Requirement of Cysteine Residues in Exons 1–6 of the Extracellular Domain of the Luteinizing Hormone Receptor for Gonadotropin Binding*

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The functional importance of cysteine residues in the extracellular domain and the extracellular loops (EL1 and EL2) to hormone binding of the rat luteinizing hormone receptor (LHR) was investigated. For this purpose, cysteines in the seven-transmembrane holoreceptor (Form A) and its hormone-binding splice variant (Form B) were replaced by serine residues, and mutant receptors were expressed in COS1 and/or insect cells. Within the extracellular domain, individual replacement of all four cysteines from Exon 1 abolished hormone binding activity, and replacement of Cys-109 and Cys-134 from exons 5 and 6 caused a 75% decrease in both cell surface and total cellular solubilized LHR hormone binding activity. Mutations of Cys-257 and -258 (Exon 9), Cys-321 and -331, and Cys-417 and -492 of EL1 and EL2, respectively (Exon 11), showed no surface hormone binding activity on intact cells, but exhibited wild type levels of total hormone binding activity when recovered from detergent-solubilized cellular extracts. This finding indicated that expression of high affinity LHR binding activity at the cell surface is independent of the acquisition of the high affinity binding conformation. Other cysteine residues, including Cys-282 (exon 10), and Cys-314 (exon 11) were not essential for hormone binding activity or plasma membrane insertion. This study demonstrates that the functional hormone binding domain utilizes all cysteines N-terminal to exon 7 and localizes the binding site to this N-terminal region of the extracellular domain.

The luteinizing hormone receptor (LHR) is a seven-transmembrane (1) G protein-coupled receptor (GPCR) with a high affinity hormone binding site on the N-terminal extracellular domain (Fig. 1) (2, 3). This hormone binding domain is composed of two Cys-rich regions in exons 1 and 9, generally conserved in the glycoprotein hormone receptor family (FSHR and TSHR), and bordering a leucine-rich domain that is repeated from exons 2 to 8 (4). A third Cys-rich region (Cys-314, -321, and -331) is located outside of the hormone binding domain although still in the extracellular region and is also conserved in the FSHR (5) and TSHR (6). The LHR contains only three cysteines within the extracellular domain that are not conserved in the FSHR and TSHR, at Cys-109, Cys-134, and Cys-282 (Fig. 1). These cysteines are within domains that have been implicated to be of importance to LH/hCG binding in chimeric studies of the glycoprotein hormones (7). Outside of the N-terminal extracellular domain, two LHR cysteines on EL-1 and EL-2 are conserved in most of the GPCR and are of structural importance to the adrenergic rhodopsin family (8, 9). Amino acid homology within the transmembrane and cytoplasmic domains of the GPCR have led investigators to propose the existence of a common mode of signal transfer and G coupling that may involve the formation or disruption of disulfide bonds (10, 11).

To study the importance of cysteines and disulfide bonding to LHR hormone binding, the indicated cysteines (SH) (Fig. 1) in the rat holoreceptor were mutated to the amino acid serine (OH) and expressed in the mammalian COS1 cell. The substitution of Cys to Ser prevents the formation of a putative disulfide bond, without significantly affecting the charge characteristics of the protein (12). The isolated hormone binding domain was studied in both the COS1 cell and the insect Sf9 cell with the soluble splice variant Form B that contains the high affinity hormone binding domain without the transmembrane, extracellular loops, or cytoplasmic domain (2). This constitutes amino acids 1–294 of the holoreceptor and a unique 22-amino acid tail that contains one additional cysteine that may functionally substitute for a Cys in the holoreceptor. Our results demonstrate that only the four Cys within exon 1 are essential for hormone binding activity and that the two unique Cys at 109 and 134 are of importance, although not essential to hormone binding. The extracellular Cys (257, 258, 321, and 331) and EL1 417 and EL2 492 that are conserved in all of the glycoprotein hormone receptors are required for membrane insertion, but they do not contribute to hormone binding activity.

MATERIALS AND METHODS

Design of LHR Form A and B Wild-type and Mutant DNA Constructs

The full-length rat ovarian LH/CG receptor (LHR Form A) cDNA was subcloned into the human cytomegalovirus promoter-driven expression vector, pCMV4 (gift from Dr. D. Russell, University of Texas Southern Medical Center), as described previously (2). LHR mutants were constructed using the recombinant circle polymerase chain reaction (13). Synthetic oligonucleotides (Midland Certified Reagent Company, Midland, TX) were designed to individually replace Cys-8, -12, -22, -109, -134, -257, -282, -314, -321, -331, -417, and -492 with serine (Fig. 1). WT and mutant Form B cDNAs were subcloned into the EcoRI site of pVL1393 (PharMingen, San Diego, CA) for infection of insect cells. The orientation of the WT and mutant Form A and Form B constructs was determined by restriction enzyme analysis, and mutations were verified by DNA sequencing.

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1 The abbreviations used are: LHR, luteinizing hormone receptor; FSHR, follicle-stimulating hormone receptor; TSHR, thyrotropin receptor; LH, luteinizing hormone; hCG, human chorionic gonadotropin; WT, wild-type; PAGE, polyacrylamide gel electrophoresis.
Importance of Extracellular Cysteines in LHR Binding

Expression of WT and Mutant LHR in Mammalian COS1 and Insect Cells

WT and mutant LHR cDNAs in pCMV4 expression vectors were transfected in mammalian COS1 cells by the LipofectAMINE (Life Technologies, Inc.) method according to manufacturer's protocol. The expressed membrane-bound receptor activity was determined by 125I-hCG binding 48 h after transfection (2). 125I-hCG (specific activity, 40.6 µCi/µg, 48% maximal bindability) was prepared by a modification of a lactoperoxidase method as described previously (14). Spodoptera frugiperda (SF9) cells (PharMingen) were co-transfected with 4 µg of WT or mutant LHR Form B (pVL1393LHRB) and 0.5 µg of BaculoGold® DNA (PharMingen) according to manufacturer's protocol. The recombinant virus titers were amplified by infection of SF9 cells (70°C for 2 h). At 48 h postinfection, the cells were harvested, washed, and homogenized in phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml of leupeptin, and 24 trypsin inhibitor units of Trasylol (aprotinin, Sigma). The cell homogenates in 20% glycerol were stored at −70°C for further studies.

Preparation of Detergent-solubilized Extracts of Transfected COS1 Cells and SF9 Cells

COS1 Cells—48 h after transfection with WT or mutant LHR cDNAs, COS1 cells were recovered, homogenized, and solubilized as described previously (2). Protein concentration was determined by BCA protein assay (Pierce, Rockford, IL). SF9 Cells—48 h postinfection with recombinant virus, SF9 cells were homogenized with 50 strokes at 1800 rpm of a tissue grinder (Potter-Elvehjem, Thomas Scientific, Swedesboro, NJ); glycerol and Nonidet P-40 were added to final concentrations of 20 and 1%, respectively. Extract was rotated for 1 h at 4°C and diluted to a final Nonidet P-40 concentration of 0.1%. The solubilized mixture was centrifuged, and protein concentration was determined as described above.

125I-hCG binding to LHR

Intact COS1 cells or solubilized extracts from transfected COS1 or insect SF9 cells were used for 125I-hCG binding assay as described previously (2, 17). Binding was performed using displacement assays by incubating intact cells or solubilized extracts with 125I-hCG and increasing concentration of unlabeled hCG. Nonspecific binding was determined in samples containing an excess of the unlabeled hCG (1 µg). The binding parameters (Kd and binding capacities) were analyzed from Scatchard plots and saturation curves using a nonlinear model curve-fitting program (15). All experiments were performed at least 3 times in triplicate. Statistical analysis for all studies were performed using analysis of variance (Supernova, Berkeley, CA) and Post Hoc Fisher LSD tests at the 99% significance level.

Western Blot Analysis of Wild-type and Mutant LH/CG Receptor

Aliquots of Form B LHR (100 ng) from detergent-solubilized SF9 cells transfected with WT and mutant cDNAs were boiled for 5 min in sample buffer (reducing conditions: final concentration, 10% glycerol, 2% SDS, 2% β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, 0.02% bromphenol blue; nonreducing conditions: final concentration, 10% glycerol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8, 0.02% bromphenol blue). Holoreceptor (−50 ng) purified from ovarian membranes as described previously (16) was denatured and prepared as above under reducing or nonreducing conditions. The truncated splice variant (Form B) or the holoreceptor (Form A) were subjected to 10% SDS-PAGE electrophoresis analysis. Following transfer to nitrocellulose membrane (Bio-Rad, Hercules, CA), the LH/CG receptor was probed with 1:800 dilution of immunoaffinity-purified rabbit polyclonal antibody raised against an peptide antigen (amino acids 36–51) of the rat LH/CG receptor (17). Secondary antibody (anti-rabbit antiserum conjugated to horseradish peroxidase (Life Technologies, Inc., Gaithersburg, MD) (1:1000 dilution) for 22°C. Membranes was washed and developed with the enhanced chemiluminescence detection kit (ECL, Amersham Corp.).

Ligand Blot

Samples processed as for Western blot analysis (nonreducing or reducing conditions; see above) were electrophoresed on 10% SDS-PAGE and transferred electrophoretically to nitrocellulose. Binding of hCG to WT and mutant receptor was performed by incubation of the

FIG. 1. The rat LH receptor. The amino acid sequence of the rat LH receptor (1). Amino acids corresponding to the cleavable signal peptide are within hexagons, and those of the mature polypeptide are within circles. The rat LHR Form B sequence diverges from holoreceptor at 294 (1) and continues with LLHGAL-PATHCLS peptide tail (2). Cysteines are indicated by shaded circles, and those we have mutated are designated by amino acid number. Exon divisions are represented by vertical lines and exons are numbered 1–11.

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Importance of Extracellular Cysteines in LHR Binding

RESULTS

Transfection of the LHR holoreceptor Form A into the COS1 cell yields a high affinity hCG hormone binding receptor that is translocated to the cell surface. Scatchard analysis of the surface wild-type holoreceptor reveals that the dissociation constant \( K_d \) of the LHR holoreceptor expressed in COS1 cell is similar to that of the rat ovarian LH receptor (2, 20). Similar hormone binding affinities were also obtained with expression of the Form B splice variant in COS1 or insect SF9 cells (17). Individual mutation of each of the cysteines in exon 1 to Ser of the holoreceptor expressed from the COS1 cell (Cys 8, 12, 14, 22) or the Form B splice variant expressed in the insect cell (Cys 8, 12, 14, 22) resulted in total loss of hormone binding activity (Table I, Figs. 2 and 3). Receptor activity was not recovered in the detergent solubilized fraction of the COS1 cell (Fig. 2), indicating that loss of activity was not due to entrapment within the cells.

To confirm that Cys-8 → Ser, Cys-12 → Ser, Cys-14 → Ser, and Cys-22 → Ser was expressed, the concentration of receptors was determined by specific radioimmunoassay. Mutant receptor levels of Cys-8, 12, 14, 22 were within 60% of wild-type, and Cys-12 → Ser and Cys-14 → Ser levels were not significantly different from wild-type (Table II).

Substitution of the unique cysteines from exon 5 and 6 (Cys-109 and Cys-134) caused a 75% decrease in hormone binding activity on the surface of the intact cell that could not be recovered in detergent-solubilized extracts (Figs. 2 and 3). Expression levels of these mutant LHRs were not significantly different from wild-type (Table II, Fig. 4), indicating that substitution of Cys-109 and -134 specifically affects hormone binding but not receptor expression. In addition, these cysteines do not appear to play a role in membrane insertion since the proportion of active mutant to wild-type LHR was the same for surface and solubilized receptors (approximately 25%). Ser-134 LHR exhibited a marked reduction in maximal hormonal activation of cAMP in comparison with the wild-type. This reduction of 80 ± 15% was consistent with the 75% decrease in hormone binding activity, and similarly substitution of Cys-109 reduced maximal hormonal stimulation (Table II).

Individual substitution of the two consecutive cysteines within exon 9 (Cys-257 and -258) (Fig. 1), which are conserved in all of the glycoprotein hormone receptors, resulted in total loss of surface binding activity (Fig. 2). However, 70–100% of the wild-type hormone binding activity was recovered following detergent solubilization. (Table I, Fig. 2). To determine whether Cys-257 functionally replaced Cys-258 in the Cys-257 → Ser mutant, the double mutant Cys-257 → Ser/Cys-258 → Ser was expressed from COS1 cells and characterized for hormone binding activity. Detergent extracts from cells expressing the double mutant also displayed 90% of the wild-type hormone binding activity. The binding affinities (\( K_d \)) of these mutant receptors were identical to that of wild-type (Table I). Thus, it does not appear that Cys-257 or -258 are important for hCG hormone binding.

Amp Determination

cAMP generation from COS1 cells transfected with wild-type or mutant LHRs in the presence of a range of hCG concentrations was determined by radioimmunoassay as described previously (19). All experiments described above were performed at least 3 times in triplicate.

Radioimmunoassay

LHR expressed from SF9 cells or COS1 cells transfected with wild-type or mutant LH receptor form B or A cDNAs, respectively, were quantitated by radioimmunoassay using affinity-purified polyclonal rabbit antiserum raised against rat LH receptor peptide antigen 36–51 as described previously (17–18). Approximately 25% of the holoreceptor expressed from the COS1 cell and 25–200 µg of particulate protein from COS1 cells were suspended in 100 µl of radioimmunoassay buffer (50 mM sodium phosphate, pH 7.4, 12 mM EDTA, 0.02% NaN₃, 0.1% Nonidet P-40, 0.1% bovine serum albumin (fraction V, Sigma) and 24 trypsin inhibitor units of tranexyl (aprotinin, Sigma) and incubated with 100 µl of LH receptor (36–51) antibody (final dilution 1:3000), 200 µl of radiomunnoassay buffer, and 50,000 cpm of 125I-labeled peptide (36–51) (specific activity, 176 Ci/mmol) for 24 h at 4°C. Separation of antibody bound from free peptide was performed as described previously (17–18). Sample values were derived from reference standard curves (ID₅₀ of displacement of 0.15 pmol/assay tube). Assays were performed in triplicate with at least three dilutions. The interassay coefficient of variation was 8%. Expressed wild-type and mutant LHR concentrations from COS1 and insect cells displayed parallelism to the standard curve, while samples from cells transfected with the expression vector only showed no displacement.

TABLE I

| Location | Mutants | Ka x 10⁶ M⁻¹ |
|----------|---------|--------------|
|          |         | Intact Cell  | Soluble     |
| WT       | C8      | 5.36 ± 0.52  | 6.71 ± 0.46 |
|          | C12S    | ND           | ND          |
| Exon 1   | C12S    | ND           | ND          |
| Exon 1   | C14S    | ND           | ND          |
| Exon 5   | C109S   | 7.11 ± 0.76  | 5.33 ± 1.38 |
| Exon 6   | C134S   | 7.46 ± 0.51  | 4.21 ± 1.52 |
| Exon 9   | C257S   | ND           | 4.06 ± 0.59 |
| Exon 10  | C258S   | ND           | 4.23 ± 0.52 |
| Exon 11  | C313S   | ND           | 6.31 ± 0.70 |
| EL 1     | C417S   | ND           | 3.67 ± 0.59 |
| EL 2     | C492S   | 4.08 ± 0.89  |

EL, extracellular loop; ND, not detectable; mean ± S.E.

FIG. 2. Percent wild-type 125I-hCG binding activity of designated CysLHRSer holoreceptor mutant proteins expressed from COS1 cells. *, no significant difference from basic vector without insert.

The blots with 125I-hCG (10⁷ cpm/ml) for 16 h at 4°C in the absence or presence of excess unlabeled hCG (1 µg/ml). The blots were processed and autoradiographed as described previously (16).
Substitution of Cys-282 → Ser (Fig. 1), which is not conserved in the FSHR or TSHR, resulted in a mutant receptor with impaired but not total loss of surface hormone binding activity (Fig. 2; Tables I and II). Cys-282 → Ser exhibited a 50% reduction in surface receptor hormone binding activity, with a binding affinity that was similar to wild-type (Table I). However, total detergent solubilization of this mutant yielded total receptor binding concentrations that were similar to wild-type (Table I and II). Hormonal stimulation caused dose-related increases in cAMP levels in cells transfected with this mutant, and maximal levels were 51 ± 2% that of wild-type. This correlated with the 50% reduction in surface receptor (Table II). Thus, Cys-282, which is unique to the LHR, is not important for either hCG hormone binding or signal transfer, but it does contribute to surface membrane expression.

Cys-314, -321, and -331; Cys-417; and Cys-492 are not contained in the Form B hormone binding domain, although these are present in the extracellular N-terminal, and EC loops 1 and 2, respectively, of the holoreceptor (Fig. 1). Substitution of Cys-314 → Ser did not affect either hormone binding affinity or membrane insertion (Table II, Fig. 2), or cAMP levels (not shown). Thus Ser-314 is functionally equivalent to Cys-314, and Cys-314 does not have to be paired in a disulfide bond in the holoreceptor for binding activity. Substitution of Cys-321, Cys-331, Cys-417, and Cys-492 by Ser resulted in mutant receptors that exhibited no surface binding activities. However, binding activity of these mutant LHRs were recovered in detergent-solubilized extracts (Table I and II; Fig. 2).

TABLE II
Effects of mutations of extracellular cysteine residues on expression and binding activity of the LH holoreceptor

| Cys Exon | RIA | Soluble | Surface | HB | SB |
|----------|-----|---------|---------|----|----|
| 8        | 1   | 60 ± 1  | —       | —  | Y  |
| 12       | 1   | 96 ± 3  | —       | —  | Y  |
| 14       | 1   | 85 ± 8  | —       | —  | Y  |
| 22       | 1   | 62 ± 2  | —       | —  | Y  |
| 109      | 5   | 100 ± 13| 21 ± 0.8| 24 ± 0.5| Y | N |
| 134      | 6   | 117 ± 18| 26 ± 0.6| 22 ± 1 | Y | N |
| 257      | 9   | 84 ± 8  | 77 ± 0.6| —  | N  |
| 258      | 9   | 79 ± 6  | 101 ± 10| —  | N  |
| 282      | 10  | 94 ± 4  | 96 ± 12 | 50 ± 7 | N | Y |
| 314      | 11  | 108 ± 5 | 98 ± 6  | 95 ± 3 | N | N |
| 312      | 11  | 85 ± 2  | 75 ± 9  | —   | N  |
| 331      | 11  | 110 ± 7 | 89 ± 11 | —   | N  |
| 417      | 11  | 106 ± 7 | 97 ± 1  | —   | N  |
| 492      | 11  | 92 ± 7  | 89 ± 2  | —   | N  |
| 12/14    | 1   | 50 ± 8  | —       | —  | Y  |
| 12/257/258| 9   | 95 ± 7  | 93 ± 9  | —  | N  |

The ability of the reduced, denatured wild-type Form B LHR to recover its active configuration and bind hormone in ligand blotting was performed with each of the exon 1 cysteine mutants (Cys-8 → Ser, Cys-12 → Ser, Cys-14 → Ser, Cys-22 → Ser) to resolve two questions. First, whether ligand binding of the reduced wild-type Form B receptor on nitrocellulose was due to renaturation that involved exon 1 cysteines, and perhaps the reformation of disulfide bonds during electroblotting, or the hormone binds to a surface that is exposed on nitrocellulose without the aid of disulfide loops. Second, does the loss of hormone binding activity by the Cys/Ser substitutions in exon 1 result from the loss of required free or disulfide bonded cysteines on the mature receptor or impairment of initial processing of the nascent LHR in the endoplasmic reticulum.

None of these cysteines in the mature LHRs (Cys-8 → Ser, Cys-12 → Ser, Cys-14 → Ser, Cys-22 → Ser) to resolve two questions. First, whether ligand binding of the reduced wild-type Form B receptor on nitrocellulose was due to renaturation that involved exon 1 cysteines, and perhaps the reformation of disulfide bonds during electroblotting, or the hormone binds to a surface that is exposed on nitrocellulose without the aid of disulfide loops. Second, does the loss of hormone binding activity by the Cys/Ser substitutions in exon 1 result from the loss of required free or disulfide bonded cysteines on the mature receptor or impairment of initial processing of the nascent LHR in the endoplasmic reticulum.

The importance of disulfide bonds to hormone binding activity was investigated in ligand blots using the Form B splice variant expressed in insect cells and the affinity-purified Form A holoreceptor isolated from ovarian membranes. Treatment of the 38-kDa Form B receptor with reducing agent prior to electrophoretic separation, and transfer to nitrocellulose, did not reduce the ability of the electroblotted receptor to bind labeled hCG (Fig. 5, LHRA, reducing and nonreducing). These results, and those reported recently (17), indicate that the initial oxidation state does not affect the activity of the Form B LHR to renature during the electroblotting transfer to nitrocellulose membrane.

In contrast, prior incubation of the Form A holoreceptor with reductant severely impaired the ability of the receptor to bind hormone after electrophoresis in ligand blots (Fig. 5, LHRA, reducing versus nonreducing). Thus, the addition of the transmembrane/cytoplasmic domains presumably has a negative effect on renaturation of the reduced LHR on nitrocellulose (see “Discussion”). A minor aggregate (>200 kDa), more pronounced under nonreducing conditions, exhibits no detectable ligand binding (Fig. 5, left, LHRA, reducing and nonreducing versus right, LHRA, reducing and nonreducing).

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None of the exon 1 Form B LHR mutant receptors exhibited hormone binding activity when immobilized on nitrocellulose (Fig. 6), indicating that in contrast to the denatured, reduced wild-type Form B LHR, the exon 1 mutants are incapable of renaturation during electroblotting (Fig. 6). This indicates that Cys-8, -12, -14, and -22 are essential for renaturation of the mature LHR and that therefore these cysteines play an essential role beyond processing of the nascent receptor, perhaps as disulfide-bonded cysteines.

**DISCUSSION**

Site-directed mutagenesis of the cysteines of the rat LHR, including residues that are unique to the LHR (Cys-109, -134, and -282), of importance to the rhodopsin/adrenergic family (Cys-417 and -492) (8, 9), and those that are conserved in the extracellular domain of the glycoprotein hormone receptor fam...
acid N-terminal extracellular hormone binding domain that can be physically separated from the transmembrane/cytoplasmic domain without loss of hCG hormone binding affinity (2). Putative disulfide bonds that connect the extracellular domain to the transmembrane/cytoplasmic domain are not required for hormone binding activity, and in this study we have found no cysteines including and following the exon 9 position 257/258 that are important for hormone binding.

The isolated high affinity hormone binding domain that is represented by the splice variant Form B and consists of exons 1 to 10 carries a unique carboxyl 22-amino acid tail with an extra cysteine (Fig. 1) that is not in the holoreceptor. This cysteine does not function as a replacement for one of the extracellular exon 11 cysteines (Cys-314, -321, -331, -417, or -492), since none of these exon 11 cysteines are important for hormone binding. However, the addition of the Form B cysteine (Cys-305) does cancel out a potential unpaired cysteine (Fig. 1) and may serve to reduce intermolecular aggregation that could result from the reactivity of an unpaired cysteine.

The four cysteines in exon 1 are each essential for hormone binding activity, and no other cysteines can replace their functions in the mutant LHRs. In the TSHR receptor, mutation of the analogous first three cysteines of exon 1 did not affect TSH binding (21), suggesting that these have a unique function in LH/hCG binding, perhaps forming disulfide bonds with the unique LHR cysteines 109 and 134, which are not conserved in the TSH receptor. Although the four cysteines of the TSHR exon 1 can substitute for the LHR exon 1 in chimeric receptor constructs, hCG binding was only achieved when the segment that includes Cys-109 and -134 of the LHR was also present in the construct (7). However, putative disulfide bonds between cysteines in exon 1 and Cys-109 or Cys-134 should yield equivalent reductions in activity upon mutation of each half cystine, which was not the case in these studies (Tables I and II). Rather, a putative disulfide bond is suggested between Cys-109 and Cys-134 in the hormone binding domain since mutation of each of these resulted in equivalent reductions of binding activity.

There is some indication that the extracellular and transmembrane domains are linked by disulfide bonds in the TSH receptor, and LHR-specific interactions between the two domains have also been proposed to account for membrane insertion of the LH receptor (22). Our experiments suggest that the LHR extracellular/transmembrane interactions may be in the form of disulfide bonds, similar to the TSHR. Cys-257, -258, -321, and -331 of the extracellular domain and the Cys-417 of EL1 and Cys-492 of EL2, all conserved in the glycoprotein hormone receptors LHR, TSHR, and FSHR, are required for membrane insertion but not hormone binding activity (Table I). Individual mutation of each of these cysteines prevented sur-
face expression, indicating that the disulfide configuration is highly specific and that other cysteines cannot functionally substitute. Most of these extracellular cysteines that are essential for insertion into the membrane may be important in signal transduction, since they are conserved in all members of the glycoprotein hormone receptor family. The acquisition of the correct disulfide configuration to initiate signal transfer may be a prerequisite for membrane insertion, although, as we have shown, acquisition of the high affinity binding conformation is not a prerequisite for membrane insertion.

In studies with bovine opsin, which does not contain an extended extracellular domain, cysteines from extracellular loop 1 and 2 (Cys-110 and -187) were required for membrane insertion (23), similar to our observation with Cys-417 and -492 mutant LHRs. Cys-621 and -622 from the cytoplasmic domain have been reported to be important for membrane insertion (24). However, another report attributes the decrease in surface Cys mutant receptors to an increase in the rate of hormone/receptor internalization (25). In addition, non-Cys mutations in the transmembrane/cytoplasmic module such as E441Q can also impair membrane insertion (26). Taken together, these studies indicate a wide range of extracellular and transmembrane/cytoplasmic amino acids that are required for membrane insertion of the LHR, suggesting that the total integrity of the exon 7–11 domain is a prerequisite for translocation to the plasma membrane.

Previous studies with tunicamycin show that folding of the nascent Form B LHR receptor that lacks the transmembrane/cytoplasmic domain, to an active configuration is dependent on the presence of N-linked carbohydrates and not on the initial oxidation state of the receptor (17). We have established that the LHR Form B receptor is only capable of spontaneous reoxidation state of the receptor (17). We have established that the LHR Form B splice variant did not depend on the initial oxidation state of the receptor, hormone binding of the Form A holoreceptor was greatly reduced when the receptor was treated with reductant prior to electrophoresis and ligand blotting (Fig. 5). The transmembrane domain appears to interfere with renaturation of the extracellular hormone binding domain only when the LHR is in the reduced, free cysteine state prior to electrophoresis/blotting. Fig. 6 suggests that renaturation of the isolated Form B hormone binding domain involves the reformation of exons 1 disulfide bonds, since the primary impact of the conservative substitution of Ser for Cys is in the loss of the ability to form disulfide bonds (12).

In the Form A holoreceptor, reactive cysteines from the transmembrane or cytoplasmic domain, exposed only under reducing conditions, may interfere with these putative disulfide interactions in the extracellular hormone binding domain, impairing ligand binding.

The observation that all but two of the cysteines characterized in this study that were determined to be functional for hormone binding or membrane insertion are conserved indicates that a common disulfide configuration may exist for the glycoprotein receptor family, designed for the general function of membrane insertion. The body of evidence that has accumulated on LHR hormone binding suggests that the extracellular hormone binding domain may be a minimal secondary structural element such as an amphipathic helix, since measurable hCG hormone binding has been surprisingly achieved with small receptor fragments and transmembrane helical domains (27). With this in mind, the “spontaneous renaturation” of the hormone binding domain that occurs in ligand blots, even under the extraordinary denaturing conditions that were used for deglycosylation (17), may instead indicate that extensive renaturation of the LHR does not have to occur for hormone binding and that a minimal Form B receptor domain, exposed on the nitrocellulose membrane, may be sufficient for binding.

In summary, the extracellular cysteines in exon 1 (Cys-8, -14, and -22) and exons 5 (Cys-109) and 6 (Cys-134) are important for hormone binding activity; those in exon 1 being essential for activity. The relatively conservative substitution of serine for cysteine suggests that the loss of binding activity may be attributed to the loss of potential disulfide bonds. Cys-257, -258, -282, -321, -331, -417, and -492 were important to various degrees for membrane insertion. Radiolmmunobassay studies indicate that while substitution of many of the LHR cysteines resulted in retention of the mutant proteins in the nonsurface membrane fraction, no changes in steady-state concentrations of the receptor were observed. These single substitution experiments demonstrate that specific cysteines in exons 1–6, perhaps through disulfide interaction, are essential/important for the active receptor configuration.

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