Purification and Protein Sequence Analysis of Rat Liver Prolactin Receptor*

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Prolactin receptors were purified from rat liver membranes by single-step immunoaffinity chromatography using a specific monoclonal antibody to the rat liver prolactin receptor. Scatchard analysis of 125I-human growth hormone binding to the purified receptor revealed two classes of specific binding sites with $K_d = 18.5 \times 10^6$ and $1.2 \times 10^6$ M$^{-1}$. Considering that both classes of binding sites are responsible for high affinity prolactin binding, the partially purified receptor preparation had a binding activity of 1.69 nmol/mg protein, representing 1000-fold purification over microsomal receptors with a recovery of 52%. From three separate purifications, 6 mg of partially purified prolactin receptor were obtained with a purity of $\sim 4$ to 6.5%. Thus, the use of monoclonal antibody for affinity chromatography resulted in a large improvement of prolactin receptor purification compared to previous hormone affinity chromatography (300-fold purification, 15% recovery). The purified receptor was run on preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis, and a homogeneous preparation of prolactin receptor was obtained by electroelution from gel slices corresponding to $M_r$ 38,000-43,000. Immunoblot analysis using a radiolabeled monoclonal antibody revealed two separated, closely located bands of $M_r$ 42,000 and 40,000 in microsomal, partially purified, and electroeluted preparations.

The homogeneous receptor protein was extensively digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone trypsin, and 10 internal amino acid sequences of the rat liver prolactin receptor were determined by gas-phase sequence analysis. Oligonucleotide probes were prepared against two of these internal sequences, and a prolactin receptor cDNA was isolated from a rat liver library using one of these probes (Boutin, J. M., Jolicoeur, C., Okamura, H., Gagnon, J., Edery, M., Shirato, M., Banville, D., Dupanter-Fourt, I., Djiane, J., and Kelly, P. A. (1988) Cell 53, 69-77). The amino acid sequence deduced from the cDNA reveals three potential sites of N-linked glycosylation, two of which were confirmed during protein sequencing.

The prolactin receptor was characterized by affinity labeling with 125I-human growth hormone. Cross-linking of microsomes revealed a single band for the hormone-receptor complex with $M_r$ 62,000. On the other hand, cross-linking of Triton X-100-solubilized or partially purified receptor with labeled hormone resulted in the appearance of two bands with $M_r$ 62,000 and 102,000, suggesting the existence of a subunit structure of the prolactin receptor, or alternatively, the existence of two types of prolactin receptor. 125I-Receptor was run on a sodium dodecyl sulfate gel, and a homogeneous radioactive preparation was obtained from gel slices of $M_r$ 42,000. When this preparation was stored for 3 weeks at $-20^\circ$ C and analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography, a larger molecular weight form ($M_r$ 84,000) was identified in addition to the original $M_r$ 42,000 band, suggesting that dimerization of the prolactin receptor occurred. This also indicates that the larger hormone-receptor complex observed in cross-linking studies could represent a dimer of the $M_r$ 42,000 subunit, only one of which is able to bind prolactin.

Prolactin, a member of the lactogenic hormone family, is present in all vertebrates and is responsible for over 85 biological actions (1). The action of PRL is mediated by a specific receptor located in membrane components and widely distributed in a number of tissues (2). In order to better understand the molecular mechanisms involved in the various biological actions induced by PRL, elucidation of the biochemical properties of the PRL receptor is of primary importance. The relative molecular mass ($M_r$) of lactogen binding subunits has been reported in the range of 32,000 -- 84,000 when analyzed on SDS-PAGE (3). A smaller form of the PRL receptor ($M_r$ 40,000), which is not linked by disulfide bonds to itself or to other subunits, has been shown in rabbit mammary gland and rat liver (4-8). On the other hand, larger molecular weight forms of the receptor also have been reported in rat ovary (9), liver (10), and rabbit mammary gland (11), and one group (9) has suggested the existence of disulfide linkages between the subunits.

Different methods of purification of the PRL receptor have been developed from various tissues. Shiu and Friesen (12) initially attempted to purify the PRL receptor from rabbit mammary gland; however, the success of this approach was limited by the low abundance of the receptor in this tissue. A more effective method for purification of the PRL receptor from rat liver has been developed by our laboratory (13). This method involves the use of immunoaffinity chromatography and affinity cross-linking to isolate and purify the receptor. The purified receptor was extensively characterized in terms of its biochemical properties and functional activity.

* This work was supported, in part, by a grant from the Medical Research Council of Canada and the National Cancer Institute of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: PRL, prolactin; hGH, human growth hormone; cPRL, ovine prolactin; BSA, bovine serum albumin; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; DSS, disuccinimidyl suberate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; PEG, polyethylene glycol; PTH, phenylthiohydantoit; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.
mammary glands using hGH-agarose chromatography. Using oPRL as an affinity ligand, receptors have been purified from mouse liver (13) and rabbit mammary gland (5, 14). Moreover, Mitani and Dufau (9) reported purification from rat ovary using a two-step affinity purification: concanavalin A-Sepharose and hGH-agarose affinity chromatography. Although a high degree of purification has been reported in several tissues (9, 14), many of the purification studies were at the analytical rather than preparative level.

Rat liver, an organ rich in PRL receptor, has the highest receptor level during pregnancy (15) or after estradiol treatment (16). Purification of the rat liver PRL receptor using oPRL affinity chromatography has been reported (6), resulting in a 300–500-fold purification over microsomes, but with a relatively low recovery. To obtain a quantity of PRL receptor sufficient to permit further structural studies, we undertook the purification of rat liver PRL receptors by immunoaffinity chromatography, which resulted in a high yield of receptor protein. A homogeneous PRL receptor obtained by electrophoresis was digested with trypsin, and the resulting peptide fragments were analyzed on their amino acid sequences. Subsequently, amino acid sequence data enabled us to prepare oligonucleotide probes and identify cDNA clones for the rat receptor (17). In this report, we also describe the characterization of the PRL receptor using immunoblot and affinity labeling techniques.

**EXPERIMENTAL PROCEDURES**

**Materials—**oPRL (NIADDK-oPRL-16; 30.5 IU/mg) and hGH (NIH hGH AFP-5180A; 2.2 IU/mg) were kindly supplied by the National Hormone and Pituitary Program. Bromocriptine (CB-154) was generously supplied by Sandoz, Basel, Switzerland. Triton X-100, CHAPS, BSA, phenylmethylsulfonyl fluoride, dithiothreitol, and TPCK-trypsin were purchased from Sigma. Sepharose and Sephadex G-25 for receptor, and Sepharose 6B for IgG. The columns were equilibrated and eluted with assay buffer containing 0.1% NaN₃ for hormones and IgG, or 25 mM Tris-HCl, pH 7.4, containing 0.1% (v/v) Triton X-100, 0.1% BSA, and 0.1% NaCl for purified receptor. Specific activities, which were calculated from recovery of radioactivity in the protein fraction, were 76-95 cpm/μg for oPRL, 90-105 cpm/μg for hGH, 4-14 cpm/μg for E2I IgG, and 43-50 cpm/μg for purified receptor.

**Electroelution—**Partially purified receptor (6 mg), extensively dialyzed against 2.5 mM Tris-HCl, pH 7.4, containing 0.1 mM CHAPS, was concentrated by lyophilization. After solubilization in 50 mM Tris-HCl, pH 8.1, 6 M guanidine hydrochloride, and 5 mM EDTA, a trace amount (300,000 cpm) of 125I-receptor was added, and the preparation was reduced and alkylated as described previously (24). Excess reagents were removed by dialysis against 1% acetic acid and the retentate lyophilized. The solubilized receptor was run on a 30-cm column at a flow rate of 1 bed volume/h at 4°C. The eluted purified receptor were analyzed by immunoblot technique.

**Immunoblot Analysis of PRL Receptor—**Rats from adult female Sprague-Dawley rats were used as the source of prolactin receptor. The animals were treated subcutaneously with estradiol valerate (0.5 mg/rat, 3 days prior to killing) and with estradiol benzoate (0.5 mg/rat, injection) for 12 h intervals beginning 24 h prior to killing. Crude membrane fractions (microsomes) were prepared from liver as previously described (6). Microsomes were centrifuged at 200,000 × g in a Soarvall rotor for 60 min at 4°C, and pellets were resuspended in 25 mM Tris-HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride up to a final protein concentration of 1.2 mg/ml. The preparation was solubilized with 1% (v/v) Triton X-100 by constant stirring for 30 min at room temperature, centrifuged at 200,000 × g for 60 min at 4°C, and the clear supernatant fraction was collected (solubilized receptor).

A specific monoclonal antibody to the rat liver prolactin receptor, E2I (6), was produced using Protein A Affi-Gel 10 (18), and 40 mg of E2I IgG was coupled to 25 ml of Affi-Gel 10 according to the manufacturer's description with a coupling efficiency of 90-100%. The Triton-solubilized receptor (1500 mg of protein) was initially passed over a 25-ml benzamidine-Sepharose 6B pre-column in order to absorb serine proteases from the preparation and then applied to E2I-Affi-Gel 10 in a 3 × 30-cm column at a flow rate of 1 bed volume/h at 4°C. The E2I-Affi-Gel 10 column was washed with 5 bed volumes of 25 mM Tris-HCl, pH 7.4, containing 1 mM CHAPS (column buffer). After equilibrating the column to room temperature, the receptor elution was achieved with 1 bed volume of 5 M MgcI inclusing 10 mM CHAPS, followed by 4 bed volumes of column buffer. Twelve fractions (10 ml/fraction) were collected from the beginning of the elution, and the active fractions were pooled (50 ml) and applied to a Sephacex G-25 column (30 × 5 cm) equilibrated with 50 mM NaCl buffer including 0.1% glycerclo to avoid repressor degradation (19). Active receptor fractions eluted in the void void volume were combined and frozen further use. Hormone affinity purification of the PRL receptor using oPRL as an affinity ligand was performed as described earlier (6).

**Receptor Binding Assay—**Microsome or solubilized receptor (100 or 40 μg protein/tube, respectively) were incubated with various concentrations of unlabeled hGH diluted in 25 mM Tris-HCl, pH 7.4, containing 10 mM MgCl₂ and 0.1% BSA (assay buffer). hGH concentration was 100-3000 ng/ml. After 1 hour at room temperature. Partially purified receptor (0.1 μg/tube) was incubated as above, except that assay buffer including 4 mM CHAPS (final concentration) was used. The assays were terminated by the addition of 3 ml of assay buffer in the case of microsomes or by adding 0.5 ml of 0.1% bovine γ-globulin and 1 ml of 24% PEG 8000 and mixing in the case of solubilized or purified preparations. Bound 125I-hGH was separated from free ligand by centrifugation at 3000 × g for 20 min at 4°C, and pellets were counted for 1 min in a LKB γ-counter with a counting efficiency of 60%. Binding data were analyzed using the LIGAND computer program (20).

**Autoradiography—**Immunoblotting—Membranes were washed extensively with phosphate-buffered saline, and subject to autoradiography.
**Protein Sequencing**—Internal sequences of the prolactin receptor were obtained by gas-phase sequence analysis of peptides generated from digestion of the electropholated receptor with TPCK-trypsin. The appropriate conditions of digestion were determined from the results of preliminary experiments in which electrophoretically purified trypsin-labeled receptor was subjected to various conditions of enzymatic digestion and analyzed on 5−20% SDS-PAGE followed by autoradiography (data not shown). Approximately 100 μg (estimated from silver-stained gel) of pure receptor was digested overnight at 37°C with 1.6 μg of TPCK-trypsin in 25 mM Tris-HCl, pH 7.4.

The tryptic peptides were separated by reverse-phase HPLC on a Hypersil ODS 5 μm (100 × 2.1 mm) column using 0.1% ammonium bicarbonate and a gradient of acetonitrile. Chromatography was performed with a Hewlett Packard System Model 1090 instrument, equipped with a diode array detector. Fractions were collected by hand, and when necessary, further purified on the same HPLC system with a shallower gradient.

Automated Edman degradations were performed on a Model 470A Gas-Phase Sequencer equipped with an on-line Model 120A phenylthiohydantoin (PTH) analyzer (Applied Biosystems) employing the general protocol of Hewick et al. (26). Samples were applied to pre-cycled filters, coated with 1.5 mg of Polyethylene plus 0.1 mg of NaCl (Bio-Rad Labs), and standard programs (03 RPRL and 03 RPHT, Applied Biosystems) were employed for pre-cycling and sequencing.

**Characterization of PRL Receptor**—Cross-linking studies were performed with tritium, Triton X-100-solubilized, and immunoaffinity-purified receptors. The receptor preparations were bound with [125I]hGH under conditions of the “binding assay,” and 50 μl of freshly prepared DSS (5.5 mM) in dimethyl sulfoxide was added to the samples to make the final concentration 0.5 mM. After 15 min on ice, the reaction was terminated by adding 0.15 volume of ice-cold 1 M Tris-HCl, pH 7.4. Aliquots of 50 μl each sample were subjected to SDS-PAGE followed by autoradiography.

Partially purified receptors were iodinated and subjected to preparative SDS-PAGE. A homogeneous radioactive sample was obtained by electrophoresis of the gel slices ranging from M, 38,000 to 43,000. The sample was reanalyzed on SDS-PAGE followed by autoradiography immediately or after 5 weeks of storage at −20°C.

**Electrophoresis and Autoradiography**—Electrophoresis was performed as follows. Samples were boiled for 2 min in 50 mM Tris-HCl, pH 6.8, containing 10% (v/v) glycerol and 2% (w/v) SDS in the presence or in the absence of 20 mM dithiothreitol, and analyzed by SDS-PAGE using the discontinuous buffer system described by Laemmli (27). Gels used were 7.5% gel for electrophoresis and cross-linking and 10% gel for immunoblot analysis and radiolabeled receptor. The gels or blots were dried and subjected to autoradiography using Kodak X-OMAT film with an intensifying screen (Dupont, Cronex) at −70°C. Standards used in electrophoresis were myosin (200,000), α-galactosidase (116,250), phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,699), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

**RESULTS**

**Purification**—The rat liver PRL receptor was purified by Triton X-100 solubilization of microsomes followed by immunoaffinity chromatography. Human GH competitive assays were performed on each receptor preparation. The results of a representative purification are summarized in Table I. Scatchard analysis (Fig. 1) of binding results indicated one class of specific binding sites with K, = 2.3 nM−1 for microsomes, whereas solubilized and partially purified receptor had two classes of specific binding sites. The high affinity sites for solubilized receptor and partially purified receptor (K, = 7.5 and 18.5 nM−1, respectively), were about 15−20-fold higher than for the low affinity sites (K, = 0.34 and 1.23 nM−1). For subsequent calculations, both high and low affinity binding sites were considered to contribute to prolactin receptor binding activity.

Triton X-100 has been shown to cause aggregation of oPRL (12). Therefore, the Triton X-100 in the solubilized-purified fraction was exchanged with CHAPS, which does not affect the PRL molecule in the same way during the chromatographic steps (13). Binding data for both 125I-oPRL and 125I-hGH to the purified receptor were similar. Solubilization of the microsomal fraction with Triton X-100 resulted in 4-fold increase in binding capacity, as has been previously reported (4, 6, 8, 12), probably due to exposure of cryptic sites. Almost 10,000 pmol of solubilized receptors were loaded onto a 40-mg E21 coupled Affi-Gel 10 column, and 40% of receptor proteins was recovered in the 5 mM MgCl2 eluate. The binding capacity of purified receptor was increased to 1700 pmol/mg protein from 1.6 pmol/mg protein of microsomes, representing ~1000-fold purification over microsomes. Assuming that the molecular weight of the rat PRL receptor is 40,000-42,000 and that there is one binding site per receptor molecule, the purity of this preparation was estimated to be 85%. Consequently, 3.5 nmol of receptor were obtained from 168 g of rat liver by the present purification. Two other preparations were carried out, resulting in receptor preparations ranging in purity from 4 to 6%.

To assess the improvement of purification of the PRL receptor, a hormone affinity purification profile is shown in Table II. A distinct difference between two purifications can be seen in the value of fold purification and binding capacity recovered. In the various separate purifications, the recovery of the PRL receptor from the affinity column was 2−4-fold greater for immunoaffinity than hormone affinity. The purity in final preparation also improved more than 3-fold by immunoaffinity chromatography. It should be noted that we also employed a benzamidine-Sepharose precolumn to remove serum proteases, thus the combined effect of removing proteases along with using a monoclonal antibody as a ligand for affinity chromatography results in a marked improvement in receptor purification and yield.

**Electroelution**—The affinity-purified receptors were reduced and alkylated and run on preparative SDS-PAGE.

### Table I

**Prolactin receptor purification by immunoaffinity chromatography**

| Sample                  | Total yield of protein | Binding capacity | Purification | Total binding capacity | Binding capacity recovered | Affinity constant K, |
|-------------------------|------------------------|------------------|--------------|------------------------|---------------------------|----------------------|
|                         | mg                     | pmol/mg protein  | −fold        | amol                   | %                         | 10^6 M⁻¹              |
| Crude microsome         | 4400                   | 1.58             | 1            | 6.94                   | 100                       | 2.3                  |
| Triton X-100-solubilized receptor | 1500 | n1 = 1.73 |               |                         |                          |                      |
|                         |                        | n2 = 4.61        |              |                         |                          | 7.5                  |
| Affinity-purified receptor | 2.14     | n1+n2 = 6.34    | 4.0          | 9.51                   | 137.0                     | 18.5                 |
|                         |                        | n1 = 561         |              |                         |                          | 1.2                  |
|                         |                        | n2 = 1132        |              |                         |                          |                      |
|                         |                        | n1+n2 = 1693     |              |                         |                          |                      |

*Values are calculated by combining both high and low affinity binding sites in solubilized and purified receptors.

*Binding capacity and affinity constants were determined from Scatchard analysis of 125I-hGH competition experiments (Fig. 1).
Prolactin Receptor Purification and Sequence Analysis

2.0

Purified

Bound hGH (fmole)

FIG. 1. Scatchard plot of $^{125}\text{I}}$-hGH binding to PRL receptors. Microsome (100 μg), solubilized receptor (40 μg), and affinity-purified receptor (0.1 μg) were incubated with $^{125}\text{I}}$-hGH as described under "Experimental Procedures." Binding data were analyzed by the method of Scatchard (28) and analyzed using the LIGAND program (20).

TABLE II

Prolactin receptor purification by hormone affinity chromatography

Values are from one representative purification out of four purifications. Binding capacity and affinity constants were determined from Scatchard analysis of $^{125}\text{I}}$-oPRL competition experiments.

| Sample                | Total yield of protein (mg) | Binding capacity (pmol/mg protein) | Purification* (fold) | Total binding capacity (nmol) | Binding capacity recovered* (%) | Affinity constant $K_a$ ($\text{M}^{-1}$) |
|-----------------------|----------------------------|-----------------------------------|----------------------|-------------------------------|---------------------------------|-------------------------------------------|
| Crude microsome       | 3879                       | 0.35                              | 1                    | 1.36                          | 100                             | 1.4                                       |
| CHAPS-solubilized     | 1448                       | $n_1 = 0.35$                      |                      |                               |                                 |                                           |
| receptor              |                            | $n_2 = 0.85$                      |                      |                               |                                 |                                           |
|                       |                            | $n_1+n_2 = 1.20$                  |                      | 3.5                           | 128                             | 1.9                                       |
| Affinity-purified     | 1.9                        | 108.64                            | 310                  | 0.21                          | 15                              | 8.0                                       |
| receptor              |                            |                                   |                      |                               |                                 |                                           |

*Values are calculated by combining both high and low affinity binding sites in solubilized and purified receptors.

Homogeneous prolactin receptor was prepared by electroeluting the $M_r \sim 38,000-43,000$ region from the gel slices. During electroelution, SDS was omitted from the elution buffer, and a minimum concentration of Triton X-100 (0.01%) was used to facilitate the following sequencing steps. Receptor recovery after each step was estimated from the radioactivity in the preparations. The band of $M_r \sim 38,000-43,000$ contained ~2% of the total radioactive counts applied on SDS-PAGE. After electroelution, 85–95% of radioactivity was recovered from the gel slices in a small volume. Approximately 100 μg of homogeneous receptor was obtained from 6 mg of affinity purified receptors in a total volume of 6 ml. This value is in agreement with the amount of peptide detected by sequence analysis (Table III), assuming a coupling efficiency of 60–80%. It was also similar to the estimate obtained by silver staining of the minigel (Phast system) (not shown).

**Immunoblot Analysis of the PRL Receptor** —By direct overlay of radiolabeled monoclonal antibody onto nitrocellulose followed by autoradiography, the detection sensitivity increased 50-fold as compared to the enzyme-substrate method. Thus, it became possible for microsomal prolactin receptor to be detected by immunoblot analysis. Analysis was performed on microsomal, hormone affinity-purified, immunoaffinity-purified, and electroeluted PRL receptor, and the autoradiogram revealed two separate but closely related bands of 42,000 and 40,000 for all preparations each with almost equal intensity (Fig. 2). Vastly different amounts of protein were loaded in each lane, which clearly reflects the degree of purity. Using this technique, it was calculated that there was 300-fold purification between microsomal and hormone affinity-purified receptor, 1250-fold between microsomal and immunoaffinity purification, and 50,000-fold between microsomal and electroeluted receptor. These values are almost identical to those obtained from Scatchard analysis of each preparation (Tables I and II). Although the relationship between two bands ($M_r \sim 42,000$ and 40,000) is still unclear, it has been suggested that isoforms of the PRL receptor may exist, and that the lower molecular weight form could be derived from the higher molecular form (6).

**Protein Sequence Analysis** —The pure prolactin receptor was extensively digested with TPCK-trypsin, and the resulting peptide fragments were separated from each other by
Oligonucleotides for screening cDNA libraries were synthesized based on the underlined protein sequence data. The sequence of the oligonucleotide probes were: a 24-mixer for the peak 13 sequence (5'-CCAGTACGTAATGACGGGCTGTAACGA-3'), two probes that were prepared from the peak 21–5 sequence, a 45-mer best predicted sequence (5'-CCTGAAGGTGCTGAGTGATCTCCACTCTC-3'), and a 23-mixer (5'-GAATGCTCCAGCGCTTCACTTC-3'). Attempts to identify a positive recombinant were negative with the 24-mixer, perhaps because for the Pro and Gly residues the codons that were selected (only two of the four possibilities were chosen) were incorrect; also, the failure with the 45-mer was certainly due to the fact that the selection of bases (based on a codon frequency table for the rat) was incorrect for 10 of the 14 choices.

Peptide sequences of the prolactin receptor

Peptide sequences of the prolactin receptor analyzed by cross-linking of solubilized or purified receptors were affinity-labeled with 125I-hGH by DSS and analyzed on SDS-PAGE followed by autoradiography. Cross-linking of microsomes with 125I-hGH revealed a single M, 62,000 band on the autoradiogram (Fig. 4, lane 1). On the other hand, cross-linking of solubilized or purified receptors resulted in the appearance of an M, 102,000 band in addition to the M, 62,000 (Fig. 3, lanes 2 and 3). Both bands disappeared when excess unlabeled hormone was added to the incubation mixture, indicating specific labeling of the PRL binding subunit. By subtracting the molecular weight of hGH (22,000), molecular weights of 80,000 and 40,000 were estimated for the higher and the lower hormone binding species, respectively. Migration and density of the M, 102,000 and 62,000 bands were not affected by the use of reducing agents (not shown). Larger forms of the PRL receptor have been reported in rat ovary (9) and more recently rat liver (10) when detergent-solubilized receptors were analyzed by cross-linking. Although our results are in good agreement with these, we failed to observe any significant change in bands between reduced and nonreduced samples.

Characterization of the PRL Receptor—Rat liver microsomes, Triton X-100-solubilized receptors, and partially purified receptors were affinity-labeled with 125I-hGH by DSS and analyzed on SDS-PAGE followed by autoradiography. Cross-linking of microsomes with 125I-hGH revealed a single M, 62,000 band on the autoradiogram (Fig. 4, lane 1). On the other hand, cross-linking of solubilized or purified receptors resulted in the appearance of an M, 102,000 band in addition to the M, 62,000 (Fig. 3, lanes 2 and 3). Both bands disappeared when excess unlabeled hormone was added to the incubation mixture, indicating specific labeling of the PRL binding subunit. By subtracting the molecular weight of hGH (22,000), molecular weights of 80,000 and 40,000 were estimated for the higher and the lower hormone binding species, respectively. Migration and density of the M, 102,000 and 62,000 bands were not affected by the use of reducing agents (not shown). Larger forms of the PRL receptor have been reported in rat ovary (9) and more recently rat liver (10) when detergent-solubilized receptors were analyzed by cross-linking. Although our results are in good agreement with these, we failed to observe any significant change in bands between reduced and nonreduced samples.

Peaks 21 and 25 in Fig. 3 were shown by protein sequence analysis to be mixtures containing more than one peptide, and in each case a major sequence could be derived from the data (Table III). The observed sequences for the two peptides in the double-sequence cycles were found to be consistent with protein sequences deduced from the cDNA. One of these mixtures, peak 21, which resolved into a single sequence after six degradations, proved to be instrumental in cloning the prolactin cDNA sequence, was accurately determined by peptide sequence analysis.

Characterization of the PRL Receptor—Rat liver microsomes, Triton X-100-solubilized receptors, and partially purified receptors were affinity-labeled with 125I-hGH by DSS and analyzed on SDS-PAGE followed by autoradiography. Cross-linking of microsomes with 125I-hGH revealed a single M, 62,000 band on the autoradiogram (Fig. 4, lane 1). On the other hand, cross-linking of solubilized or purified receptors resulted in the appearance of an M, 102,000 band in addition to the M, 62,000 (Fig. 3, lanes 2 and 3). Both bands disappeared when excess unlabeled hormone was added to the incubation mixture, indicating specific labeling of the PRL binding subunit. By subtracting the molecular weight of hGH (22,000), molecular weights of 80,000 and 40,000 were estimated for the higher and the lower hormone binding species, respectively. Migration and density of the M, 102,000 and 62,000 bands were not affected by the use of reducing agents (not shown). Larger forms of the PRL receptor have been reported in rat ovary (9) and more recently rat liver (10) when detergent-solubilized receptors were analyzed by cross-linking. Although our results are in good agreement with these, we failed to observe any significant change in bands between reduced and nonreduced samples.

Radiolabeled receptor was reduced with 20 mm dithiothreitol and subjected to preparative SDS-PAGE. The homogeneous sample was electroeluted from gel slices ranging between M, 38,000 to 43,000 and analyzed on SDS-PAGE the following day (Fig. 5, lane 1). When this sample was stored at -20 °C for 3 weeks and then rerun on SDS-PAGE, a larger molecular weight form, M, 84,000 was identified on the autoradiogram in addition to the original M, 42,000 form (Fig. 5, lane 2). This clearly demonstrates that the M, 42,000 binding subunit dimerized into a larger form.

DISCUSSION

In order to obtain preparative amounts of receptor, it is necessary to begin purification with a tissue containing high...
concentrations of receptor. For this reason, female rat liver was chosen as a source of PRL receptor (15). Moreover, receptor levels were further enhanced by estradiol treatment (16).

The PRL receptor is a membrane protein existing in small quantities and its isolation requires the proper detergent and highly specific methodologies. Affinity chromatography is the method of choice, since it bases the purification on the functional rather than on overall chemical and physical properties, which are similar among most detergent-solubilized membrane proteins. By using PRL affinity chromatography, we previously purified the PRL receptor from rat liver (6). Partially purified receptor preparations obtained by hormone affinity chromatography were useful for immunization and antibody production and for characterization of the PRL receptor molecule, while neither purity nor final recovery of receptor were satisfactory for protein sequencing.

Similar results using hormone affinity chromatography have been reported for the purification of rabbit (4, 12) and pig mammary gland (8) receptor. A possible explanation for the low recovery could be receptor degradation during the various purification steps and/or a strong interaction between receptor and ligand that does not permit separation by the dissociating agent. Recently, Berthon et al. (29) demonstrated an improvement in purification by immunoaffinity chromatography of pig mammary gland receptor.

Imunoaffinity chromatography employing a specific monoclonal antibody offers several advantages compared to hormone affinity chromatography. First, microsomes can be solubilized with Triton X-100, which is a more efficient detergent for solubilizing rat liver membranes, but is not compatible for use with oPRL affinity chromatography (12). Second, chromatography is performed at 4 °C rather than 20 °C, which should decrease degradation and/or loss of receptor binding activity. Finally, the binding characteristics between E21 and the prolactin receptor allow much larger amounts of receptor to be eluted from the affinity column by 5 M MgCl₂ without loss of specific binding activity in the eluate. Almost 40% of the receptor applied to the column was eluted, resulting in a 50% final receptor recovery over microsomes. Consequently 9 nmol of the PRL receptors were obtained from three purifications.

**FIG. 2. Immunoblot analysis of PRL receptors.** Receptor preparations were analyzed by 10% SDS-PAGE under reducing conditions and blotted onto a nitrocellulose membrane. The blots were incubated with 125I-labeled monoclonal antibody (10⁶ cpm), dried, and autoradiographed for 18 h at -70 °C. The amount of protein loaded into each lane is indicated at the bottom of figure: lane 1, microsomes; lane 2, hormone affinity-purified receptor; lane 3, immunoaffinity-purified receptor; lane 4, receptor purified by electroelution.

**FIG. 3. Reverse-phase HPLC of PRL receptor digested with TPCK-trypsin.** Approximately 2.2 nmol of receptor was digested with TPCK-trypsin in a volume of 500 μl. One hundred and twenty-five microliters of the digest were injected on a Hypersil ODS 5 μm column. Elution solvents were 0.1% ammonium bicarbonate (solvent A) and 100% acetonitrile (solvent B). Peptide fragments were eluted with a linear gradient of 5-55% solvent B in 60 min with a flow rate of 0.1 ml/min. The absorbance was measured at 210 nm. Arrows show the peptide peaks successfully sequenced (Table III).
or aggregation of the receptor proteins during purification steps that resulted in a loss of binding activity. Two attempts to obtain NH₂-terminal sequence data using intact PRL receptor were unsuccessful, presumably because the NH₂ terminus of this protein is blocked. This agrees with the predicted NH₂-terminal amino acid deduced from the cDNA sequence, which suggests that Gln may be present as a pyro-Glu residue. By microsequence analysis, two of the three potential N-linked glycosylation sites were confirmed in the receptor molecule. The biological significance of the oligosaccharide residues on the PRL receptor is far from clear. We are currently characterizing the type of oligosaccharide linkage found in the rat liver PRL receptor.  

A larger molecular weight (M, 80,000) binding species was found in addition to the M, 42,000 binding subunit when detergent-solubilized or partially purified receptors were analyzed by cross-linking (9, 10). Whether this larger form represents a different type of PRL receptor or is formed by association of the M, 42,000 subunits with each other or with another membrane component is still unclear. However, the appearance of the M, 84,000 form in the homogeneous receptor preparation (Fig. 5) suggests that the M, 42,000 form is able to dimerize. Moreover, when membranes from Chinese hamster ovary cells transfected with the cDNA clone encoding the M, 42,000 PRL receptor (17) were subjected to immunoblot analysis using several monoclonal antibodies to the PRL receptor molecule, the biological significance of the oligosaccharide residues on the PRL receptor is far from clear. We are currently characterizing the type of oligosaccharide linkage found in the rat liver PRL receptor.  

Acknowledgments—We thank Joe Zachwieja for excellent technical assistance in the preparation of monoclonal antibodies, Réjean Melanson for preparation of figures, and Nirmala Brunel for typing the manuscript.

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