin (BSA) as the standard.

Total cellular lysates were prepared by solubilizing cells directly in buffer B or in the case of coimmunoprecipitation experiments in buffer C (0.5% [wt/vol] DDM, 25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1 mM $CaCl_2$, 20 mM *N*-ethylmaleimide, complete EDTA-free protease inhibitors; Roche). Insoluble material was removed by centrifugation at 16,000 × *g* for 30 min, and protein was assayed as above. The supernatant fraction was diluted to 125 μg of protein/ml with buffer B or to 1200 μg of protein/ml with buffer C and supplemented with 0.1% (wt/vol) BSA. Culture medium samples (200 μl) were diluted 1:1 with buffer D (buffer B containing 1% instead of 0.5% [wt/vol] DDM). The solubilized membrane extracts or lysates (50–600 μg) and diluted medium samples were subjected to immunoprecipitation or ligand affinity chromatography, or lysates (100 μg) were directly analyzed by SDS-PAGE after precipitation with methanol, as described (Wessel and Flugge, 1984).

Membrane Extractions

Total cellular membranes were subjected to sequential extraction with high-salt (1 M NaCl) and alkaline (0.1 M Na_2CO_3, pH 11.5) buffers for 30 min at 4°C. For controls, only buffer A was used. Membrane particles were collected by centrifuging the samples for 20 min at 45,000 × *g*. After extractions, DDM was supplemented to extracts at a final concentration of 0.5% (wt/vol). Proteins in the high-salt and alkaline extracts were precipitated with methanol as described above adding STI (20 μg/ml) as a carrier.

Immunoprecipitation and Ligand Affinity Chromatography

Before immunoprecipitation, samples were precleared for 60 min with 20 μl of protein G-Sepharose that was equilibrated with buffer B containing 0.1% (wt/vol) BSA. The mouse anti-cMyc and anti-HA antibodies were used at a dilution of 1:100. They were incubated with the supernatant for 60 min and 20 μl protein G-Sepharose was then added and incubated overnight at 4°C with gentle agitation, pelleted, and washed twice with 500 μl of buffer A and four times with 500 μl of buffer E (buffer B containing 0.1% [wt/vol] instead of 0.5% [wt/vol] DDM). The bound proteins were eluted by incubating the resin for 5 min at 95°C in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% [wt/vol] SDS, 10% [vol/vol] glycerol, 0.001% [wt/vol] bromophenol blue). Alternatively, the mouse anti-HA and anti-cMyc antibodies were replaced with agarose-immobilized antibodies (20–25 μl) and the anti-cMyc immunoprecipitates were eluted as described (Petäjä-Repo *et al.*, 2000) replacing the FLAG peptide with cMyc peptide. Calnexin and calreticulin were immunoprecipitated using Affi-Gel 10–coupled anti-C-terminal calnexin and anti-calreticulin antibodies, respectively. Antibody coupling was performed according to the instructions of the manufacturer using 50 μl of antisera for 100 μl of resin (Bio-Rad). Ligand affinity chromatography was performed using Affi-Gel 10–coupled hCG, as described previously (Apaja *et al.*, 2004). Glycerol 20% (vol/vol) was added to all buffers and 140 mM NaCl to buffer E instead of buffer B.

Deglycosylation of the Immunoprecipitated Proteins

Immunoprecipitated receptors from cell lysates were deglycosylated after elution from protein G-Sepharose with 1% (wt/vol) SDS, 50 mM Na-phosphate, pH 7.5. Before the enzyme reaction, eluates were diluted 10-fold with 0.5% (wt/vol) DDM, 50 mM Na-phosphate, pH 5.5, 50 mM EDTA, 0.2 mM PMSF, 2 mM 1,10-phenantroline, 5 μg/ml leupeptin, 5 μg/ml STI, and 10 μg/ml benzamidine (Endo H) or 0.5% (wt/vol) DDM, 50 mM Na-phosphate, pH 7.5, 50 mM EDTA, 0.2 mM PMSF, 2 mM 1,10-phenantroline, 5 μg/ml leupeptin, 5 μg/ml STI ,and 10 μg/ml benzamidine (PNGase F). Endo H was added to a final concentration 50 mU/ml and PNGase F to 50 U/ml. Samples were incubated at 30°C for 16 h and the reaction was terminated by adding SDS-sample buffer.

Real-Time Quantitative PCR

Total RNA was extracted using the Trizol reagent (Invitrogen), according to the manufacturer's instructions. RT reactions were carried out using the MuLV-reverse transcriptase (Finnzymes, Espoo, Finland or Fermentas, Hanover, MD; 10 or 100 U, respectively) in a final volume of 10 μl for 0.5 μg of total RNA according to the manufacturers' instructions. The synthesis product was used for real-time quantitative PCR that was performed with the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA) using TaqMan chemistry and primers specific to the human BiP (GenBank accession no. X87949). The results were normalized to 18S RNA quantified from the same samples.

[^{35}S]methionine/cysteine Incorporation into Trichloroacetic Acid–precipitable Proteins

Cells were labeled with [^{35}S]methionine/cysteine as described previously (Pietilä *et al.*, 2005) and equal amount of cell lysates from noninduced and induced cells were subjected to precipitation with 10% (vol/vol) trichloroacetic acid. The precipitated proteins were dissolved in 0.1 M NaOH and after adding scintillant (PerkinElmer, Norwalk, CT) the radioactivity was measured with the Wallac MicroBeta TriLux scintillation counter (PerkinElmer).

Western Blot Analysis and SDS-PAGE

Immunoprecipitated and ligand affinity-purified receptors and cell lysates were heated for 2 min at 95°C in the absence or presence of 50 mM dithiothreitol before SDS-PAGE (4% stacking gels and 10% separating gels). For Western blot analysis, the separated proteins were electroblotted onto Immobilon B membrane (Millipore, Bedford, MA) and probed with the appropriate antibodies. Horseradish peroxidase–conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) or goat anti-mouse antibody (Invitrogen) and enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech) were used to reveal the blotted proteins. The molecular mass markers (Bio-Rad) were detected by staining with Ponceau S. The relative intensities of the bands on immunoblots were analyzed by densitometric scanning with the Agfa Duoscan HiD (Ridgefield Park, NJ), and β-actin was used as a loading control for the UPR samples. The data were quantified using the Scion image, version 4.0.2 (Frederick, MD), subtracting the local background from each lane.

Confocal Microscopy

Cells were cultured on poly-L-lysine (100 μg/ml)-coated glass coverslips and fixed in 2% (wt/vol) paraformaldehyde for 10–20 min. After fixation, cells were permeabilized with 0.1% (vol/vol) Triton X-100, 0.5% (wt/vol) BSA, PBS for 30 min and incubated with the appropriate primary antibodies (1:100) for 45 min. After washing with PBS, fluorescent secondary antibody combinations (1:200) in the permeabilization buffer were added and incubations were continued for 30 min. After final washes with PBS, coverslips were mounted on glass slides with Immu-mount (Thermo Electron, Waltham, MA) for confocal microscopy. Alternatively, cell surface receptors were labeled under nonpermeabilizing conditions by adding the primary antibody to the medium for 30 min before fixation and permeabilization. All incubations were performed at 22°C. The specificity of the anti-cMyc staining was confirmed by blocking the antibody with the cMyc peptide (100 μg/ml). To visualize the nuclei, cells were incubated with the DNA-binding fluorochrome Hoechst 33258 (Sigma, 1 mg/ml stock diluted 1:800 in PBS) for 10 min. The specimens were viewed with the Zeiss LSM 510 (Thornwood, NY) or Olympus Fluoview FV1000 confocal microscope (Melville, NY). The Chariot (Active Motif, Carlsbad, CA) protein delivery kit was used for antibody transfections following the instructions of the manufacturer. Recombinant protein expression was induced with tetracycline (0.5 μg/ml) 7 h before antibody transfections and lactacystin (10 μM) or brefeldin A (5 μg/ml) were added 3 and 1 h after tetracycline addition, respectively.

Flow Cytometry

To label plasma membrane receptors, cells were harvested in PBS and 1×10^6 cells/sample were incubated for 30 min at 4°C in the dark with monoclonal anti-cMyc antibody (1:1000) in buffer F (PBS, 1% [vol/vol] FCS). The residual antibody was removed by washing with buffer F and sedimenting the cells at $200 \times g$ for 5 min at 4°C before incubation with 0.5 μg/ml phycoerythrin-conjugated rat anti-mouse antibodies in buffer F for 30 min. To detect damaged cells, 7-amino-actinomycin D (Becton Dickinson; 0.25 $\mu g/1 \times 10^6$ cells) was added into the cell suspension 10 min before flow cytometry. Ten thousand cells of each sample were analyzed on the Becton-Dickinson FACSCalibur flow cytometer. Data analysis to quantitate changes in mean surface receptor fluorescence values were performed using the CellQuestPro4.02 software (Becton-Dickinson). Mean fluorescence of live cells minus mean fluorescence of cells stained only with the phycoerythrin-conjugated secondary antibody, was used for calculations.

Transmission and Immunoelectron Microscopy

Cells were cultured on 100-mm plates and induced for 16 h, after which lactacystin (10 μM) or vehicle was added 6 h before harvesting. For immunoelectron microscopy, cells were fixed in 4% (wt/vol) paraformaldehyde, 2.5% (wt/vol) sucrose, 0.1 M phosphate buffer for 30 min, immersed in 2% (wt/vol) agarose, 2.3 M sucrose, and frozen in liquid nitrogen. Thin cryosections were cut with the Leica Ultracut UCT microtome (Deerfield, IL). For immunolabeling, thin sections were first incubated in 0.05 M glycine, PBS followed by incubation in 5% (wt/vol) BSA, 0.1% (wt/vol) CWFS (cold water fish skin) gelatin (Aurion, Wageningen, The Netherlands), PBS. The sections were incubated with mouse anti-cMyc antibody (Stressgen) and then with protein A-gold complex (10 nm; Slot and Geuze, 1985). Antibodies and the gold conjugate were diluted in 0.1% (wt/vol) BSA, 0.1% (wt/vol) CWFS gelatin, PBS. The sections were embedded in methylcellulose. For transmission electron microscopy, cells were fixed in 1% (vol/vol) glutaraldehyde, 4% (vol/vol) formaldehyde, 0.1 M phosphate buffer for 5 min, harvested, pelleted, and immersed in 2% (wt/vol) agar, PBS. Pellets were postfixed in 1% (wt/vol) OsO_4, 0.1 M PIPES (pH 7.4) for 1 h, dehydrated in acetone, and embedded in Embed 812 (Electron Microscopy Sciences, Hatfield, PA). All incubations were performed at 22°C. Thin sections were cut with the Reichert Ultracut ultramicrotome. Both immunolabeled and osmium treated sections were examined using the Philips CM100 transmission electron microscope (Mahwah, NJ).

Data Analysis

Data were analyzed using GraphPad Prism version 4.02 (San Diego, CA). For the statistical *t*-tests, the limit of significance was set at $p < 0.05$, and data are presented as mean ± SEM.

RESULTS

LHRvariant Is a Soluble Protein But Is Not Secreted to the Medium

To facilitate immunological detection and purification of the receptor proteins, a *Myc*-epitope tag was introduced to the N-terminus of the rLHRvariant. Similarly, the full-length receptor was modified to contain an N-terminal *HA*- or *Myc*-tag and a C-terminal *Flag*-tag. The constructs were stably transfected into a tetracycline-inducible $HEK293_i$ cell line that allowed expression of the receptor proteins in a controlled manner in an identical genetic background. Alternatively, the constructs were transfected into $HEK293_c$ cells that expressed the receptor proteins constitutively.

The $HEK293_i$-*Myc*-rLHRvariant cells were induced for recombinant protein production with 0.5 μg/ml tetracycline for 24 h and the proteins in the DDM-solubilized total cellular lysates and medium were subjected to Western blot analysis. As seen in Figure 2A, lane 2, the anti-cMyc antibody recognized two protein species with an apparent molecular weight of 52,000 and 54,000 from the lysates of induced cells, the relative intensities of which varied from one experiment to another. Neither one of these species was detected in the non-induced cells (Figure 2A, lane 1), suggesting that they represent the expressed protein. When the same samples were analyzed under nonreducing conditions, the two variant species were less distinguishable from each other and migrated faster (Figure 2A, lane 4), suggesting that they contain disulfide bonds.

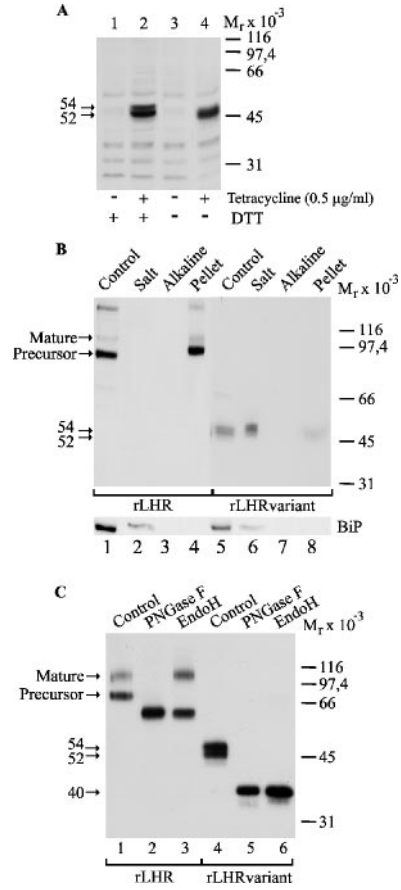

Figure 2. The LHRvariant is a soluble protein containing high-mannose-type N-linked oligosaccharides. (A) Total cellular lysates from noninduced (lanes 1 and 3) and tetracycline induced (lanes 2 and 4) $HEK293_i$-*Myc*-rLHRvariant cells were precipitated with methanol, subjected to SDS-PAGE under nonreducing (lanes 3 and 4) or reducing (lanes 1 and 2) conditions and analyzed by Western blotting. (B) Membrane vesicles from tetracycline-induced $HEK293_i$-*Myc*-rLHR-*Flag* (lanes 1–4) and $HEK293_i$-*Myc*-rLHRvariant (lanes 5–8) cells were subjected to high-salt (lanes 2 and 6), alkaline (lanes 3 and 7), or buffer (lanes 1 and 5) extractions. The extracts supplemented with DDM (lanes 2, 3, 6, and 7) and the DDM-solubilized membranes (lanes 1, 4, 5, and 8) were analyzed by reducing SDS-PAGE and Western blotting. An aliquot of the samples was subjected to Western blot analysis using mouse anti-BiP antibody (bottom panel). (C) Lysates from induced $HEK293_i$-*Myc*-rLHR-*Flag* (lanes 1–3) and $HEK293_i$-*Myc*-rLHRvariant (lanes 4–6) cells were subjected to immunoprecipitation with mouse anti-cMyc antibody and immunoprecipitates were incubated for 16 h at 30°C with Endo H (50 mU/ml; lanes 3 and 6), PNGase F (50 U/ml; lanes 2 and 5) or without enzymes (lanes 1 and 4) before reducing SDS-PAGE and Western blotting. The blotted proteins were probed with mouse anti-cMyc antibody (A and B, lanes 1–4, and C) or rabbit anti-receptor antibody directed against the N-terminus of the receptor (B, lanes 5–8). DTT, dithiothreitol. Molecular weight markers used to calibrate the gels are indicated on the right.

Surprisingly, the antibody was unable to detect any specific bands from the culture medium after induction, and no specific proteins were purified from the medium by immunoprecipitation or hCG affinity chromatography (unpublished data). Similar results were also obtained, when the epitope-tagged variant was expressed in Chinese hamster ovary cells or the untagged protein with its endogenous signal sequence was expressed in HEK293 cells (Supplementary Figure 1). These results, therefore, indicate that in contrast to expectations, the

variant is retained intracellularly and is not secreted to the medium.

One explanation for the inability to detect secreted forms of the variant could be that the protein is associated with membranes. Although the variant lacks the transmembrane domains of the full-length receptor, it has a unique C-terminal tail of 22 amino acids (Tsai-Morris *et al.*, 1990; Aatsinki *et al.*, 1992). Thus, membrane vesicles from induced HEK293$_i$ cells expressing either the full-length receptor or the variant were subjected to sequential high-salt and alkaline extractions before Western blot analysis. These treatments are known to disrupt weak electrostatic and hydrophobic interactions, respectively, both of which mediate association of peripheral membrane proteins with lipid bilayers (Fujiki *et al.*, 1982). The full-length receptor that was expressed as a M_r 94,000 mature species and a M_r 76,000 precursor form (Pietilä *et al.*, 2005) was resistant to both treatments (Figure 2B, lanes 2 and 3). In contrast, the variant behaved as the soluble ER protein BiP and dissociated from the membranes after high-salt treatment (Figure 2B, lane 6, top and bottom panels, respectively). Thus, this excludes the possibility that the variant could be a tightly bound peripheral or an integral membrane protein.

The identities of the two variant forms that were expressed in the induced HEK293$_i$-*Myc*-rLHRvariant cells were studied further by subjecting immunoprecipitated samples to glycosidase treatments. As seen in Figure 2C, lane 5, both the M_r 52,000 and M_r 54,000 species were sensitive to PNGase F and removal of the glycans resulted in the appearance of a single protein species of M_r 40,000. This suggests that the two variant forms are likely to carry different number of N-glycans. In addition, the sensitivity of the two variant species to Endo H (Figure 2C, lane 6) indicates that they contain high-mannose–type *N*-linked glycans that are typical for glycoproteins residing in the ER (Parodi, 2000). In contrast, only the M_r 76,000 precursor form of the full-length receptor was sensitive to Endo H (Figure 2C, lane 3).

LHRvariant Has Difficulties in Finding the Correct Hormone-Binding Conformation, Interacts with ER Chaperones, and Is a Substrate for ERAD

Because the LHRvariant was not found to be secreted to the medium, it may have difficulties in folding in the ER. Thus, we tested whether it is able to gain hormone-binding ability, because it contains the critical amino acids that are required for hormone binding (Ascoli *et al.*, 2002; Vassart *et al.*, 2004). To compare the binding ability of the variant to that of the full-length receptor, the same amount of protein from lysates of induced HEK293$_i$-*Myc*-rLHR-*Flag* and HEK293$_i$-*Myc*-rLHRvariant cells were subjected to immunoprecipitation and hCG affinity chromatography. As expected, the two receptor species of M_r 94,000 and M_r 76,000 were purified from cells that express the full-length receptor (Figure 3A, lanes 4 and 10), and no such species were purified from noninduced (Figure 3A, lanes 3 and 9) or nontransfected cells (Figure 3A, lanes 1–2 and 7–8). As has been reported previously (Pietilä *et al.*, 2005), more mature receptors were purified by hCG affinity chromatography than by immunoprecipitation, whereas the reverse was true for the receptor precursor. Because the cell surface and intracellular receptors are both able to bind hormone with high affinity (Fabritz *et al.*, 1998), the most likely explanation for this finding is that only a small fraction of the receptor precursors is able to bind hormone. In comparison, the two variant forms that were purified by ligand affinity chromatography were detectable only after a very long exposure time (Figure 3A, compare lanes 12 and 14, exposed for 4 and 20 min, respectively), suggesting that a yet smaller fraction of the variant was capable of hormone binding.

Because the LHRvariant has apparent difficulties in finding the correct conformation for hormone binding, it is likely to be retained in the ER by its quality control components and targeted for degradation. Therefore, we tested whether the variant might interact with ER molecular chaperones calnexin and calreticulin that are known to interact with newly synthesized glycoproteins (Helenius and Aebi, 2004). For that purpose, lysates from HEK293$_i$-*Myc*-rLHRvariant cells were subjected to immunoprecipitation with anti-cMyc, anti-calnexin, and anti-calreticulin antibodies and the immunoprecipitates were analyzed by Western blotting. As seen in Figure 3B, calnexin was detected in the anti-cMyc immunoprecipitates from induced but not from noninduced cells (compare lanes 2 and 4). Similar findings were obtained for calreticulin (Figure 3B, lanes 6 and 8). Thus, these data indicate that the rLHRvariant interacts with the two molecular chaperones that may retain the misfolded molecules in the ER.

To test the possibility that the variant is targeted for ERAD, a proteasomal inhibitor lactacystin (Lee and Goldberg, 1998) was added to the culture medium 6 h before lysates were prepared and the variant was subjected to Western blot analysis. As can be seen in Figure 3C, proteasomal blockade led to a 1.6 ± 0.4-fold increase (mean ± SEM; n = 4) in the amount of the two variant forms. This indicates that it is a substrate for ERAD and is directed to proteasomal degradation, a finding that has also been reported for the full-length receptor (Pietilä *et al.*, 2005). However, no degradation intermediates or high-molecular-weight aggregates were detected (Figure 3C, lane 2). Similarly, no DDM-insoluble, SDS-soluble variant forms were observed (unpublished data), which is in contrast to many other proteins that are targeted for proteasomal degradation (Johnston *et al.*, 1998; Illing *et al.*, 2002; Junn *et al.*, 2002; Saliba *et al.*, 2002).

LHRvariant Accumulates in the ER and Causes Redistribution of Calnexin and Calreticulin

To further characterize the subcellular localization of the LHRvariant and to identify the site where it is retained, the noninduced and induced HEK293$_i$-*Myc*-rLHRvariant cells were permeabilized, labeled with anti-cMyc antibody, and subjected to immunofluorescence microscopy. No specific staining was detected in noninduced cells (Figure 4, A–C and M–O) but the induced ones displayed distinct punctuate perinuclear staining (Figure 4, D–L and P–R) that colocalized with ER resident proteins calreticulin (Figure 4, D–F) and calnexin (Figure 4, G–I). No significant colocalization was observed with the Golgi protein GM130 (Figure 4, J–L) and colocalization with the ER chaperone BiP was only partial (Figure 4, P–R). Intriguingly, the variant appeared to accumulate in juxtanuclear regions (Figure 4, D–L and P–R) and, furthermore, its expression led to redistribution of calnexin and calreticulin that also accumulated in the same locations close to the nucleus (see arrowheads in Figure 4, F and I). No such redistribution was observed in nontransfected HEK293$_i$ cells that were treated with tetracycline (unpublished data). Similarly, no detectable redistribution of BiP was detected (Figure 4, M–R), suggesting that the ER morphology in general remained unaltered.

A few misfolded membrane-bound or secreted proteins have been shown to accumulate in pericentriolar aggresomal structures in the cytosol, especially if their proteasomal degradation is compromised (Johnston *et al.*, 1998; Notter-

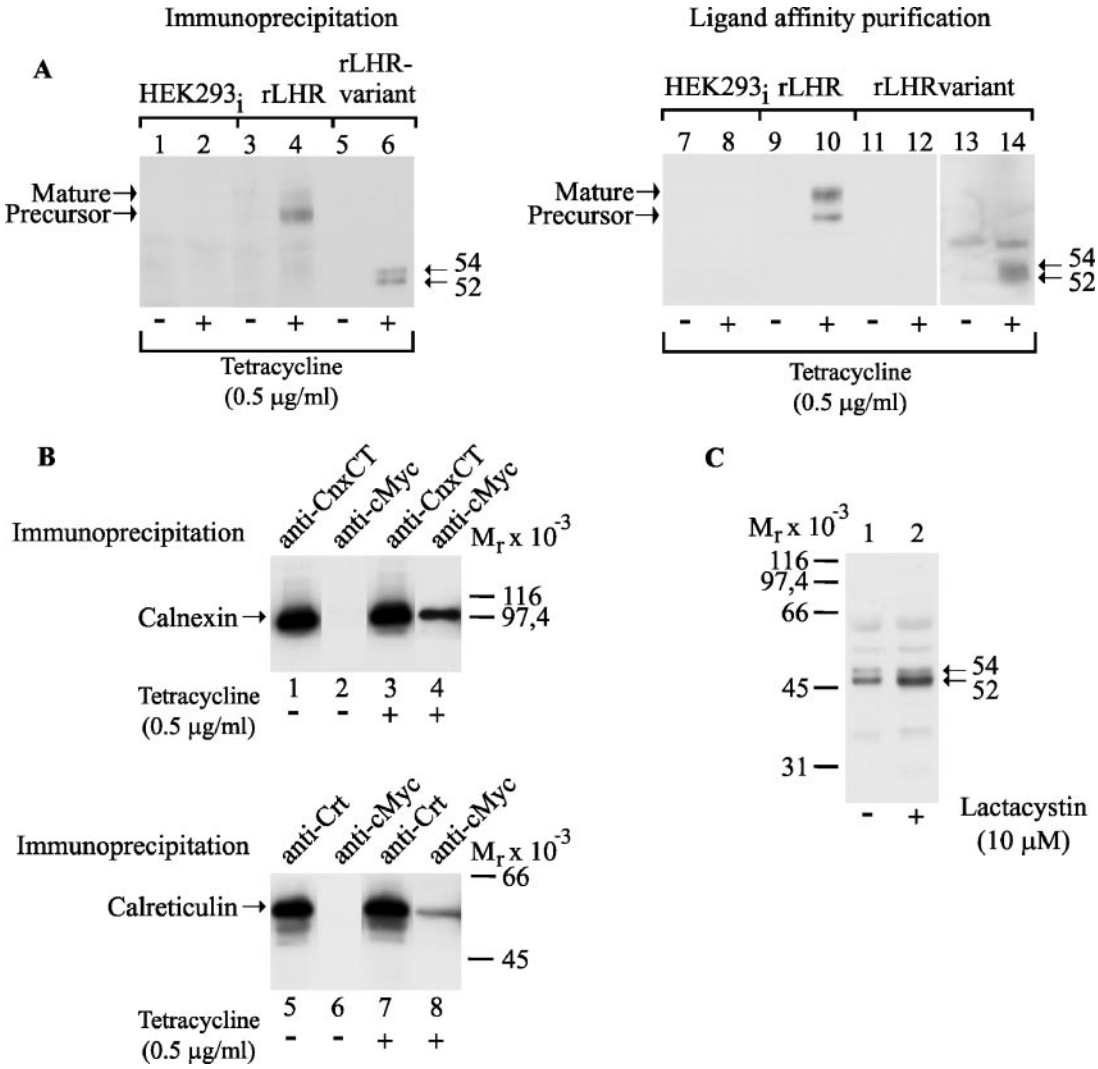

Figure 3. The LHRvariant has difficulties in finding the correct conformation for hormone binding, interacts with calnexin and calreticulin and is a substrate for ERAD. (A) Equal aliquots of lysates from noninduced (lanes 1, 3, 5, 7, 9, 11, and 13) and tetracycline induced (lanes 2, 4, 6, 8, 10, 12, and 14) HEK293$_i$-*Myc*-rLHR-*Flag* and HEK293$_i$-*Myc*-rLHRvariant cells and nontransfected HEK293$_i$ cells were subjected to immunoprecipitation and hCG affinity chromatography, as indicated. The purified samples were subjected to reducing SDS-PAGE and Western blotting and probed with mouse anti-cMyc antibody. Lanes 13 and 14 represent a longer exposure of lanes 11 and 12, respectively. (B) Equal aliquots of lysates from noninduced (lanes 1–2 and 5–6) and induced (lanes 3–4 and 7–8) HEK293$_i$-*Myc*-rLHRvariant cells were subjected to immunoprecipitation using polyclonal anti-C-terminal calnexin (lanes 1 and 3) or anti-calreticulin (lanes 5 and 7) antibodies or monoclonal anti-cMyc antibody (lanes 2, 4, 6, and 8). The immunoprecipitates were analyzed by reducing SDS-PAGE and Western blotting using anti-N-terminal calnexin (lanes 1–4) or anti-calreticulin (lanes 5–8) antibodies. anti-CnxCT, anti-C-terminal calnexin antibody; anti-Crt, anti-calreticulin antibody. (C) HEK293$_i$-*Myc*-rLHRvariant cells were treated with tetracycline for 16 h and lactacystin (10 μM; lane 2) or vehicle (lane 1) was added to the culture medium 6 h before harvesting. Cellular lysates were prepared and methanol-precipitated samples were subjected to reducing SDS-PAGE and Western blotting using mouse anti-cMyc antibody.

pek *et al.*, 1999; Kabore *et al.*, 2001; Illing *et al.*, 2002; Junn *et al.*, 2002; Saliba *et al.*, 2002). Thus, we explored the possibility that the juxtanuclear accumulations in the induced HEK293$_i$-*Myc*-rLHRvariant cells might represent cytosolic aggresomes. We reasoned that if the variant is retrotranslocated from the ER and accumulates in aggresomes, the N-terminal *Myc*-epitope should be available in the cytosol. This was studied by delivering antibodies into the cells using the Chariot protein transfection reagent. The HEK293$_i$-*Myc*-rLHRvariant cells were first transfected with anti-calnexin antibodies that were directed against either the C- or N-terminus of the protein in the cytosolic and lumenal sides of the ER membrane, respectively (Helenius and Aebi, 2004). After fixation and permeabilization, the Alexa488-conjugated secondary antibody was able to label cells that were transfected with the C-terminal antibody (Figure 5B; 46–58% of cells labeled) but not with the N-terminal antibody (Figure 5A; 0% of cells labeled). This indicates that the antibodies are delivered into the cytosol but are not able to the reach the lumen of the ER. As an additional control, HEK293$_i$-*Myc*-rLHR-*Flag* cells were transfected with either anti-cMyc or FLAG antibodies and the availability of the corresponding N- and C-terminal epitopes of the full-length receptor were studied following blockade of receptor transport in the ER with brefeldin A (Lippincott-Schwartz *et al.*, 1989). As seen in Figure 5, C and D, only the anti-FLAG

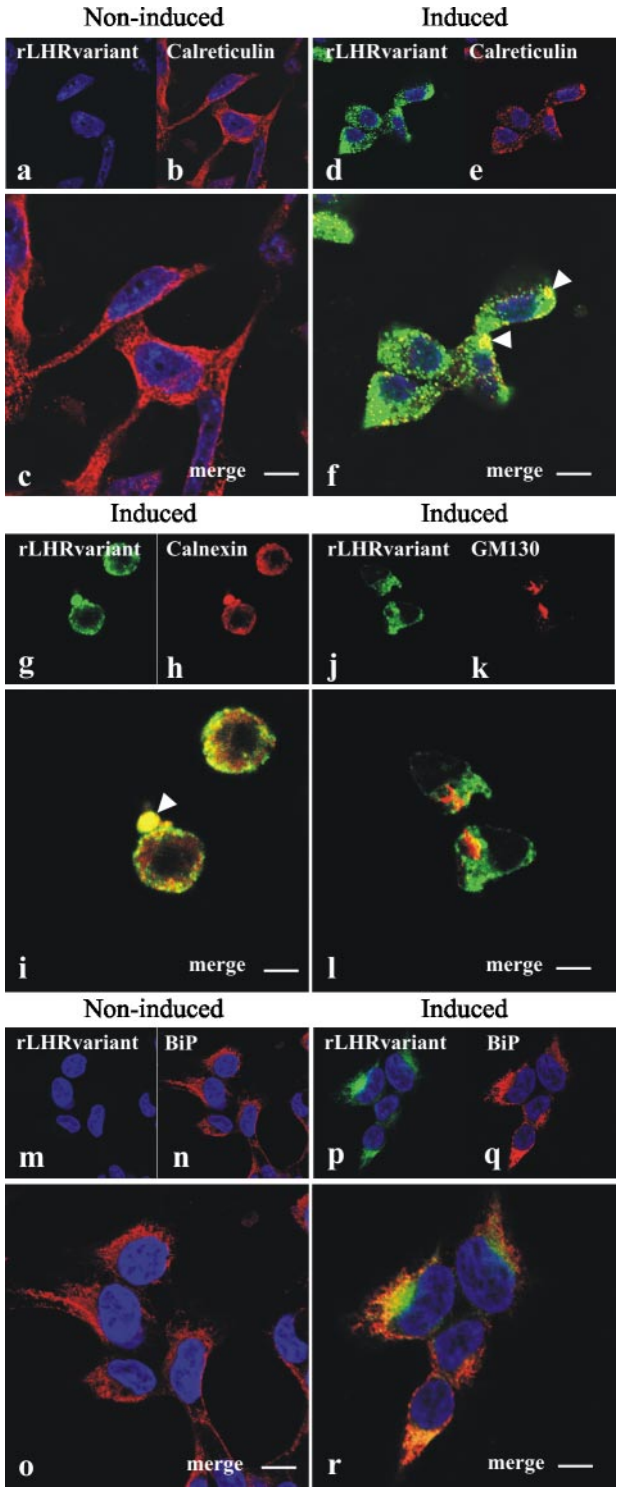

Figure 4. The LHRvariant colocalizes with ER markers calnexin and calreticulin and causes their redistribution. Noninduced (A–C and M–O) and tetracycline induced (D–L and P–R) HEK293$_i$-*Myc*-rLHRvariant cells were cultured on coverslips, fixed, and permeabilized before processing for double label indirect immunofluoresence. The ER proteins calreticulin (A–F) and calnexin (G–I) were labeled with rabbit anti-calreticulin and anti-C-terminal calnexin antibodies, respectively, and the ER protein BiP (M–R) and Golgi protein GM130 (J–L) with mouse anti-KDEL and anti-GM130 antibodies, respectively. The LHRvariant was stained in parallel using either mouse (A–I) or rabbit (J–R) anti-cMyc antibody. Secondary antibodies were Alexa 488- or Alexa 568-conjugated goat anti-rabbit and anti-mouse IgG. Colocalization of the variant and the ER markers calnexin and calreticulin in the juxtanuclear region is indicated by arrowheads (F and I). The nuclei were stained with Hoechst in A–F and M–R. Bar, 10 μm.

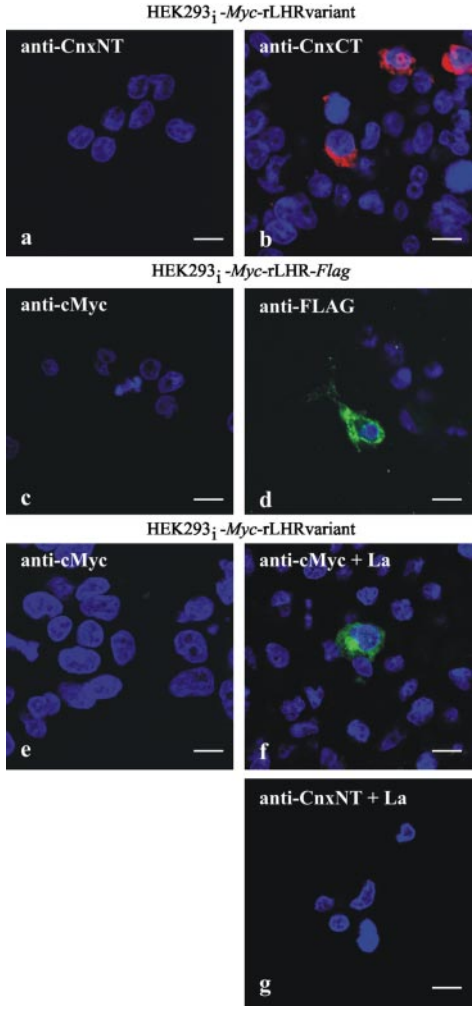

Figure 5. The LHRvariant accumulates in the ER and upon proteasomal blockade in the cytosol. Tetracycline induced HEK293$_i$-*Myc*-rLHRvariant and HEK293$_i$-*Myc*-rLHR-*Flag* cells were transfected with anti-N-terminal-calnexin (A and G), anti-C-terminal-calnexin (B), anti-cMyc (C and E-F) or anti-FLAG (D) antibodies. After fixation and permeabilization, cells were labeled with secondary antibodies, Alexa 488-conjugated anti-mouse or Alexa 568-conjugated anti-rabbit IgG. The HEK293$_i$-*Myc*-rLHR-*Flag* cells in C and D were treated with brefeldin A (5 μg/ml) during induction to retain the full-length receptor in the ER and proteasomal degradation was inhibited with lactacystin (10 μM) in HEK293$_i$-*Myc*-rLHRvariant cells in F and G. Positive cells were counted from 170 to 485 cells/coverslip: 0, 51 $\pm$ 7, 0, 64 $\pm$ 5, 0, 20 $\pm$ 9, 0% (mean $\pm$ SEM) for A, B, C, D, E, F, and G, respectively. Bar, 10 μm. anti-CnxCT, anti-C-terminal calnexin antibody; anti-CnxNT, anti-N-terminal calnexin antibody; La, lactacystin.

antibody was able to recognize the receptor, indicating that the protein is inserted into the membrane according to expectations with the C-terminus in the cytosol and the N-terminus in the ER lumen. When the HEK293$_i$-*Myc*-rLHRvariant cells were then transfected with anti-cMyc antibody

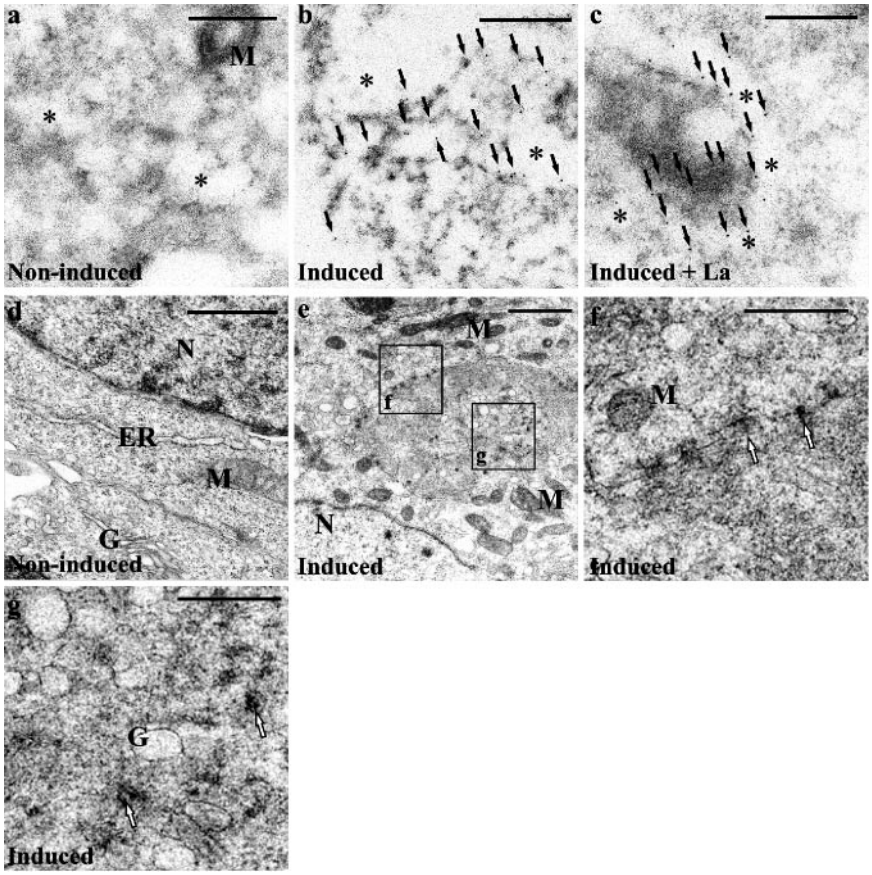

Figure 6. The LHRvariant is located in the ER but proteasomal inhibition leads to its appearance in the cytosol. (A–C) Noninduced (A) and tetracycline induced (B and C) HEK293$_i$-*Myc*-rLHRvariant cells that were treated or not with lactacystin (10 μM) were fixed, and thin cryosections were labeled with mouse anti-cMyc antibody followed by protein A-gold complex (10 nm). The ER lumen is marked with an asterisk and the arrows indicate gold particles. (D–G) Noninduced (D) and tetracycline induced (E–G) HEK293$_i$-*Myc*-rLHRvariant cells were fixed and embedded in plastic, and thin sections were analyzed. Arrows indicate electron-dense material in the compact juxtanuclear tubulo-vesicular body of the induced cells that includes Golgi vesicles and is surrounded by mitochondria. The areas enclosed by squares in E are shown in larger magnification in F and G. Bars, 500 nm (A–C, F and G), 1000 nm (D and E). ER, endoplasmic reticulum; G, Golgi vesicles; La, lactacystin; M, mitochondria; N, nucleus.

and treated with tetracycline to induce variant expression, no specific labeling with the secondary antibody could be detected (Figure 5E; 0% of cells labeled), indicating that the variant must be in the lumenal side of the ER membrane. However, when proteasomal degradation was inhibited with lactacystin, the *Myc*-epitope became available (Figure 5F; 18–23% of cells labeled). The N-terminus of calnexin remained, however, inaccessible to the N-terminal antibody (Figure 5G), indicating that the ER membrane remained intact in the lactacystin-treated cells.

To verify the subcellular localization of the LHRvariant, the HEK293$_i$-*Myc*-rLHRvariant cells were subjected to ultrastructural analysis by immunoelectron microscopy. Thin cryosections of fixed cells were labeled with mouse anti-cMyc antibody followed by protein A–conjugated gold particles (10 nm). As seen in Figure 6B, gold particles were detected in induced cells within the ER tubular network, close to the lumenal membrane, whereas no such particles were apparent in noninduced cells (Figure 6A). In contrast, proteasomal blockade with lactacystin led to appearance of gold particles also in aggregates outside the ER lumen (Figure 6C). When the induced HEK293$_i$-*Myc*-rLHRvariant cells were subjected to transmission electron microscopic analysis, prominent juxtanuclear tubulo-vesicular structures with electron-dense material were observed (Figure 6E-G) that were absent in the noninduced cells (Figure 6D). Taken together, these data indicate that the LHRvariant is retained in the ER in a specialized compartment and although it is targeted for proteasomal degradation, it accumulates in the cytosol only if the proteasomal function is inhibited.

LHRvariant Interferes with LHR Biogenesis by Inducing Misrouting of the Receptor in the ER

A few splice variants and ER-retained mutant forms of GPCRs have been shown to interfere with normal processing and transport of the corresponding wild-type receptors (Benkirane *et al.*, 1997; Grosse *et al.*, 1997; Karpa *et al.*, 2000; Brothers *et al.*, 2004; Kaykas *et al.*, 2004; Nakamura *et al.*, 2004; Sarmiento *et al.*, 2004). Thus, we next investigated whether the LHRvariant that was apparently sequestered by the ER quality control components might have an effect on the biogenesis of the full-length receptor. An N-terminally *HA*-tagged rLHR construct was stably transfected into HEK293$_i$-*Myc*-rLHRvariant cells to create a cell line that expresses the variant under induction but the full-length receptor in a constitutive manner. After treatment of the cells with tetracycline or vehicle, total lysates were prepared and equal aliquots were subjected to immunoprecipitation and hCG affinity chromatography followed by Western blot analysis. As can be seen in Figure 7, A and B, coexpression with the variant led to a clear decrease in the amount of both mature and precursor forms of the receptor, whereas no such change was observed in tetracycline-treated HEK293$_c$ cells that express only the *Myc*- and *Flag*-tagged full-length receptor (Figure 7C). When the number of cell surface receptors was analyzed by flow cytometry using intact anti-HA/phycoerythrin-conjugated anti-mouse antibody stained cells, a significant reduction in the number of cell surface receptors was detected (Figure 7D). Conversely, expression of the full-length receptor did not lead to cell surface targeting and secretion of the variant (unpublished data). Importantly, the decrease in the number of receptor precursors

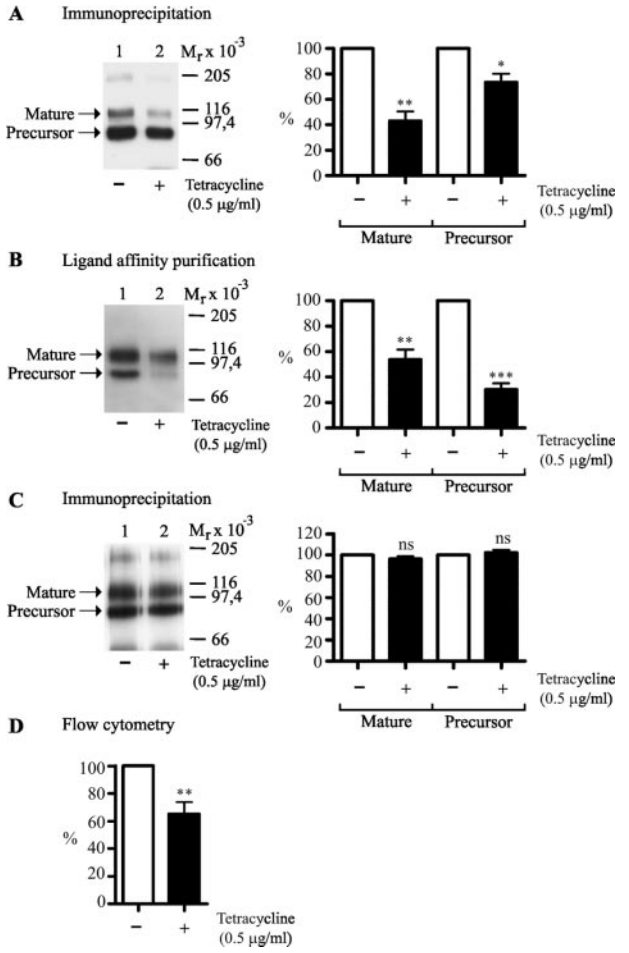

Figure 7. Coexpression with the LHRvariant leads to a decrease in the number of full-length receptors. (A and B) Lysates from noninduced (lane 1) and tetracycline induced (lane 2) HEK293$_i$-*Myc*-rLHRvariant cells expressing constitutively the HA-tagged full-length LHR were prepared and receptors were subjected to immunoprecipitation with mouse anti-HA antibody (A) or hCG affinity chromatography (B) and analyzed by reducing SDS-PAGE and Western blotting with anti-HA antibody. (C) HEK293$_c$ cells constitutively expressing the *Myc*- and *Flag*-tagged full-length LHR were treated (lane 2) or not (lane 1) with tetracycline for 24 h and lysates were subjected to immunoprecipitation with mouse anti-cMyc antibody. The immunoprecipitates were analyzed by reducing SDS-PAGE and Western blotting using polyclonal anti-cMyc antibody for detection. Panels on the right in A–C show the relative intensities of the mature and immature receptor bands as revealed by densitometric scanning and represent the mean ± SEM of three independent experiments. The intensities in cells not treated with tetracycline were set to 100%. (D) Intact noninduced and induced HEK293$_i$-*Myc*-rLHRvariant cells expressing constitutively the HA-tagged full-length LHR were labeled with mouse anti-HA antibody followed by phycoerythrin-conjugated rat anti-mouse antibody and subjected to flow cytometry. The values (mean ± SEM of four independent experiments) indicate the relative mean cell fluorescence compared with the values in noninduced cells that were set to 100%. ***$p < 0.0001$; **$p < 0.009$; *$p < 0.03$; ns, not significant.

that were purified by hCG affinity chromatography (Figure 7B, lane 2) was more pronounced than that purified by immunoprecipitation (Figure 7A, lane 2). This suggests that the newly synthesized full-length receptors have more pronounced difficulties in finding their correct conformation for hormone binding in the variant expressing cells.

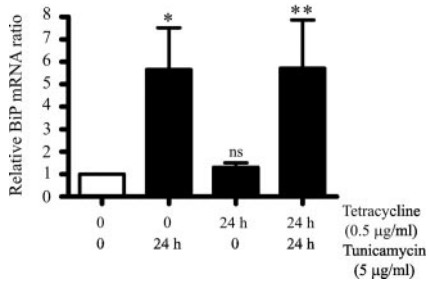

Figure 8. The rLHRvariant expression in HEK293$_i$ cells does not induce unfolded protein response. HEK293$_i$-*Myc*-rLHRvariant cells were treated or not with tetracycline and/or tunicamycin, as indicated. The human BiP mRNA expression was analyzed by quantitative PCR. The values indicate the mean relative values (±SEM of three independent experiments) of BiP/18S. The noninduced cells were given a ratio value of 1. **$p < 0.01$; *$p < 0.02$; ns, not significant.

To unambiguously demonstrate that coexpression of the LHRvariant caused the observed reduction in the number of full-length receptors, the possible secondary effects that induction might create have to be ruled out. Therefore, we tested whether expression of the variant might cause UPR. The UPR is a typical cellular response to accumulating proteins in the ER and results in upregulation of ER foldases and chaperones and also in proteins that are involved in ERAD. Eventually, it also leads to a decrease in protein synthesis (Schröder and Kaufman, 2005). No changes, however, were observed in [^{35}S]Met/Cys incorporation to trichloroacetic acid–precipitable proteins in the noninduced and induced HEK293$_i$-*Myc*-rLHRvariant cells expressing the full-length receptor (unpublished data). Similarly, no changes were detected in the variant expressing cells in the amount of BiP or protein disulfide isomerase by Western blotting (unpublished data) and the BiP mRNA levels analyzed by real-time quantitative PCR remained unaltered (Figure 8). In contrast, the UPR-inducing reagent tunicamycin added 24 h before harvesting, caused a significant increase in BiP mRNA levels (Figure 8). Thus, the lowered receptor number observed in cells that express the variant is unlikely to result from impaired protein synthesis or changes in the ER quality control apparatus.

Because the LHRvariant appears to accumulate in a specialized ER subcompartment, it may also cause misrouting of the full-length receptor shortly after synthesis. Thus, we examined intracellular location of the LHR with immunofluorescence microscopy in cells that coexpress the variant. In noninduced cells that did not express the variant the full-length receptor was apparent in a diffuse perinuclear network with a clear staining of the nuclear envelope (Figure 9, A–C), consistent with the fact that a substantial proportion of receptors exist as precursors in the ER (see Figure 2B, lane 1, and 3A, lane 4; Pietilä *et al.*, 2005). The cell surface receptors could be clearly detected only when intact cells were labeled with anti-cMyc antibody (Figure 9M). When the variant expression was induced, the cell surface staining decreased slightly (Figure 9N), but the changes in the distribution of the intracellular LHRs were more extensive. The receptors lost partially the weblike distribution and were relocated to a more compact location in the juxtanuclear area together with the variant (Figure 9, D–F). No such changes in receptor distribution were observed in tetracycline-treated HEK293$_c$ cells that express constitutively the epitope-tagged full-length receptor (Figure 9, O and P), but the same redistribution was observed when the HA-tagged LHRvariant was transiently transfected into HEK293$_i$-*Myc*-

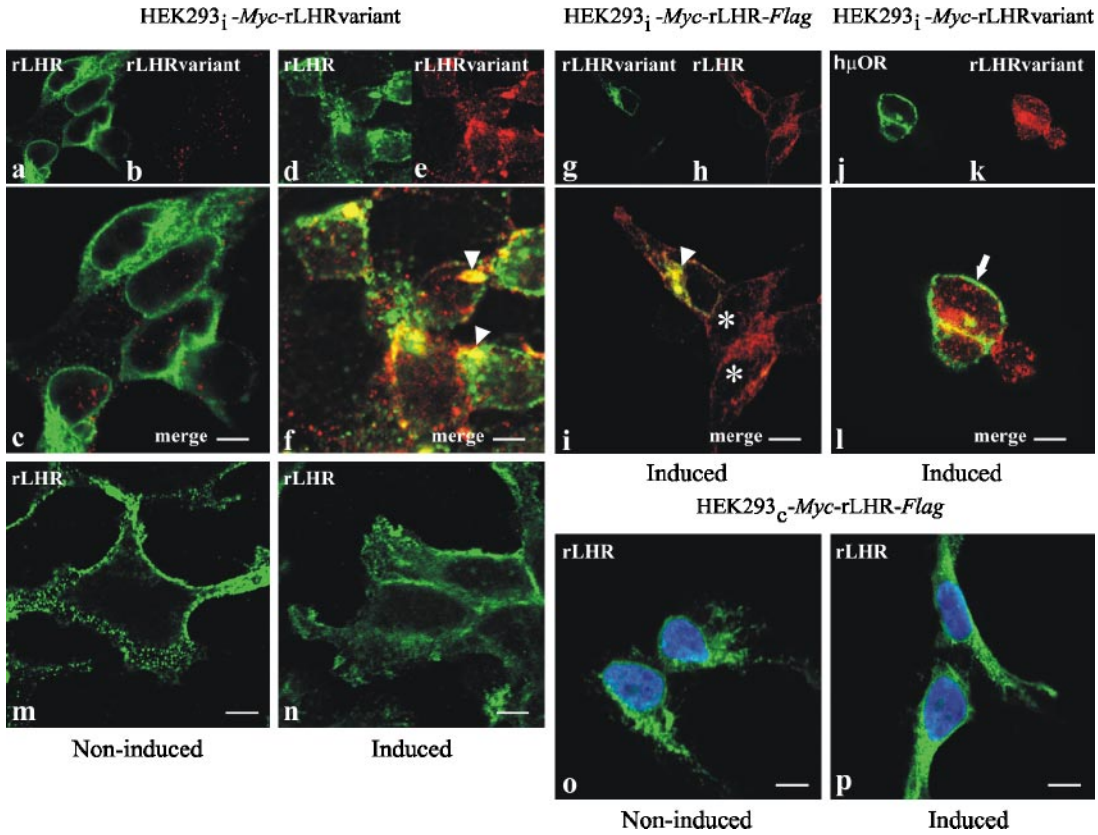

Figure 9. The rLHRvariant redistributes the full-length LHR into a subcompartment of the ER. HEK293$_i$-*Myc*-rLHRvariant cells expressing constitutively the *HA*-rLHR (A–F, M, and N) or transiently the *HA*-hμOR-*Flag* (J–L), or alternatively HEK293$_i$-*Myc*-rLHR-*Flag* cells transiently transfected with the *HA*-rLHRvariant (G–I) or HEK293$_c$-*Myc*-rLHR-*Flag* cells (O and P) were treated or not with tetracycline, as indicated. After permeabilization the HA-tagged rLHR (A–F) and rLHRvariant (G–I) and *HA*- and *Flag*-tagged hμOR (J–L) were labeled with mouse anti-HA antibodies and the *Myc*-tagged rLHRvariant (A–F and J–L) and *Myc*- and *Flag*-tagged rLHR (G–I, O, and P) with rabbit anti-cMyc antibody. Alternatively, the cell surface *Myc*- and *Flag*-tagged rLHRs (M and N) were labeled before permeabilization with rabbit anti-cMyc antibody. Secondary antibodies were Alexa 488- or Alexa 568-conjugated goat anti-rabbit and anti-mouse IgG. Colocalization of the variant and the full-length receptor in the juxtanuclear region is indicated by arrowheads (F and I). The LHRvariant mediated redistribution of the full-length receptor is clearly evident in the HEK293$_i$-*Myc*-rLHR-*Flag* cells transiently transfected with the *HA*-rLHRvariant (arrowhead in I), but in nontransfected cells (asterisks in I) the receptor is apparent in a more dispersed reticular pattern. The hμOR did not colocalize significantly with the variant and was transported to the cell surface (arrow in l). The nuclei were stained with Hoechst in O and P. Bar, 10 μm.

rLHR-*Flag* cells and the expression of both proteins was induced concomitantly with tetracycline (Figure 9, G–I). The vector alone did not cause any redistribution (unpublished data). To rule out the possibility that overexpression of the variant might nonspecifically interfere with protein transport, the HEK293$_i$-*Myc*-rLHRvariant cells were transiently transfected with the HA-tagged hμOR. As seen in Figure 9, J–L, the ER staining of the hμOR did not colocalize significantly with the variant, suggesting that its intracellular trafficking route may be different. Furthermore, the variant did not interfere with transport of the hμOR to the cell surface. Taken together, these data indicate that the LHRvariant does not only decrease the number of full-length receptors but also redistributes them into an ER subcompartment, a phenomenon that is not a result of nonspecific misrouting of proteins after overexpression of a misfolded protein.

DISCUSSION

The LHR is a GPCR that is subject to extensive alternative splicing but the biological role of the numerous splice variants has remained largely unknown. In the present study we demonstrate that the common splice variant comprising a large portion of the N-terminal extracellular domain of the receptor can regulate expression of the full-length receptor in a heterologous expression system in mammalian cells. Similar findings have been reported in the past for a few other GPCRs (Grosse *et al.*, 1997; Karpa *et al.*, 2000; Sarmiento *et al.*, 2004) and recently also for another LHR variant (Nakamura *et al.*, 2004). Thus, regulation of GPCRs by their corresponding splice variants may be a more common phenomenon than has been anticipated.

In contrast to expectations, the LHRvariant was not found to be a secreted protein but was retained in specialized juxtanuclear areas of the ER before being targeted for ERAD. It also caused misrouting of the full-length receptor in the ER and decreased the number of receptors that were capable of hormone binding and reaching the plasma membrane. These results thus question the possibility that the variant could act as a soluble quencher of LH and hCG in the circulation and is therefore more likely to have a functional role in LH/hCG target cells by regulating the number of functional receptors. Thus it can be argued that the most prominent changes detected in transgenic mice overexpressing the variant (Apaja, Poutanen, Aatsinki, Petäjä-Repo, and Rajaniemi, unpublished results), namely alterations in pitu-

itary-gonadal functions and steroidogenesis and morphological changes in the adrenals and kidneys, are likely to be caused by variant-mediated changes in the intracellular trafficking of the full-length receptor in the corresponding target cells.

The LHRvariant contains all the amino acids that have been shown to be required for hormone binding (Ascoli *et al.,* 2002; Vassart *et al.,* 2004). Thus, it was not unexpected that it could be purified by hCG affinity chromatography when expressed in HEK293 cells. This is in line with previous studies that have shown that the variant displays similar high affinity in saturation binding assays as the full-length receptor (Tsai-Morris *et al.,* 1990; Zhang *et al.,* 1995). Nevertheless, because only a very small proportion of the variant was purified and no secretion to the medium was observed, it is apparent that a majority of the protein is not able to find the correct conformation. The reason for this inability to fold correctly is not known but is unlikely to result from the absence of amino acids in the C-terminal cysteine-knot domain of the ectodomain, as also the full-length LHR ectodomain has been shown to be retained intracellularly in mammalian cells (Xie *et al.,* 1990; Braun *et al.,* 1991). However, the 22-amino acid C-terminal tail of the variant contains an additional cysteine (Figure 1B) that might interfere with the formation of the correct disulfide bonds. Interestingly, when the truncated ectodomain is coexpressed with the C-terminal portion of the receptor containing the transmembrane and cytoplasmic domains, they form a functional receptor that is delivered to the cell surface (Bozon *et al.,* 2002). In contrast, coexpression of the extracellular variant and the full-length receptor did not lead to secretion of the variant but resulted in the intracellular retention of the receptor.

The results of the present study indicate that the LHRvariant was retained intracellularly and appeared to accumulate in a special juxtanuclear subcompartment of the ER. It also led to relocation of the ER chaperones calnexin and calreticulin into the same cellular location. This variant-containing ER subcompartment resembles ER membrane structures that have been shown to form in response to a few other misfolded or unassembled proteins, both in yeast and in mammalian cells. For example, misfolded human asialoglycoprotein receptor H2a and free heavy chains of murine class I major histocompatibility complex accumulate in a specialized "quality control" compartment of the ER adjacent to the centrosome, Golgi, and ER-to-Golgi intermediate compartment when proteasomal degradation is inhibited (Kamhi-Nesher *et al.,* 2001), and some mutant forms of Ste6p in yeast accumulate in specialized structures, called ER-associated compartments (ERAC; Huyer *et al.,* 2004). Similarly, the olfactory receptors have been found to be sequestered in ER aggregates together with calnexin (Lu *et al.,* 2003). These ER subcompartments have been suggested to represent holding sites, into which misfolded or unassembled proteins are delivered before being eventually retrotranslocated to the cytosol (Kamhi-Nesher *et al.,* 2001; Frenkel *et al.,* 2004; Huyer *et al.,* 2004).

Blockade of proteasomal degradation with lactacystin was found to increase the amount of the LHRvariant, indicating that it is an ERAD substrate. However, only proteasomal blockade resulted in the appearance of the variant in the cytosol. This was suggested by the finding that antibodies delivered into the cytosol of variant expressing cells were able to recognize the N-terminal epitope of the protein only when the proteasomal degradation was blocked. Furthermore, immunoelectron microscopy revealed that the variant could be seen outside the ER only after lactacystin treatment. These findings are in contrast to many other misfolded or unassembled proteins that have been shown to accumulate in the cytosol even without proteasomal blockade (Huppa and Ploegh, 1997; Bebök *et al.,* 1998; Petäjä-Repo *et al.,* 2001). The highly hydrophobic nature of the variant makes it very prone to aggregation and it is therefore reasonable to speculate that it is advantageous for the cell to maintain such proteins in a soluble form in the ER lumen by chaperones. This notion is supported by the finding that calnexin and calreticulin were found to interact with the variant and relocated together to the same ER subcompartment and no high-molecular-weight aggregates of the variant were detected by Western blotting. The juxtanuclear ER subcompartment may thus provide a mean, by which cells can cope with aggregation-prone misfolded proteins.

Coexpression of the LHRvariant with the full-length receptor led to substantial change in the behavior of the latter. There was a significant decrease in the number of both cell surface and intracellular receptors and a larger fraction of receptor precursors were incapable of hormone binding. In addition, the intracellular receptors redistributed in a more juxtanuclear area of the ER. These changes were not a consequence of altered protein synthesis or changes in the components of the ER quality control apparatus, as expression of the variant was not found to cause UPR or general change in the ER morphology. Furthermore, the LHRvariant had no effect on the distribution of another GPCR, the hμOR that was transported to the cell surface, indicating that it did not cause a general block in ER export. Therefore, the most likely cause for the decrease in the receptor number is misfolding of the newly synthesized receptors and their misrouting into a specialized ER subcompartment. Whether these relocated receptors are eventually retrotranslocated to the cytosol and degraded by proteasomes needs to be assessed in future studies. This is, however, very likely as the variant did not lead to accumulation of the receptor precursors. An attractive possibility is that the variant functions as a closed circuit chaperone or degradation assistant of the full-length receptor in enhancing degradation of the receptor precursors. It can be hypothesized that misrouting of the full-length receptor might be a consequence of its interaction with the variant. In agreement with this possibility, Nakamura *et al.* (2004) have shown that the human LHR splice variant lacking exon 9 interacts with and can alter expression of the full-length LHR as well as that of another glycoprotein hormone receptor, the follicle-stimulating hormone receptor (Yamashita *et al.,* 2005). Furthermore, the recently reported crystal structure of the truncated follicle-stimulating hormone receptor ectodomain and its cognate hormone reveal that the ectodomain is able to dimerize in the absence of the C-terminal half of the protein (Fan and Hendrickson, 2005). Future studies are necessary to test the possibility that the LHRvariant might form a heterodimer with the full-length receptor. However, our preliminary trials to detect dimerization by coimmunoprecipitation have failed, suggesting that stable interaction between the variant and the full-length receptor is unlikely to take place (unpublished data).

As a summary, we have demonstrated that the LHRvariant is a nonsecreted intracellularly retained protein that can regulate the number of full-length receptors by redistributing the newly synthesized receptor molecules in the ER into a subcompartment that probably diverts them away from the normal secretory pathway. Thus, the LHRvariant may have a role in posttranslational regulation of the full-length receptor. Whether such a regulatory role could be assigned to other splice variants of the LHR in general is not known. However, this is a very likely possibility, because changes in physiological stimuli (Lakkakorpi *et al.,* 1993; Licht *et al.,* 2003), cellular differentiation (Sokka *et al.,* 1992; Tena-Sempere *et al.,* 1994; Zhang *et al.,* 1994; Apaja *et al.,* 2004, 2005)

and tumorigenic cell growth (Lin *et al.*, 1994; Reinholz *et al.*, 2000; Jiang *et al.*, 2002; Steinmeyer *et al.*, 2003) have all been shown to regulate the splicing pattern of the receptor.

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