Purification, Characterization, and Amino-terminal Sequence of Rat Ovarian Receptor for Luteinizing Hormone/Human Choriogonadotropin*

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The luteinizing hormone (LH)/human choriogonadotropin (hCG) receptor of rat ovary was solubilized with Lubrol PX in the presence of 20% glycerol and protease inhibitors, and purified by one-step affinity chromatography. Purified receptor had a specific hCG binding capacity of 4900 pmol/mg protein, and displayed a single class of high affinity binding sites (K_a = 6.20 x 10^9 M^-1). An 11,200-fold purification over the starting crude homogenate was achieved. The purified LH/hCG receptor was identified by sodium dodecyl sulfate-gel electrophoresis and silver staining as a single protein of 92 kDa. The ability of the purified 92-kDa protein to specifically bind hormone was demonstrated by electroblotting onto Immobilon P membrane, incubation with 125I-labeled hCG, and autoradiography of the blot. In addition to a 92-kDa band, ligand blotting also yielded a 170-kDa band representing receptor dimer. Covalent cross-linking of hCG, with isotope in either the α- or β-subunit, to membrane-bound receptor produced complexes that contained a single receptor component of approximately 92 kDa. The cross-linking studies indicated that both subunits interact with receptor and also suggested receptor dimer formation. Following sodium dodecyl sulfate-electrophoresis, purified receptor was electroblotted onto polyethyleneimine-treated glass fiber filters for direct microsequencing in a gas-phase sequenator. Eleven cycles of sequence analysis yielded the unique sequence:

NH2-Arg-Glu-Leu-Ser-Gly-Ser-Leu-
XXX-Pro-Glu-Pro-COOH.

These results indicate that the rat ovarian LH/hCG receptor is a protein of 92 kDa which can be easily purified in microgram amounts. This study also describes a relatively simple technique for electroblotting and microsequencing that should be applicable to other membrane-bound hormone receptors.

The effects of LH and hCG on gonadal tissues are initiated by binding of circulating hormone to specific, high affinity receptors in the plasma membrane. LH/hCG receptors are coupled to intracellular effector systems, most notably adenylate cyclase, through guanine nucleotide-binding regulatory proteins or G-proteins (1-4). The molecular mechanisms involved in the dynamic coupling of LH/hCG receptors and G-proteins, however, are not known. In recent years knowledge of G-protein structure and function has increased tremendously and numerous G-proteins have been purified and cloned (5-7). The catalytic subunit of adenylate cyclase has also been purified (8, 9) and reconstituted in phospholipid vesicles to produce a hormonally responsive enzyme (9, 10). To study transmembrane signaling in gonadotropin-responsive tissues, isolation and characterization of the LH/hCG receptor is essential.

Purification of the LH/hCG receptor has been hampered by its low concentration in tissues and its lability in solution. Little can be done about the former, but the addition of glycerol (11) and protease inhibitors (12-14) to buffers has been found to greatly enhance the stability of solubilized receptors. Several reports on the purification of LH/hCG receptor from various sources have thus recently appeared (15-20). The consensus among these different studies is that the receptor is a single "subunit," transmembrane glycoprotein that may noncovalently associate to form oligomers. The size reported for the receptor "monomer" ranges from 60,000 to 100,000 daltons. Rajaniemi's group (15, 21) and Dufau's group (20) have affinity purified receptor from rat ovary to high specific activity and have demonstrated by ligand blotting that their purified protein (M_r = 90,000 and 78,000, respectively) specifically binds 125I-labeled hCG. Several other investigators have reported more complex structures for the receptor involving smaller, multiple subunits joined by disulfide (22, 23) or noncovalent (24, 25) bonds, but the ability of subunits to bind hormone has not been demonstrated.

Elucidation of the structure of the LH/hCG receptor and further study of signal transduction in gonadotropin-responsive tissues will be greatly facilitated by the complete amino acid sequence of the receptor. Toward this end we have affinity purified LH/hCG receptor from rat ovaries to near homogeneity and high specific activity. We demonstrate by ligand blotting that the purified protein specifically binds 125I-hCG and have used chemical cross-linking to show similarity with the membrane-bound receptor. Furthermore, we describe a technique that enabled us to determine the amino-terminal sequence of electroblotted receptor.

EXPERIMENTAL PROCEDURES

Materials—Lubrol PX, Tween 20, diisuccinimidyl suberate, and dithiothreitol (DTT) were obtained from Pierce Chemical Co. Polyethyleneimine (PEI, 50% free base), phenylmethylsulfonyl fluoride,
and N-ethylmaleimide (NEM) were purchased from Sigma. 4-Ethylmorpholine was from Aldrich, and 3,3′-dipentyloxacarbocyanine iodide was a product of Molecular Probes, Eugene, OR. Polyestradiol phosphate (Estradurin) was obtained from Ayerst, New York, NY. Sodium dodecyl sulfate (SDS) was from ICN, Costa Mesa, CA, and cyanogen bromide (CNBr) from Pharmacia LKB Biotechnology Inc. Polyvinylidene difluoride immobilobin P membranes were purchased from Millipore, Bedford, MA. Human chorionic gonadotropin and human luteinizing hormone (hLH) were obtained from the National Pituitary Agency, Bethesda, MD. Pregnant mare serum gonadotropin (PMSG) was obtained from Diosynth, Chicago, IL.

**Tissue Preparation**—Twenty-three-day-old Holtzman rats were rendered pseudopregnant by administration of pregnant mare serum gonadotropin (PMSG) and rendered pseudopregnant by administration of pregnant mare serum gonadotropin. The ovaries were removed, freed of extraneous tissue, frozen on dry ice, and stored at −80 °C. Membrane-enriched 2000 × g pellet fractions were prepared as previously described (27).

**Receptor Solubilization**—Receptor was purified from four batches of 200 ovaries. The ovaries were thawed in 10 volumes of 10 mM potassium phosphate, 140 mM KCl, 5 mM EDTA, 5 mM NEM, 0.2 mM phenylethanesulfonfluoride, pH 7.4 (Buffer A), finely minced with a polytron, and homogenized in a motor-driven glass-Teflon homogenizer (three complete strokes). The brei was centrifuged at 16,000 × g for 20 min and the supernatant decanted. The pellet was washed twice with a Teflon homogenizer in the dark. The pellet was resuspended to half the original volume of Buffer A and recentrifuged. The supernatant was decanted, and the pellet resuspended to half the original volume. This was repeated for 3 h at 4 °C and then centrifuged at 20,000 × g for 60 min. The supernatant (solubilized receptor) was removed, added to an equal volume of chilled petroleum ether, and vigorously shaken. The two phases were separated by centrifugation at 1500 × g for 5 min, and the aqueous layer was recovered. The aqueous layer was recovered and the pH adjusted to 4.0. The eluate contained 20% glycerol, 10 mM potassium phosphate, 5 mM EDTA, 5 mM NEM, 0.2 mM phenylethanesulfonfluoride, pH 7.4, and filtered through a 0.45-μm nylon membrane. The filtration solution was assayed for the affinity purification. The affinity chromatography purification was performed as described by Keinanen et al. (14) with some modifications. Solubilized receptor (200 μl) was mixed with the hCG-Sepharose and incubated overnight on a rotor platform at 4 °C. The slurry was poured onto a sintered glass filter and the filtrate recovered. The gel was washed three times with 500 ml of Buffer A containing 0.5% Lubrol, 20% glycerol, and then with 500 ml of Buffer A containing 500 mM KCl, 0.1% Lubrol, 20% glycerol. The gel was then resuspended in Buffer A containing 0.1% Lubrol, 20% glycerol, and poured into a column (2.6 × 4.4 cm). The column was washed with 300 ml of Buffer A containing 0.1% Lubrol, 20% glycerol, and receptor was then eluted with 100 mM potassium acetate, 0.1% Lubrol, 20% glycerol, pH 4.0. Acid-eluted receptor fractions (4.5 ml) were immediately neutralized by collection into tubes containing 0.5 ml of 1.5 M potassium phosphate, 20% glycerol, pH 8.0. Pooled active fractions were dialyzed overnight against 2 liters of 100 mM potassium phosphate, 5 mM EDTA, 5 mM NEM, pH 7.4, and concentrated by ultrafiltration on a YM-30 membrane (Amicon Corp.). Purified receptor was stored frozen at −80 °C.

**Binding Assays**—The hCG binding activity of fractions from the purification steps was measured using PEI-treated membrane filters as previously described (26). 125I-Labeled hCG was prepared according to Lee and Ryan (27) and contains isotope predominantly in the α-subunit. Maximum binding capacity and equilibrium binding constants for receptor were determined from competition binding assays performed as described (26). SDS-Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed according to Laemmli (28) in 7.5% or 10% polyacrylamide gels. Receptor samples for electrophoretic analysis were precipitated with methanol/chloroform/water as described by Wessel and Flurkey (29). Purified receptor was dialyzed overnight in the presence of 100 mM DTT, and then incubated overnight at room temperature in the milk buffer containing 1 μg of 125I-hCG in the presence or absence of excess unlabeled hCG, LH, or hFSH. Blots were then washed extensively with ice-cold milk buffer, briefly rinsed with distilled water, and air dried. Dried blots were exposed to Kodak XAR or XRP film for 2-5 days at −80 °C.

**Microsequencing of Purified Receptor**—Purified receptor (10 μg) was precipitated with methanol/chloroform/water, redissolved in SDS-sample buffer containing 100 mM DTT, heated for 3 min at 100 °C, and subjected to electrophoresis in a 10% gel as described above. Samples were then electrophoretically transferred to PEI-treated GF/F (Whatman) glass fiber filter using the same buffer and conditions as described for ligand blotting. PEI-treated GF/F filters were prepared by soaking the filters in 0.3% aqueous solution of PEI for 4 h at room temperature and then allowing them to dry for 24 h. The filters were then treated with transfer buffer before use. After transfer, PEI-treated filters were rinsed briefly with distilled water, and protein bands were visualized by staining with 3,3′-dipentyloxacarbocyanine iodide as described by Aebesold et al. (31). Blotted protein bands were removed with a sharp razor blade and placed directly in an Applied Biosystems 470A-gas-phase sequencer. Phenyliothydantoin-amino acid derivatives were identified by reverse-phase HPLC.

**Coulant Cross-linking of 125I-hCG to Membrane-bound Receptor**—The 125I-hCG preparation described above contains the isotope in the α-subunit and the β-subunit. 125I-hCG with isotope in the α-subunit was radiolabeled by delipidation of isolated β-subunit, recombination with excess unlabeled α, and purification of the reconstructed hormone by gel filtration according to Canfield et al. (32). Luteal membranes were incubated with 40 ng of radiolabeled hCG (125I-labeled αβ or 125I-labeled β) for 2 h at 30 °C in Buffer A, washed twice in ice-cold Buffer A by centrifugation, and then cross-linked with 0.3 μmol disuccinimidyl suberate in Buffer A for 15 min at 30 °C. Cross-linked membranes were washed once with 40 mM Tris-HCl buffer, pH 7.4, and solubilized in SDS-sample buffer containing 100 mM DTT with heating for 3 min at 100 °C. Samples were subjected to electrophoresis on 7.5% polyacrylamide gels. Solubilized receptor was then transferred to nitrocellulose membranes and visualized using the antibody to the human LH receptor (an IgM produced in the mouse). Microsequencing of the purified receptor was performed in a 10% gel as described above. 125I-hCG samples containing 2.5 × 106 cpm were electrophoresed and stained with methanol/chloroform/water as described by Wessel and Flurkey (29). Purified receptor was dialyzed overnight in the presence of 100 mM DTT, and then incubated overnight at room temperature in the milk buffer containing 1 μg of 125I-hCG in the presence or absence of excess unlabeled hCG, LH, or hFSH. Blots were then washed extensively with ice-cold milk buffer, briefly rinsed with distilled water, and air dried. Dried blots were exposed to Kodak XAR or XRP film for 2-5 days at −80 °C.

**RESULTS AND DISCUSSION**

**Purification of LH/hCG Receptor**—Table I summarizes results from the purification of receptor from 800 rat ovaries. Four separate batches of 200 ovaries were processed by the purification scheme described above. Purified receptor fractions were pooled and concentrated. The crude ovarian homogenate exhibited a binding capacity for 125I-hCG equivalent to approximately 3 pmol/ovary or 300 fmol/mg tissue (1 ovary = 100 mg wet weight). Solubilization of receptor with Lubrol PX resulted in a modest increase in total binding activity and a 2-fold increase in specific activity. Delipidation of the receptor with petroleum ether caused no change in binding activity or protein concentration but resulted in improved binding of solubilized receptor to the hCG-Sepharose. Routinely 85-90% of hormone binding activity was removed by overnight incubation with the hCG-Sepharose at 4 °C. After extensive washing of the gel, both batchwise and in the column, receptor binding activity was eluted as a sharp peak by lowering the pH to 4.0. The eluate routinely contained 18-
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TABLE I
Receptor purification

The data for crude membrane homogenate and lubrol-soluble extract were derived from four batches of 200 ovaries each, and the standard errors were derived from these four batches, although the data are the sums of the four batches. All batches were Dooled for the concentration step.

<table>
<thead>
<tr>
<th></th>
<th>Total protein</th>
<th>¹²⁵I-hCG binding</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>pmol</td>
<td>pmol/mg protein</td>
<td>-fold</td>
<td>%</td>
</tr>
<tr>
<td>Crude membrane homogenate</td>
<td>6,130 ± 182</td>
<td>2,676 ± 123</td>
<td>0.437 ± .017</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Lubrol PX-soluble extract</td>
<td>3,080 ± 106</td>
<td>2,944 ± 147</td>
<td>0.956 ± .040</td>
<td>2</td>
<td>110</td>
</tr>
<tr>
<td>Affinity purified concentrate</td>
<td>0.110</td>
<td>540</td>
<td>4,900</td>
<td>(45% pure)*</td>
<td>11,200</td>
</tr>
</tbody>
</table>

*Assuming molecular weight of 92,000 and 1 mol of hormone/mol of receptor, the specific activity of pure receptor = 10,870 pmol/mg protein.

22% of the binding activity present in the crude homogenate.

Fig. 1 shows a representative Scatchard plot (34) of binding data obtained with ¹²⁵I-hCG and purified LH/hCG receptor. The data show a single class of high affinity binding sites with a $K_d = 6.20 \pm 0.71 \times 10^{-9}$ M$^{-1}$ ($n = 3$).

The specific activity of our purified receptor was 4909 pmol/mg protein, which represents an 11,000-fold purification over the crude homogenate. If the receptor is a polypeptide of 92 kDa (see below) and binds 1 mol of hormone/mol of receptor, the specific activity of pure receptor would be 10,870 pmol/mg protein. The specific activity of our preparation is therefore approximately 45% of the theoretical maximum. Kusuda and Dufau (17) purified rat ovarian receptor to a similar specific activity (5100 pmol/mg protein), whereas Keinanen et al. (14) achieved a specific activity of 7920 pmol/mg protein. Failure to come closer to the theoretical maximum may be due to contaminating protein, denaturation of receptor during purification, or to dimerization as suggested by Kusuda and Dufau (19). These workers have reported that soluble receptors readily dimerize and have proposed that receptor dimers may be capable of binding only 1 hormone molecule.

Characterization of Purified Receptor—Purified receptor (400 ng) was analyzed by SDS-PAGE under reducing and nonreducing conditions (Fig. 2). Silver staining of the gel revealed a broad major band of 92 kDa both in the presence and absence of reducing agent, indicating the absence of disulfide-linked subunits. Although not demonstrated by us, several investigators (14, 17, 21, 35) report that the receptor is a glycoprotein based upon its ability to bind to lectins. The appearance of receptor as a broad band on our SDS-gels suggests microheterogeneity and this may be due to different degrees of glycosylation. In addition to the 92-kDa protein, a minor band of approximately 200 kDa was sometimes detected as were several lower molecular mass bands in the 45–68 kDa range. The high molecular mass band may be receptor dimer/aggregates and the lower ones proteolytic fragments of the 92-kDa protein or contaminants not related to receptor. We do not believe that these proteins are non-hormone binding...
subunits as they are not present in the same amount as the 92-kDa protein.

To establish that the 92-kDa protein we purified does indeed have hormone binding activity, ligand blotting was performed with samples of purified receptor (75 ng). Receptor was subjected to SDS-PAGE under nonreducing conditions and without heating in SDS sample buffer. If samples were reduced or heated to 100 °C, hormone binding activity was destroyed. After electrophoresis, resolved samples were electroblotted onto Immobilon P membranes and remaining active sites were blocked with a milk-containing buffer. Initial blotting experiments utilized Tris-glycine buffer at pH 8.3 as the transfer buffer and nitrocellulose as the transfer membrane. Subsequently, we found increased retention of binding activity if transfers were performed at pH 7.8. A similar observation has been made by Kusuda and Dufau (19). Immobilon P membranes were employed rather than nitrocellulose because they retained more receptor-hormone complexes during the multiple washes to remove unbound labeled hormone.

Incubation of blots with 125I-labeled hCG followed by autoradiography demonstrated labeling of a 92-kDa band (Fig. 3, lane I). This molecular mass for receptor monomer agrees with the findings of Keinänen et al. (20) who reported purified ovarian receptor to be 90 kDa, and Kim et al. (35) who immunoprecipitated a 92-kDa LH/hCG receptor. Dufau and co-workers (17, 19) and Wimalesena et al. (15, 16) have reported slightly smaller molecular masses for rat and porcine ovarian receptor, respectively. These differences may be due to anomalous behavior of the glycoprotein in different electrophoretic systems, or minor proteolytic damage. We also observed labeling of a band of 170 kDa (Fig. 3, lane 1). In agreement with Kusuda and Dufau (19) who observed a similar high molecular weight band on ligand blots, we believe that this band represents receptor dimer.

The appearance of bands on our ligand blots could be prevented by incubation in the presence of excess unlabeled hCG and hLH (Fig. 3, lanes 2 and 3), but not by hFSH (lane 4). The decrease in labeling intensity observed in the presence of excess unlabeled hFSH may be due to contamination with hLH.

**Formation of Covalently Cross-linked hCG-Receptor Complexes**—The autoradiograph of Fig. 4 shows the electrophoretic pattern of cross-linked hCG-receptor complexes formed with 0.3 mM disuccinimidyl suberate. No bands were formed when membranes were incubated with 125I-hCG in the presence of excess unlabeled hormone (not shown). A 134- and >200-kDa band are formed when either the α (lane A) or β (lane B) subunit of hCG contained the isotope, indicating α-β-receptor complexes. Since α-β complexes migrated as 46 kDa, subtraction of this mass from 134 kDa yields a receptor component of 88 kDa. The >200-kDa band may represent...
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hormone-receptor dimer cross-links. With label in the α-subunit, additional bands of 110, 73, and 65 kDa were formed. With isotope in the β-subunit, only an additional band of 122 kDa could be resolved. The 110-kDa band is concluded to be an α-receptor cross-link and the 122-kDa band a β-receptor cross-link. Subtraction of the appropriate subunit mass (α = 22 kDa, β = 30 kDa) again yields a receptor component of 88–92 kDa. Ascoli and Segaloff (13) observed cross-linked complexes of similar size with mouse Leydig tumor cells and porcine granulosa cells. In further agreement with these authors, our results also demonstrate that both subunits can be cross-linked to receptor and suggests close association of both subunits with the receptor. These findings are contrary to those of Kusuda and Dufau (17) who observed no cross-linking of β-subunit to receptor and concluded that major interaction of hCG with receptor occurs through the α-subunit.

The 73- and 65-kDa complexes, formed with isotope in the α-subunit, may be proteolytic fragments. Subtraction of α mass (22 kDa) from these bands yields proteins of 51 and 43 kDa. No bands of this size were detected by ligand blotting, but their sum (94 kDa) is very close to 92 kDa. The results of our cross-linking experiments provide evidence that the membrane-bound LH/hCG receptor is also composed of a hormone-binding protein of approximately 92 kDa and indicate that purified receptor is not a proteolytic fragment of a larger molecule.

Microsequencing of Purified Receptor—Initially we tried to obtain amino-terminal sequence information by the method of Matsudaira (36) utilizing purified receptor that had been electroblotted onto Immobilon P membranes. We found this method to be unsatisfactory because receptor would not bind to the Immobilon P membrane at the high pH (11.0) used for electrotransfer (results not shown). Changing transfer buffer and lowering the pH to 7.8 improved binding to Immobilon P membranes but receptor still migrated through several sheets, and a sufficient amount of protein for microsequencing was not retained. Coomassie Brilliant Blue R-250 was used to stain and localize protein bands on Immobilon P membranes according to Matsudaira (36). Unexpectedly, this stain detected a protein of 44 kDa that we had not previously seen in our silver-stained gels. Reexamination of purified receptor by SDS-gel electrophoresis and silver staining with five times as much protein (2 μg versus 400 ng) revealed the presence of this band (Fig. 5). Contamination with this protein may account for our ability to achieve receptor purity of only 45%.

We have attempted to microsequence this protein but the amino terminus appears blocked. Antibodies specific for the α-subunit of Gx did not recognize this protein by immunoblotting. Intense staining of this 44-kDa protein with the anionic dye Coomassie Blue suggests a basic pl, but its nature remains unknown.

We previously reported that PEI-treated cellulose acetate membranes tightly bind hormone-receptor complexes and could be used to separate bound from free hormone in assays of soluble receptor binding (26). We therefore treated GF/F glass-fiber filters with PEI as described under “Experimental Procedures” and tried them as the transfer membrane. We found that the 92-kDa receptor was quantitatively bound to the PEI-treated filters. A recent report has shown that PEI can be used as an efficient carrier for protein sequencing and results in lower backgrounds when compared to Polybrene (37). The fluorescently stained 92-kDa band was visualized under UV light (254 nm), excised from the glass-fiber filter with a razor blade, and subjected to 11 cycles of sequence

2 P. C. Roche, unpublished results.

analysis in a gas-phase sequenator. Ten amino acids were identified as follows:

\[
\text{NH}_2-\text{Arg-Glu-Leu-Ser-Gly-Ser-Leu-XXX-Pro-Glu-Pro-COOH}
\]

The eighth amino acid (XXX) could not be conclusively identified, but is most likely a Cys. This peptide sequence was analyzed with software developed by the Genetics computer Group (38), and no identity to other entries in the database was found. We conclude that this is the amino-terminal sequence of the rat ovarian LH/hCG receptor. This is the first reported amino-terminal sequence information for a gonadotropin receptor from any source, and we look forward to comparison with receptor from other species. We believe that the blotting technique that we have reported will be useful for obtaining additional sequence information that can be utilized for isolation and cloning of the gene for the LH/hCG receptor and determination of the complete amino acid sequence. Our blotting technique may also be useful for performing microsequencing of other membrane receptors such as the FSH or TSH receptor.

In conclusion, we have purified the rat ovarian LH/hCG receptor to a specific activity of 4900 pmol of hCG bound/mg of protein. The purified receptor is a 92-kDa protein, and purity is calculated to be approximately 45% of the theoretical maximum. Amino-terminal sequencing of electroblotted receptor can be performed. Molecular biology techniques can now be employed for determination of the complete amino acid sequence thus enabling further study on the structure/function of the LH/hCG receptor.

REFERENCES

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