Evidence for monomeric and oligomeric hormone-binding domains in affinity-purified gonadotropin receptor from rat ovary
(subunit composition/disulfide linkage/ligand blotting/lutropin/choriogonadotropin)

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ABSTRACT Rat ovarian lutropin/choriogonadotropin receptor was purified from a Triton X-100-solubilized membrane preparation by affinity chromatography with Affi-Gel 10 coupled to purified human choriogonadotropin. The affinity-purified receptor preparations contained a single class of high-affinity binding sites for $^{125}$I-labeled human choriogonadotropin, with an equilibrium dissociation constant ($K_d$) of 2.5 $\times 10^{-9}$ M, which is comparable to the $K_d$ values for membrane-bound and solubilized receptors. The purified receptor appeared as two dominant bands with molecular weights of 135,000 and 92,000 after sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) under nonreducing conditions. These two bands were also detected in subsequent direct ligand blotting analysis when the purified receptor was electrophoretically transferred to a nitrocellulose membrane after SDS/PAGE under nonreducing conditions. When the individual affinity-purified receptor bands were electroeluted from the gel and analyzed again by SDS/PAGE under nonreducing conditions, the Mr 92,000 and the 135,000 proteins retained their original molecular form even when 8 M urea was included in the gel. However, when the electrophoretically purified Mr 92,000 and 135,000 bands were subjected to SDS/PAGE under reducing conditions, the Mr 135,000 species was almost completely converted to a Mr 92,000 band, but the Mr 92,000 species did not undergo any alteration in molecular weight. The results suggest that the lutropin/choriogonadotropin receptor from rat ovary exists in two molecular forms, and the higher molecular weight form appears to be composed of disulfide-linked Mr 92,000 subunit, which comprises the hormone-binding domain.

The stereoiodenic function of the ovary is regulated by gonadotropins [luteinizing hormone (LH) and chorionic gonadotropin (CG)]. The action of gonadotropins is mediated by their interaction with specific receptors located on the ovarian plasma membrane, followed by activation of adenylate cyclase to increase the intracellular cyclic AMP level (1). Reports have appeared regarding the structure of the ovarian LH/CG receptor as determined by chemical crosslinking (2-4), by photoaffinity labeling (5-7), and by floation in sucrose density gradients combined with gel filtration chromatography (8). Recently, more information about the ovarian LH/CG receptor has resulted from attempts by several laboratories to purify the receptor by affinity chromatography (9-15). The conclusions of these studies suggested that the receptor could be either a heteropolymer or a single polypeptide. However, there appears to be a consensus that the molecular weight of one of the binding domains of the receptor is in the range of 70,000-95,000 (2-15). In the present studies we have examined the possible subunit structure of the affinity-purified receptor by a combination of SDS/PAGE, direct ligand blotting, and electrophoretol. The results clearly indicate that the receptor is an oligomeric protein of Mr $\approx$135,000 that contains a Mr 92,000 subunit comprising the ligand-binding domain. In addition, evidence indicates that the Mr 92,000 subunit is linked through disulfide bonds to form the higher molecular weight species.

MATERIALS AND METHODS

Materials. Human CG (hCG; lot CR125, 11,900 international units/mg) was a gift from the Center for Population Research, National Institute of Child Health and Human Development, Bethesda, MD. Carrier-free Na$^{125}$I was obtained from Amersham. hCG was radioiodinated by the chloramine-T method (16), to yield a specific activity of 40--60 $\mu$Ci/µg (1 $\mu$Ci = 37 kBq). Affi-Gel 10, enhanced colloidal gold total protein detection kit, nitrocellulose membrane, Triton X-100, and reagents for SDS/PAGE were obtained from Bio-Rad. All other chemicals were purchased from Sigma.

Animals and Tissues. Twenty-one-day-old female Sprague-Dawley rats (Harlan--Sprague--Dawley) were rendered pseudopregnant as described (17). The animals were sacrificed on day 7 following hCG injection. The ovaries were dissected free of extraneous tissue and processed immediately.

Preparation of Crude Membrane Fraction. All experiments were performed at 4°C. The membranes were isolated from the ovaries as described (18) with the inclusion of 0.01% soybean trypsin inhibitor, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, and 25% (vol/vol) glycerol in phosphate-buffered saline (0.15 M NaCl/0.05 M phosphate, pH 7.4) (buffer A). The 700 $\times$ g supernatant was further centrifuged at 100,000 $\times$ g for 30 min in a Beckman L5-65B ultracentrifuge. The 100,000 $\times$ g pellets, representing a crude preparation of the plasma membranes, were combined for further processing. Although this fraction also includes other subcellular components, no attempt was made to purify the plasma membranes, to avoid the loss of receptor yield.

Solubilization of the Receptor. The crude membrane preparation was resuspended in ice-cold buffer A, in a volume equivalent to 0.4 ml per ovary, with gentle homogenization. Solubilization was achieved by the addition of Triton X-100 to a final concentration of 1% (vol/vol). The mixture was stirred on ice for 45 min and then centrifuged for 30 min at 160,000 $\times$ g. The supernatant, which represents the solubilized receptor, was used for the affinity purification.

Affinity Chromatography. hCG-Affi-Gel 10 was prepared by following the manufacturer's instructions (Bio-Rad), using a ratio of 5 mg of highly purified hCG to 5 ml of Affi-Gel 10 beads. A 10-ml aliquot of the Triton X-100-solubilized membrane fraction was mixed with 5 ml of hCG-Affi-Gel 10, and

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Abbreviations: CG, chrorionic gonadotropin; hCG, human CG; LH, luteinizing hormone.
the suspension was mixed end-over-end at 4°C for 16 hr. The gel was then packed into a column and the flowthrough was collected. The column was washed sequentially with the following: 30 ml of 10 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM N-ethylmaleimide, 0.05% Triton X-100, and 25% glycerol (buffer C); 5 ml of 50 mM glycine/HCl (pH 4.5) containing 0.1% Triton X-100; and 40 ml of buffer C containing 0.5 M NaCl. The bound material was then eluted from the column with 25 mM acetic acid (pH 4.0) containing 0.5 M NaCl, 0.05% Triton X-100, 25% glycerol, and the protease inhibitors as noted above, and the eluate was neutralized immediately with 1.5 M Tris/HCl (pH 8.8). Aliquots from each fraction were assayed for 125I-hCG binding activity. The fractions with hCG-binding activity were combined and then concentrated and dialyzed, with the use of a Micro-ProDiCon apparatus (Bio-Molecular Dynamics), against 50 mM Tris/HCl (pH 7.4) containing 20% glycerol and protease inhibitors. The eluate from the affinity column was then packed into the discontinuous buffer system (22). SDS/PAGE method of Wray and Menon (22). Ligand Blots. After SDS/PAGE, proteins were transferred to a nitrocellulose membrane (23). The membrane was rinsed in phosphate-buffered saline and then cut into two parts. One part was stained with enhanced colloidal gold for total protein (Bio-Rad protocol), and the other part was subjected to ligand blotting. In brief, the nitrocellulose sheets were incubated in phosphate-buffered saline containing 3% bovine serum albumin, 0.1% Triton X-100, and 10 mM MgCl₂ for 4 hr at room temperature, and then the addition of 5283-hCG (10⁷ cpm in a 30-ml volume), an additional 12-hr agitation was carried out in the absence or presence of excess unlabeled hCG. The blots were then washed thoroughly and air-dried. Autoradiography was performed using Kodak X-Omat film with an intensifying screen, and the films were exposed at −70°C for 5–7 days.

**Electroelution.** Affinity-purified receptor preparations were first subjected to SDS/PAGE under nonreducing conditions. The receptor bands were localized after staining gel strips from each side of the gel with silver. Individual bands were then removed from the gel with a razor blade and were stored at −20°C until further use. Electroelution was performed with an ISCO electrophoretic concentrator (model 1750) with 0.04 M Tris/acetate buffer (pH 8.6) in the electrode chamber and 0.01 M Tris/acetate buffer (pH 8.6) in the inner chambers and sample cups. Each buffer contained 2 mM EDTA and 0.01% SDS. Gel pieces were preincubated in 5% SDS for 30 min, rinsed three times with the same buffer as that in the sample cups, and then loaded in the sample wells. Electroelution was carried out at 3 W total output for 3–4 hr. Aliquots of recovered samples were analyzed by SDS/PAGE.

**Protein Assay.** Protein was determined by the method of Smith et al. (24) with bovine serum albumin as the standard. To minimize interference in the assay, trichloroacetic acid was used to precipitate protein (25).

## RESULTS

**Characterization of Affinity-Purified Receptor.** Rat ovarian LH/hCG receptor was purified by two cycles of affinity chromatography on hCG-Affi-Gel 10 columns. The results from a representative purification procedure starting with ovaries from 40 rats are summarized in Table 1. The first cycle of affinity chromatography resulted in >1300-fold purification. The second affinity chromatography resulted in further purification of the receptor with 12% recovery of the binding activity. Thus, about 3200-fold purification of LH/hCG receptor was achieved by sequential hCG affinity chromatography, and the overall recovery of activity from the Triton X-100-solubilized preparation was ~7.5%. The specific activity of the receptor after two successive affinity chromatographies was 4735 pmol/mg of protein, which is still lower than the theoretical maximum. However, since the presence of Triton X-100 in the binding assay caused a concentration-dependent inhibition of the binding activity (data not shown), the actual specific activity could be even greater than what is reported here. The purified receptor was stable when stored at −70°C for several months and could be thawed and refrozen at least twice without significant loss of activity.

Scatchard plot (20) analysis of the binding data revealed a single class of high-affinity binding sites in the purified receptor (Fig. 1). The equilibrium dissociation constant (Kₐ) was calculated to be 2.5 × 10⁻⁸ M, which is comparable to the Kₐ value of membrane-bound or Triton X-100-solubilized receptor. The data indicate that no notable changes took place in the hormone-binding characteristics of the receptor during the course of purification.

<table>
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<th>Preparation</th>
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<th>Activity, pmol</th>
<th>Specific activity, pmol/mg</th>
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<th>Yield, %</th>
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Fig. 1. Scatchard analysis of purified LH/hCG receptor. Purified LH/hCG receptors were incubated with a constant amount of 
\(^{125}\text{I}-\text{hCG}\) and various quantities of unlabeled hCG. Each data point represents the average of triplicate determinations. B/F, bound/bound-
free. (Inset) The same data, expressed as the binding of hCG as a function of the ligand concentration.

When the purified receptor was analyzed by SDS/PAGE under nonreducing conditions, two dominant broad protein bands appeared corresponding to \(M_r\) 135,000 and 92,000, respectively. Additionally, three minor bands corresponding to \(M_r\) 65,000, 55,000, and 42,000 were also detected (data not shown). To establish the identity of the receptor bands, the proteins were transferred electrophoretically to nitrocellulose membrane and subjected to direct ligand blotting analysis. Incubation of the blot with \(^{125}\text{I}-\text{hCG}\) followed by autoradiography revealed two labeled bands at \(M_r\) 135,000 and 92,000 (Fig. 2A). The label was displaced when incubations were performed in the presence of excess unlabeled hCG (Fig. 2B). The specificity of binding is further illustrated by the fact that the protein bands in the lower molecular weight region were not labeled. Occasionally, a faint band at \(M_r\) 42,000 was detected from which label could also be displaced by unlabeled hCG. This band may represent a degradation product that retained the hormone-binding activity. The inconsistency in the detection of the \(M_r\) 42,000 band may be a result of the variation in the amount of low molecular weight bands present in different receptor preparations. When the receptor was subjected to SDS/PAGE under reducing conditions and electropho-
ted onto nitrocellulose, ligand blotting revealed very poor binding of the receptor compared to that observed under nonreducing conditions (data not shown).

Receptor Components Purified by Electrophoresis. To examine the relationship between the \(M_r\) 135,000 and 92,000 polypeptides, the two bands were removed from the gel and the proteins were electroeluted separately. When the two proteins were individually submitted to SDS/PAGE under nonreducing conditions, they migrated to their original corresponding positions (Fig. 3A). However, when the electroeluates were analyzed under reducing conditions, the major-
ity of the protein from the \(M_r\) 135,000 band migrated to the \(M_r\)
92,000 position and the migration of the \(M_r\) 92,000 protein remained unchanged (Fig. 3B), suggesting the possible involvement of disulfide linkages in the higher molecular weight species. The involvement of disulfide bonds in the linkage between receptor subunits was further explored by urea/SDS/PAGE under nonreducing conditions. In the presence of 8 M urea, both \(M_r\) 92,000 and 135,000 bands were still detected and the electroeluate from the \(M_r\) 135,000 band did not dissociate into \(M_r\) 92,000 protein (data not shown), indicating that the subunits are probably not linked through hydrophobic interaction. A smaller polypeptide was not

Fig. 2. Ligand blotting of purified LH/hCG receptors. Purified receptors were subjected to SDS/PAGE under nonreducing conditions and transferred electrophoretically onto nitrocellulose membranes. The blots were incubated with \(^{125}\text{I}-\text{hCG}\) in the absence (A) or presence (B) of excess unlabeled hCG for 12 hr at 25°C. The blots were washed and dried prior to autoradiography. Lanes 1, purified receptor after first hCG-Affi-Gel chromatography (3 \(\mu\)g of protein); lanes 2, purified receptor after second hCG-Affi-Gel chromatography (0.5 \(\mu\)g of protein). Molecular weight estimates of the receptors (\(M_r\) \(\times 10^3\)) are indicated at left.

Fig. 3. SDS/PAGE analysis of LH/hCG receptor subunits purified by electroelution. Affinity-purified receptors were subjected to SDS/PAGE under nonreducing conditions. Gel slices at positions corresponding to \(M_r\) 92,000 and 135,000 were removed after electrophoresis, and proteins electroeluted from the gel slices were analyzed by SDS/PAGE under nonreducing (A) or reducing (B) conditions.
observed on the gels when the Mr 135,000 band was transformed into the Mr 92,000 band. Thus it appears that the Mr 135,000 protein may be a homodimer or an aggregate of the Mr 92,000 polypeptide species. To examine the possible presence of lower molecular weight proteins following the reduction of Mr 135,000 to Mr 92,000 species, the Mr 135,000 protein was iodinated with chloramine T and subjected to SDS/PAGE and autoradiography. While the Mr 135,000 band was transformed to Mr 92,000 under reducing conditions, no corresponding lower molecular weight band was detectable on the autoradiogram (data not shown). This further confirms that the larger molecular form of the receptor may represent a dimer of Mr 92,000 subunit.

**Stability of the Receptor Subunit.** The electroeluates from the Mr 92,000 bands were stored in the electroelution buffer at -70°C for up to 3 months before SDS/PAGE analysis. The Mr 92,000 material was partially degraded during storage to two polypeptides of Mr 65,000 and 55,000 (Fig. 4, lane 2). Complete degradation of the Mr 92,000 protein to Mr 65,000 and 55,000 species was observed after prolonged storage of the receptor at -20°C (Fig. 4, lane 3). This finding suggests that the two lower molecular weight proteins present in the affinity-purified receptor preparations are degradation products of the receptor.

**DISCUSSION**

In the present study we have isolated LH/hCG receptor from rat ovary with at least 3200-fold purification by two cycles of affinity chromatography on hCG-Affi-Gel 10 columns. The purified receptor exhibited an equilibrium dissociation constant similar to that of the membrane-associated receptor and was detected as two discrete bands upon direct ligand blotting with 125I-hCG following SDS/PAGE. In addition, evidence is presented for the smaller receptor band being derived from the larger receptor band upon reduction of the disulfide bonds.

There is considerable disagreement in the literature about the molecular size and composition of the LH/hCG receptor (2–13). For example, whereas Kusuda and Dufau (10) identified the purified LH/hCG receptor as a single protein of Mr 73,000, Bruch et al. (11) reported that the purified receptor consists of four nonidentical subunits of Mr 79,300, 64,400, 55,300, and 46,700, although in a subsequent study it was concluded that the receptor may exist as a single polypeptide (14). Saxena et al. (12) reported that a Mr 120,000 protein represents the functional unit of the hCG receptor and that this Mr 120,000 protein is composed of Mr 85,000 and 38,000 proteins linked covalently through disulfide linkages. More recently, Rebois et al. (8), on the basis of hydrodynamic properties, reported that the receptor is composed of two dissimilar subunits held together noncovalently: a hormone-binding subunit of Mr 76,000 and a Mr 50,000 component.

In the present study we have attempted to unravel the molecular structure of the affinity-purified receptor by a combination of direct ligand blotting and silver staining to detect the protein band under different electrophoretic conditions. We found that the receptor consists of two dominant bands corresponding to Mr 135,000 and 92,000 when SDS/PAGE was conducted under nonreducing conditions. When the proteins were electrophoretically transferred to nitrocellulose membranes and subjected to direct ligand blotting with 125I-hCG, two discrete bands were detected corresponding to the same molecular sizes. Further, when the electroeluates of the Mr 92,000 and 135,000 proteins were analyzed by SDS/PAGE under nonreducing conditions, both bands retained their original molecular sizes even when 8 M urea was included in the gel, suggesting that the Mr 92,000 protein and other components of the receptor were held together by covalent interactions. When the electrophoretically purified Mr 92,000 and 135,000 bands were subjected to SDS/PAGE under reducing conditions, the Mr 135,000 species was almost completely transformed into a Mr 92,000 band, but the Mr 92,000 protein did not show any change in its electrophoretic mobility. This observation suggests the involvement of disulfide interactions in the linkage between the receptor subunits. The presence of the Mr 92,000 band under nonreducing conditions indicates that the receptor may be partially reduced in the native state or during the purification process. Interestingly, a recent study (26) suggests that the native receptor is a dimer of identical hormone-binding subunits associated by noncovalent interactions. The molecular weights for the dimer and monomer were reported to be 150,000 and 78,000, respectively, and the dissociation of the dimer was attributed to the presence of SDS. Since different methods were used in the present study, it is not clear whether the dimeric receptor structure reported by Kusuda and Dufau (26) is similar to that suggested by our results.

Our data suggest that the molecular weight of the LH/hCG receptor in the rat ovary is in the range of 135,000 and that this receptor species undergoes dissociation under reducing conditions to yield a Mr 92,000 species possessing the hormone-binding domain of the receptor. Since we were not able to detect a lower molecular weight receptor species consistently, we conclude that the Mr 135,000 species might be a homodimer of Mr 92,000 subunit. It is conceivable that the discrepancy in molecular size of the dimer, predicted on the basis of the size of the monomer, may be due to the glycoprotein nature of the receptor (27, 28). The Mr 135,000 protein appeared as a broad band with a molecular weight estimated within the range of ≥10,000–15,000 Da. Thus an accurate estimation of the molecular weight cannot be assessed from the SDS/polyacrylamide gels. Amino-terminal analysis of the individual bands and peptide mapping may

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Fig. 4. SDS/PAGE analysis of electroeluted LH/hCG receptor under various storage conditions. The electroeluted Mr 92,000 receptor subunit was prepared as described in the legend to Fig. 3 and analyzed by SDS/PAGE under reducing conditions. Lane 1, freshly prepared Mr 92,000 protein; lane 2, Mr 92,000 protein after storage at -70°C for 3 months; lane 3, the same preparation after continued storage at -20°C for 1 month. Positions of molecular weight standards (Mr × 10^5) are indicated at left.
help to reveal the structural relationship between the two receptor species. The demonstration of the existence of the LH/hCG receptor as a disulfide-linked complex may have relevance with respect to attempts to isolate the cDNA of the receptor for further structural characterization.

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