Purification and Characterization of Prolactin Receptors from Rat Ovary*

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Prolactin receptors were purified to homogeneity by two affinity chromatography steps using concanavalin A-Sepharose and human growth hormone (hGH)-Sepharose. The purified receptors showed specificity and high affinity for lactogenic hormones and a binding capacity of 20 n mole/mg of protein. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis revealed that purified receptors were composed of two major protein bands of Mr = 41,000 and 88,000, which were identified as radioactive bands by binding of 125I-hGH to blotted renatured receptors and by autoradiogram of free and 125I-radiolabeled purified receptors. Autoradiographic analysis of SDS-polyacrylamide gel electrophoresis of cross-linked 125I-hGH-receptor or hGH-125I-labeled receptor complexes showed two radioactive bands of Mr = 63,000 and 106,000. Analysis of the free receptors by high performance liquid chromatography using Superose 12 revealed two peaks of binding activity for 125I-hGH eluting in the positions of Mr ~ 150,000 and 250,000. After cross-linking with 125I-hGH, SDS-polyacrylamide gel electrophoresis analysis revealed that both peak fractions contained two binding species with Mr = 63,000 and 106,000. Chromatography of 125I-hGH-receptor complexes showed two radioactive fractions with approximate Mr ~ 180,000 and 300,000. The treatment of 125I-labeled receptors with SDS and reductant resulted in the dissociation of the higher Mr form into the lower Mr form upon gel filtration. Chromatofocusing of free receptors showed three isoforms with pI 4.0, 5.0, and 5.3. These results indicate that detergent-solubilized prolactin receptors appear to be aggregated forms of holoreceptor containing two binding species of Mr = 41,000 ± 2,000 and 88,000 ± 3,000.

Lactogenic hormones such as prolactin have been described to participate in a number of biological processes including reproduction, metabolism, development, and regulation of electrolyte balance (1-4). A large body of evidence has been accumulated to indicate that these hormones initiate their actions by binding to specific binding sites on the target cell surface. The fact that antibodies against prolactin receptors from mammary gland mimic prolactin actions in this organ both in vitro as well as in vivo indicates a possible role of the microaggregation of prolactin receptors in the transmission of the hormone signal across the membranes (5-7). However, the molecular mechanism between hormone binding to the cell surface receptors and the expression of its biological response is far from clear. Since the binding sites for lactogenic hormones have been identified in various tissues including mammary gland, liver, ovary, kidney, prostate, and brain (8-11), it is necessary to characterize the receptor in order to understand the signal transmission mechanism across the membranes as the initial event of hormone action.

A role for prolactin in modulation of steroid production and luteinizing hormone receptor number of the ovary has been described (12, 13). Luteinized rat ovaries are a richer source of lactogenic hormone receptors than lactating mammary gland and are of value for receptor purification (14, 15). The detergent solubilization of prolactin receptors from the membranes caused a 7-fold increase in their binding capacity, which was considered to be due to unmasking of nascent receptors unavailable for hormone binding in the intact membrane (15, 16). In our previous study using bifunctional cross-linking reagents, we identified for ovarian membrane lactogen receptor a Mr = 80,000 species containing a binding unit of Mr = 40,000 (17). In this study, we have further characterized the molecular species and subunit structure and purified prolactin receptors solubilized from luteinized ovarian membranes to homogeneity by affinity chromatography. For these studies, the purified free and complexed prolactin receptors with hGH1 were analyzed by SDS-PAGE, electrophoretic blotting, and high performance liquid chromatography using gel filtration and chromatofocusing columns. Furthermore, 125I-radiolabeled receptors were also used to assess the receptor structure. The present results indicate that Triton X-100 solubilized native prolactin receptors appear to be aggregated forms of holoreceptor containing two binding subunits of Mr = 41,000 ± 2,000 and 88,000 ± 3,000.

EXPERIMENTAL PROCEDURES

Materials—hGH (NIADDK-hGH-I), ovine prolactin (NIH-P-S-10), human placental lactogen, bovine growth hormone (NIH-GH-B-18), and ovine luteinizing hormone (NIAMDD-oLH-23) were obtained through the Hormone Distribution Program, National Institute of Arthritis, Metabolism and Digestive and Kidney Diseases and national chorionic gonadotropin (CR 121, 12,000 IU/mg) through the Center for Population Research, National Institute of Child Health and Human Development. DSS and DSP were purchased from Pierce Chemical Co. Soybean trypsin inhibitor, lactoperoxidase, bovine-

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The abbreviations used are: hGH, human growth hormone; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DSS, dicycinnidyl suberase DSP, 3,3'dithiobis(succinimidyl propionate); PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[3-cholamidopropl]dimethylammonio]-1-propane sulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.
globulin, bovine albumin, PMFS, N-ethylmaleimide, and Triton X-100 were from Sigma. CNBr-activated Sepharose, concanavalin A-Sepharose, high performance liquid chromatography Superose 12, and Mono P columns and molecular weight standards were obtained from Pharmacia Fine Chemical Co. [125I]iodine was from New England Nuclear. 0.1 M bicarbonate buffer for Sf-96 was obtained from Bio-Rad Laboratories. [125I]hGH (specific radioactivity, 40-60 μCi/μg) was prepared by a modification of a lactoperoxidase method (18) as described previously for hCG (19). hGH-Sepharose 4B used for affinity chromatography was prepared as follows. hGH (1 mg) was coupled to CNBr-activated Sepharose 4B (0.1 ml gel volume) by incubating the mixture in 0.1 M bicarbonate buffer (pH 8.8) with 1% (w/v) cyanogen bromide for 2 h at 25 °C. After incubation, the residual active groups remaining on the gel were blocked by the addition of 0.2 mM glycine (pH 8.0), followed by washing four times alternately with 50 ml of 0.1 M bicarbonate buffer (pH 8.3) and 0.1 M acetate buffer (pH 4.0) each containing 0.5 mM NaCl. All other reagents used were of analytical grade.

Animals and Preparation of Tissues—Female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories. Superovulated ovaries were removed from pseudopregnant rats treated with progesterone male's gonadotropin (Gestyl, Organon Diagnostics) and human chorionic gonadotropin (Pergonal) as described previously (14, 15). Ovaries were dissected, finely minced, and homogenized in ice-cold Dulbecco’s phosphate buffered saline or 20 mM HEPES (pH 7.4) with 0.25 mM sucrose containing 0.1 mg/ml soybean trypsin inhibitor, 0.1 mM PMSF, and 0.2 mM sodium molybdate by means of a Teflon pestle and glass homogenizer fitted with a Teflon pestle (10 strokes of 1.900 rpm). The homogenate was centrifuged for 15 min at 1,000 × g and the supernatant was centrifuged for 30 min at 35,000 × g. The pellet was resuspended in 5 ml of homogenization solution without sodium molydate/gram of ovary, and solubilized with Triton X-100 at a final concentration of 1% (v/v). The suspension was homogenized in homogenizer with Teflon pestle (15 strokes) and allowed to rest on ice for 40 min. This suspension was diluted 4-fold with homogenization solution without sodium molydate and then centrifuged for 90 min at 220,000 × g (Beckman SW 40 rotor). The lipid floating on top was removed and the supernatant was decanted and stored at −70 °C (solubilized membranes).

Purification of Solubilized Prolactin Receptors by Affinity Chromatography—Triton X-100-solubilized membranes (50-60 μg of protein) obtained from 30 ovaries were chromatographed on a concanavalin A-Sepharose column (1.5 × 15 cm) equilibrated with PBS (pH 7.4) containing 10 mM MgCl2, 0.1% Triton X-100, and 0.2 mM PMSF. The column was washed with 1% Triton X-100 in PBS containing 0.2 M glycine (pH 8.0), followed by washing four times alternately with 50 ml of 1% Triton X-100, 10 mM MgCl2, 0.5 mM PMSF, and 0.5 M NaCl. The bound receptors were eluted with 0.15 M α-methylmannoside, and the eluted fractions were assayed for [125I]hGH binding activity. The active fractions eluted with 0.15 M α-methylmannoside were pooled and then subjected to hGH-Sepharose chromatography. The receptor fractions (1 mg of protein/40 ml) were incubated with hGH-Sepharose 4B (2 ml gel volume) for 2 h at 25 °C by agitation with end-over-end tube rotator (Scientific Equipment Products, Baltimore, MD), followed by a further 2-h incubation at 4 °C using a Dupont Cronex Lightning Plus intensifying screens and Eastman Kodak X-Omat AR5 film.

Electrophoretic Blotting—Purified receptors were electrophoresed under nonreducing conditions using a 10% SDS-polyacrylamide gel. Electrophoretic transfer of peptides from acrylamide gel to the nitrocellulose sheet was carried out according to Towbin et al. (24). The nitrocellulose sheet was blocked by the addition of 0.2 M glycine (pH 8.0), followed by washing four times alternately with 50 ml of 0.1 M Tris-HCl (pH 7.4) containing 10 mM α-methylmannoside. The blots were then washed sequentially with gelatin solution, 0.1% Triton-X-100 and 0.15 M NaCl and 10 mM MgCl2, 20 mM Tris-HCl (pH 7.4), and distilled water, and dried for autoradiography.

Purification of Detergent-Solubilized Prolactin Receptors—Prolactin receptors were solubilized from ovarian membranes with 1% (w/v) Triton X-100. This preparation exhibited a 7-fold higher binding capacity for [125I]hGH (6 pmol/mg of protein) than that of particulate membranes, consistent with the results reported previously (15). The solubilized membrane fraction was then subjected to concanavalin A-Sepharose chromatography. The receptor binding activity bound to the column was eluted as a broad peak at 0.15 M 2-mercaptoethanol.

RESULTS

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Affinity-purified receptors bound [125I]hGH with a KD of 1.5-
Purification and Characterization of Prolactin Receptors

Fig. 1. Purification of solubilized prolactin receptors by concanavalin A-Sepharose and hGH-Sepharose affinity chromatography. A, Triton X-100-solubilized membranes (60 mg of protein) were chromatographed on a concanavalin A-Sepharose column as described under “Experimental Procedures.” B, the active fractions eluted from concanavalin A-Sepharose with 0.15 M α-methylmannoside were pooled and then subjected to hGH-Sepharose chromatography (see “Experimental Procedures”). The receptors adsorbed to the gel were eluted with 5 M MgCl₂ containing 0.1% Triton X-100 and 0.1 mM PMSF.

Table I

<table>
<thead>
<tr>
<th>Sample</th>
<th>Kᵦ (× 10⁻⁷ M⁻¹)</th>
<th>Capacity (pmol/mg protein)</th>
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</thead>
<tbody>
<tr>
<td>Triton X-100-solubilized membranes</td>
<td>3.5 ± 2.1</td>
<td>6.0 ± 1.4</td>
</tr>
<tr>
<td>Concanavalin A-Sepharose-purified receptors</td>
<td>3.9 ± 1.3</td>
<td>39.8 ± 5.1</td>
</tr>
<tr>
<td>hGH-Sepharose-purified receptors</td>
<td>3.5 ± 1.0</td>
<td>20,440 ± 4,790</td>
</tr>
</tbody>
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4.5 × 10⁻⁷ M⁻¹ and binding capacity of 20 nmol/mg of protein. About 4,000-fold purification was obtained by two steps of affinity chromatography and the overall recovery of activity was in the range of 15%.

Affinity-purified receptors were resolved by SDS-PAGE under reducing and nonreducing conditions. Silver staining of polyacrylamide gel under reducing conditions revealed two major peptide bands of Mr = 45,000 and 88,000 (Fig. 3). In some preparations, the higher Mr, receptor species was observed as a triplet indicating that the variants of the higher molecular species possibly correlated with differences in carbohydrate moiety and/or phosphorylation states of the receptor. Additional minor protein bands were also observed in the range of Mr = 60,000 and 200,000. The former bands corresponded to the previously described artificial bands observed on SDS-PAGE under reducing conditions (27). The latter band is likely to be an aggregated form of receptor protein observed also in some experiments on the SDS-PAGE analysis of cross-linked hormone-receptor complexes, (see Fig. 4c, and Fig. 7).

Fig. 2. Scatchard analysis derived from binding inhibition studies. Points are the mean of closely agreeing triplicate incubations. Each tube contained 0.1 ml of the purified receptor preparation, 0.1 ml of ¹²⁵I-hGH tracer (87,000 cpm/1.3 ng), and 0.3 ml of assay buffer containing radioinert hGH (0–50 ng). The final volume of the assay was 0.5 ml/tube. The concentration of the receptor preparation prior to dilution for assay was of 1.975 nM, and its protein content determined by [³H]dinitrofluorobenzene assay was 107 µg/liter, giving a specific activity of 18.458 nmol/mg of protein.

Fig. 3. Silver staining of purified ovarian prolactin receptors. Receptor preparations were electrophoresed on a 10% SDS-polyacrylamide gel under reducing conditions. Proteins were visualized by a silver staining technique. Lane A, Triton X-100-solubilized membranes; lane B, concanavalin A-Sepharose-purified receptors; lane C, hGH-Sepharose-purified receptors. The positions of the molecular weight markers are indicated on the left of the gel.

Cross-linking of ¹²⁵I-hGH to Prolactin Receptors—In order to characterize the receptor-binding species, ¹²⁵I-hGH was chemically cross-linked to the receptors using the bifunctional cross-linker DSS and DSP, and the cross-linked hormone receptor complexes were resolved by SDS-PAGE analysis. Autoradiograms revealed the two characteristically labeled bands corresponding to Mr = 63,000 and 106,000 (Fig. 4). Densitometric measurement showed that the intensity of the former band was 4–5-fold greater than that of the latter band. When solubilized membranes and concanavalin A-Sepharose purified receptors were cross-linked and analyzed by SDS-PAGE, two minor additional bands corresponding to Mr = 78,000 and 120,000 were also labeled (Fig. 4, lanes A and C). Another band of Mr = 44,000 was observed upon resolution
of cross-linked samples in SDS-PAGE. Since hGH has a tendency to form aggregation (28), this band is likely to be a dimer of $^{125}$I-hGH. Therefore, it is likely that the two additional minor bands ($M_r = 78,000$ and 120,000) observed only during the analysis of crude soluble receptors and partially purified preparations may correspond to receptor cross-linked to the $^{125}$I-hGH dimer. Furthermore, two additional minor high molecular weight bands of 200 $\times$ 10$^3$ and greater than 200 $\times$ 10$^3$ were observed. These more likely are aggregated forms of receptor not dissociated by the treatment prior to gel electrophoresis.

SDS-PAGE (under nonreducing conditions) of $^{125}$I-hGH cross-linked to receptors using the thio-cleavable cross-linker DSP resolved two labeled bands corresponding to $M_r = 60,000$ and 105,000, coincident with those of DSS cross-linked complexes. An additional broad radioactive area of aggregated materials migrated in the range of $M_r = 200,000$. When SDS-PAGE using 5% polyacrylamide gel was performed to identify the high molecular form, only this diffuse aggregated form was detected. As expected, SDS-PAGE under reducing conditions abolished these labeled bands (not shown).

Incubation of the receptors with labeled $^{125}$I-hGH hormone in the presence of ovine prolactin, human placental lactogen, and hGH prior to cross-linking abolished the labeled bands, whereas bovine growth hormone, human chorionic gonadotropin, and ovine luteinizing hormone did not inhibit the labeling of the binding sites to $^{125}$I-hGH, indicating that the purified receptors retained their specificity for lactogenic hormones (not shown).

Electrophoretic Blotting of Purified Prolactin Receptors—To further characterize the molecular size of prolactin-binding species, electrophoretic blotting experiments were carried out. A representative example is shown in Fig. 5. Affinity-purified receptors were electrophoresed on a 10% SDS-polyacrylamide gel and then transferred electrophoretically onto a nitrocellulose membrane. Incubation of the blot with $^{125}$I-

Fig. 4. Cross-linking of $^{125}$I-hGH-receptor complexes from solubilized membranes, concanavalin-A, and hGH-Sepharose-purified receptors. Triton X-100-solubilized membranes (lanes A, B), concanavalin-A-Sepharose (lanes C, D) or hGH-Sepharose (lanes E, F)-purified receptors were incubated with $2 \times 10^5$ cpn of $^{125}$I-hGH for 5 h at 25 $^\circ$C in the absence (lanes A, C, E) or presence (lanes B, D, F) of 2 $\mu$g of unlabeled hGH. The hGH-receptor complexes were cross-linked with 0.5 mM DSS for 15 min at 0 $^\circ$C. Samples were subjected to SDS-PAGE under reducing conditions using 7.5% polyacrylamide gel followed by autoradiographic analysis. The positions of the $M_r$ standards are indicated on the left of the autoradiogram.

Fig. 5. Blotting of purified prolactin receptors. Affinity-purified receptors were electrophoresed on a 10% SDS-polyacrylamide gel under nonreducing conditions and transferred electrophoretically onto a nitrocellulose sheet. After quenching the remaining protein binding sites and renaturation of the receptor proteins on the nitrocellulose sheets with gelatin (see "Experimental Procedures") the blot was incubated with $10^5$ cpn of $^{125}$I-hGH in the absence (A) or presence (B) of 20 $\mu$g of unlabeled hGH for 12 h at 25 $^\circ$C. After washing, the blots were dried for autoradiography. The positions of the molecular weight markers are indicated on the left of the autoradiogram.

The results obtained from SDS-PAGE analysis of purified free and cross-linked receptors to hGH consistently indicate that the detergent-solubilized prolactin receptors are composed of two binding subunits with $M_r = 41,000 \pm 2,000$ and 88,000 $\pm 3,000$. However, the results were not consistent with the findings shown in Fig. 4. The efficiency of the cross-linking reaction was estimated to be 10% under this experimental condition and explained the relatively low intensity of these complexes. Thus, $^{125}$I-iodinated receptors retained their binding activity.
The present study has demonstrated that prolactin receptors, purified from luteinized ovarian membranes to homogeneity, retained binding specificity for lactogenic hormones such as ovine prolactin, human placental lactogen, and hGH. Furthermore, based on the binding capacity of the receptors...
previous studies, several major protein bands were observed were chromatographed on a Superose 12 column equilibrated with peak fractions preparations followed by protein silver staining or autoradiors.

The analysis of prolactin receptors by SDS-gel electrophoresis after SDS-PAGE fractionation of the partially purified receptors was aimed at the characterization of its molecular structure and amino acid sequence. The analysis of prolactin receptors by SDS-gel electrophoresis and gel filtration has confirmed the presence of two binding components. In any case, the $M_r$ of the prolactin binding subunit reported in the above studies was similar to that of lower molecular weight subunit found in this and our previous studies (17, 36), but the higher $M_r$, species revealed by chemical cross-linking in solubilized receptors of ovary, testis, mammary gland, and kidney, and in purified ovarian receptors was not observed. The finding of the $M_r = 41,000$ binding subunit and its complexed form with hGH ($M_r = 63,000$) when the preparation of receptors and the binding reaction were carried out in the presence of proteinase inhibitors, does not rule out the possibility that the lower $M_r$ subunit is a proteolytic product of the higher $M_r$ species. In any case, the $M_r = 41,000$ peptide represents the smallest binding entity isolated (17, 31-36). It is of interest to note that analogous prolactin binding subunits with a similar $M_r$ value have been identified in various tissues and species. In contrast, discrepancies in the size and antigenic determinants of the growth hormone receptors are well known (37-39), and the growth hormone receptor appears to be highly species-specific (39). The fact that the lower $M_r$ species was released partially from the higher $M_r$ form of crude ovarian solubilized receptors under reducing conditions indicated that the binding component seemed to be associated partially via disulfide bonds (17). Thus, the present evidence indicates that the lower binding component ($M_r = 41,000$) is probably an integral part of the larger form containing the binding site. Also, this lower $M_r$ form could be a proteolytic cleavage product of the larger form, $M_r = 88,000$. Furthermore, the possibility that the higher $M_r$ subunit is a distinct entity from the lower $M_r$ subunit cannot be completely ruled out.

Superose 12 chromatography of the free receptors showed that the binding activity for $^{125}$I-hGH eluted in the positions of apparent $M_r \sim 150,000$ and 250,000 (Fig. 7). When the receptors were incubated with $^{125}$I-hGH and then subjected to Superose 12 chromatography, two peaks associated specifically with $^{125}$I-hGH were found in the position of apparent $M_r$.
$180,000$ and $300,000$. It is possible that the larger chromatographic form of the receptor could result from association of the lower form. This is supported by the fact that when treated with SDS and 2-mercaptoethanol, the higher form dissociated into the lower form(s) (Fig. 8). The results also indicate that the receptor subunits are cross-linked by both disulfide bonds and noncovalent interactions in the membranes.

Previous gel filtration studies on the Triton-solubilized liver and mammary lactogen receptors have reported $M_r$ values that ranged from $150,000$ to $300,000$ (31, 32-40). More recently, $M_r$ values of $320,000-340,000$ were reported for prolactin receptors. In contrast, lower $M_r$ species of $37,000$ were reported for CHAPS-solubilized receptor from mouse liver membranes (42). In conclusion, we have demonstrated the biochemical evidence for the presence of two prolactin-binding species in Triton X-100-solubilized prolactin receptors (17, 36). The mechanism by which aggregation or clustering of prolactin receptors would trigger the biological responses remains to be elucidated. The techniques described in this study allow purification of microgram quantities of biologically active receptor subunits available for further molecular characterization and sequence studies.

REFERENCES