

Interaction of lactogenic hormones with purified recombinant extracellular domain of rabbit prolactin receptor expressed in insect cells

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The extracellular domain of rabbit prolactin receptor (rbPRLR-ECD) expressed in an insect/baculovirus expression system was purified by affinity chromatography on immobilized PRL followed by gel filtration. The purified protein was over 90% homogeneous as indicated by SDS-PAGE in the presence or absence of reducing agent, and by chromatography on a Superdex column. Its molecular mass determined by SDS-PAGE was 32 kDa, and by gel filtration, 27 kDa. Both values are higher than the 22.8 kDa deduced from the cDNA sequence, indicating extensive glycosylation. The K_d value for interaction with ovine (o) PRL was 25.4 nM⁻¹, but even at high rbPRLR-ECD:hormone molar ratios, the stoichiometry of interaction with oPRL or human growth hormone indicated formation of only 1:1 complexes, in contrast to human growth hormone (hGH)-ECD which forms 2:1 complexes with hGH.

Extracellular receptor domain; Prolactin; Human growth hormone; Rabbit

1. INTRODUCTION

Numerous indirect studies have indicated that receptor dimerization is an initial step in the prolactin (PRL) signal transduction that occurs subsequent to hormone binding [1–4]. Direct evidence for dimerization of recombinant, non-glycosylated human growth hormone (hGH) receptor's extracellular domain (hGHR-ECD) containing amino acids 1–238 out of a total of 246 due to interaction with hGH, has been recently provided by biochemical [5] and X-ray crystallographic studies [6], in which two non-symmetrical binding sites in hGH were identified. A similar 2:1 stoichiometry of hGHR-ECD:hGH interaction was also observed in our lab using full-size (1–246) hGHR-ECD [7]. Hormone-induced receptor dimerization may be characteristic of an entire family of GH/PRL/cytokine receptors [8]. This is indicated by a recent report that in a leukemia cell line expressing a hybrid receptor composed of the ECD of hGHR linked to the transmembrane and intracellular domains of murine granulocyte colony-stimulating factor receptor, monoclonal antibodies to hGHR stimulated DNA synthesis while their Fab fragments were devoid of mitogenic activity [9].

Since GHs and PRLs [10], as well as the ECDs of their receptors, exhibit a high degree of similarity [11],

we decided to study whether hormone-induced receptor dimerization also occurs in the PRL:PRLR interaction. Recently, we have produced and expressed the ECD of rabbit (rb) PRLR using an insect/baculovirus expression system [12]. In the present work, we describe its purification and interaction with two lactogenic hormones, ovine (o) PRL and hGH.

2. MATERIALS AND METHODS

2.1. Materials

Ovine PRL (NIDKK AFP-8277E) and human GH (AFP-4793 B) were received from the National Hormone and Pituitary Programs (Bethesda, MD, USA). Recombinant hGHR-ECD (hGH binding protein) containing 1–246 amino acids and expressed in *E. coli* was prepared as described previously [7]. Reagents for tissue culture are listed in a former publication [12]. All other reagents were of analytical grade.

2.2. Preparation of insect cells' conditioned medium

The growth of *Spodoptera frugiperda* (SF 9) insect cells has been described previously [12]. Confluent cell cultures (grown in spinner flasks to 3–4 × 10⁶ cells/ml) were transferred to 175-cm² tissue culture flasks (Nunc, Kamstrup, Denmark) and seeded at 55 × 10⁶ cells/flask. After infection with a recombinant baculovirus containing the pGM Ac3 E rb PRLRL [12], the cells were cultured at 27°C in serum-free SF-900 medium. After 3 days, the conditioned medium containing the PRLR-ECD was collected, centrifuged for 15 min at 1,200 × g and stored at –20°C.

2.3. Preparation of oPRL-Affigel column

The column was prepared according to the method of Shiu and Friesen [13]. One ml of resin contained 0.5–0.6 mg of oPRL. The same column was used for five successive rbPRLR-ECD preparations, after reequilibration with the running buffer.

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2.4. SDS-polyacrylamide gel electrophoresis and Western blot analyses
SDS-PAGE was carried out according to Laemmli using 10% gels [14]. The gels were stained with Coomassie blue. The protocol for Western blot analyses was as described previously [12].

2.5. Binding assays

The binding assays and Scatchard analyses were performed as described previously, using goat anti-rbPRLR polyclonal antibody 46 for immunoprecipitation of the hormone-ECD complex [12]. 0.022 pmol of [¹²⁵I]oPRL were added/tube. Preparation of iodinated hormones has been described previously [15].

2.6. Determination of the molecular size of rbPRLR-ECD lactogenic hormone complexes by gel filtration

The molecular size of the oPRL-ECD complexes was determined by gel filtration, as described previously [7]. The concentration of oPRL in the incubation mixture was 0.5 nmol/ml and that of ECD varied from 0 to 2.5 nmol/ml. The reaction was carried out in 25 mM Tris-HCl buffer, pH 8.0, containing 10 mM MgCl₂ and 150 mM NaCl (TMN buffer). After a 60-min incubation at room temperature, 200- μ l aliquots were applied to a Superdex-75HR 10/30 column (Pharmacia, Uppsala, Sweden), connected to an HPLC (Waters, USA), which had been preequilibrated with the same buffer, prior to sample application. The column was developed at 1.0 ml/min. at room temperature. Protein content was monitored by absorbance at 280 nm. The interaction between rbPRLR-ECD or hGHR-ECD and iodinated oPRL or hGH was determined in 25 mM Tris-HCl buffer, pH 8.0, containing 10 mM MgCl₂ and 0.1% bovine serum albumin. Concentration of each of the radiolabelled hormones was 0.1 pmol/ml and of the ECDs, 0.0–2.0 pmol/ml. After a 16-h preincubation, 200 μ l aliquots were applied to a Superdex-75HR 10/30 column, preequilibrated with the incubation buffer and connected to an FPLC (Pharmacia, Uppsala, Sweden). The column was developed at 1.0 ml/min at room temperature, and 0.5 ml fractions were collected and monitored for radioactivity at LKB gamma-counter.

3. RESULTS

3.1. Purification and characterization of rbPRLR-ECD

Conditioned medium (1,200 ml) obtained from SF9 cells infected with pGmAc3 E rbPRLR [12] was adjusted to pH 8.0, with 25 mM Tris-HCl, 10 mM MgCl₂, left for 60 min on ice and then centrifuged at 100,000 \times g for 30 min. Clear supernatant was applied to an oPRL-Affigel column (3.5 \times 2.0 cm) at 100 ml/h. The column and the 80 ml of overlaying supernatant were kept at room temperature, while the rest of the supernatant and the eluate were kept on ice. The column was subsequently washed with 60 ml of TMN buffer, 60 ml of TMN buffer containing 1 M NaCl, 30 ml of TMN, then

30 ml of TMN containing 1 M MgCl₂. Finally 8 ml of TMN containing 4.5 M MgCl₂ were applied to the column and the elution was stopped. After 15 min, the elution was started again with TMN buffer containing 4.5 M MgCl₂, at the rate of 30 ml/h. Fractions (2.5 ml) were collected in siliconized polypropylene tubes containing 2.5 ml of TMN buffer and immersed in ice. Over 70% of the total binding activity was recovered in the first 12 tubes of 4.5 M MgCl₂ eluate (Table I). Very little or no binding activity was found in the earlier eluates or in the breakthrough fraction (not shown). The 4.5 M MgCl₂ eluate was concentrated to 1.1 ml in an Amicon concentrator using a YM3 membrane pretreated with 0.01% polyvinylpyrrolidone (MW 360,000). The concentrated fraction was further separated on Superdex-75HR 10/30 column equilibrated with TMN buffer, in four successive injections of 0.25 ml each. The column was developed at 1 ml/min at room temperature, and 0.5 ml fractions were collected in siliconized polypropylene tubes. Seventy-five percent of the protein and all binding activity was found in fractions eluted in tubes 23–25 (Fig. 1). The overall yield was 25% (Table I). The purified fraction was over 90% homogeneous as indicated by SDS-PAGE in the presence or absence of reducing agent (Fig. 2), and by rechromatography on the Superdex column (Fig. 3E). Its molecular mass estimated by SDS-PAGE, was 32 kDa, in agreement with that determined previously by immunoblotting [12]. Western blot analysis revealed the purified ECD to be recognized by goat anti-PRLR polyclonal antibody 46 (not shown). A parallel determination of molecular mass, from the of Superdex column profile (Figs. 3 and 4E) yielded a value of 27 kDa. The molar extinction coefficient at 280 nm (63,198) was calculated using the molar extinction coefficients of tyrosine (1,197) and tryptophan (5,559), as suggested previously [16].

The purified rbPRLR-ECD exhibited high affinity for oPRL. Scatchard analysis yielded a linear plot (not shown). The K_a value calculated from three separate preparations was 25.4 ± 7.4 mM⁻¹ (mean \pm S.E.M.), consistent with the previously published value for non-purified ECD in conditioned medium [12]. The purified fraction retained over 85% of its binding activity when stored at 4°C or –20°C for 4 weeks in siliconized pol-

Table I
Yield of baculovirus produced rbPRLR-ECD

Fraction	ml	Specific binding* (pmol/ml)	Total pmol bound	Yield (%)
Conditioned medium	1,200	13	15,600	100.0
4.5 MgCl ₂ eluate	60	183	10,980	70.4
Concentrated 4.5 MgCl ₂ eluate	1.1	7,113	7,824	50.2
Superdex eluate (tubes 23–25)	7.5	516	3,870	24.8

*Calculated from the specific binding of [¹²⁵I]oPRL. The specific radioactivity of the ligand was 70 μ Ci/mg, which corresponds to 3.54×10^6 dpm or 2.3×10^6 cpm/pmol. Since 0.022 pmol of [¹²⁵I]oPRL were added/tube, 1% of specific binding represents 0.22 fmol of specifically-bound radioactive ligand.

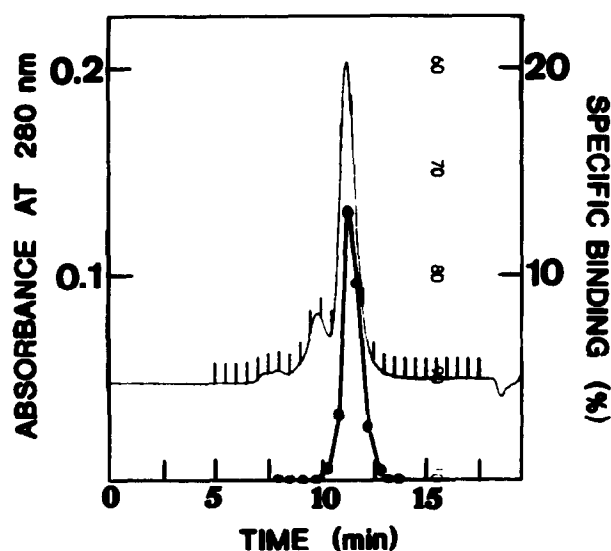


Fig. 1. Chromatography of 0.25 ml concentrated 4.5 M MgCl_2 affinity-column eluate, on a Superdex-75HR 10/30 column equilibrated with TMN buffer. The column was developed at 1 ml/min at room temperature and 0.5 ml fractions were collected in siliconized polypropylene tubes. Diluted aliquots corresponding to 0.004 μl were taken for determination of the specific binding (●—●).

propylene tubes. Chromatography of the stored fractions on the Superdex column indicated that the slight loss of binding activity most probably results from absorption of the purified fraction to the tube, and not from dimerization or oligomerization of the monomeric rbPRLR-ECD (not shown).

3.2. Interaction with lactogenic hormones

The stoichiometry of the interaction between purified rbPRLR-ECD and oPRL was studied using non-iodinated oPRL (Fig. 3). The hormone:ECD complex was clearly separated from its components. Its molecular mass was calculated using bovine serum albumin (67 kDa), ovalbumin (43 kDa) and oPRL (23 kDa) as markers. At a 1:1 ratio of rbPRLR-ECD:oPRL, over 90% of the applied protein appeared as a complex with a molecular mass of ca. 50 kDa, consistent with the predicted value. Raising the rbPRLR-ECD:oPRL ratio to 5:1 did not change the MW of the complex, but the size of the 27 kDa peak, consisting of excess rbPRLR-ECD, was gradually increased.

Because of the limited amount of purified protein higher rbPRLR-ECD:oPRL or rbPRLR-ECD:hGH ratios could be obtained only by using [^{125}I]oPRL or [^{125}I]hGH as ligands and detecting the complex or the excess of the labelled hormone by measuring radioactivity. The elution profile of the complex did not change at increased rbPRLR-ECD:labelled hormone ratios (Fig. 4A and B), and the radioactive peak eluted out in the 43–52 kDa range. Excess of the rbPRLR-ECD could not be seen, but at the 1:1 ratios over 85% of the radioactivity already appeared in the complex. We also

tested the interaction of [^{125}I]hGH with hGHR-ECD (amino acids 1–246), to validate our results (Fig. 4C). Increasing the hGHR-ECD:[^{125}I]hGH ratio clearly shifted the peak of the complex to higher MWs, indicating a shift from the formation of 1:1 to 2:1 hGHR-ECD:[^{125}I]hGH complexes. It should be noted that even at 1:0.5 and 1:1 ratios, simultaneous formation of 2:1 and 1:1 complexes was observed.

4. DISCUSSION

Over 90% pure rbPRLR-ECD was obtained using a two-step protocol consisting of affinity chromatography on oPRL-Affigel and gel filtration. Inclusion of 10 mM MgCl_2 in the conditioned medium applied to the affinity column and the use of siliconized polypropylene tubes significantly contributed to binding efficiency and prevented non-specific absorption of the eluted rbPRLR-ECD to the collection tubes. Overall yield was 25% (Table I). The apparent value of the rbPRLR-ECD concentration in the conditioned medium (Table I) was ~10-fold lower than that reported previously [12], for two reasons: (i) the cells were grown in a serum-free medium that indeed yields less rbPRLR-ECD; (ii) the present value was determined from the specific binding, which is known to underestimate the amount of recep-

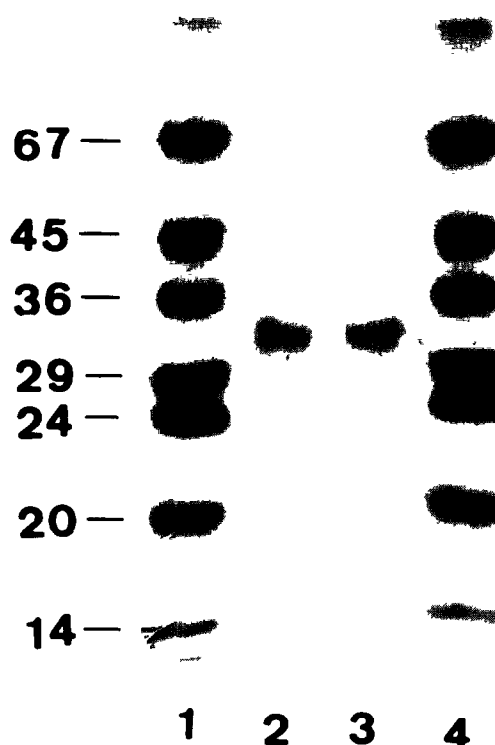


Fig. 2. Electrophoretic patterns of purified rbPRLR-ECD. SDS-PAGE (10%) was carried out in absence (lane 2) or presence (lane 3) of β -mercaptoethanol. Lanes 1 and 4 show protein molecular weight markers. The gel was stained with Coomassie brilliant blue.

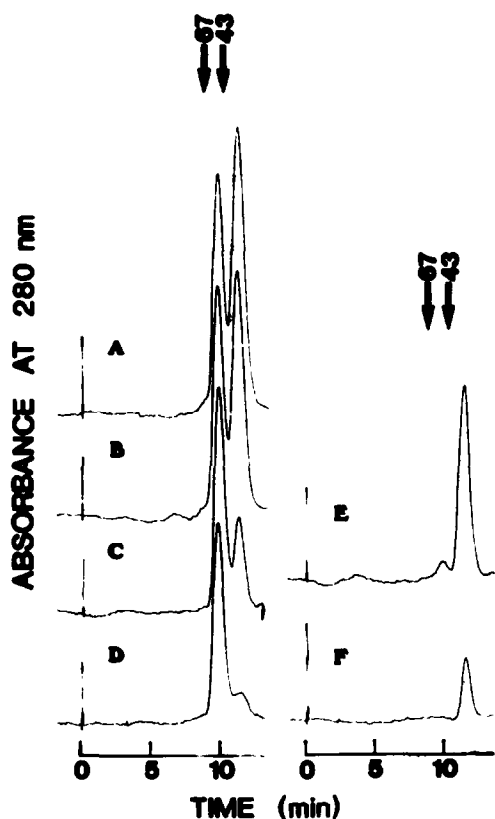


Fig. 3. Gel filtration of rbPRLR-ECD:oPRL complexes on a Superdex-75HR 10/30 column. Complex formation was carried out during 30 min of incubation at room temperature, using the following rbPRLR-ECD:oPRL ratios: (A) 5:1; (B) 4:1; (C) 3:1; (D) 2:1; (E) 1:0; (F) 0:1. Aliquots (200 μ l) of the incubation mixture were applied to the column and complex formation was monitored by absorbance at 280 nm. For more details see text.

tor, as compared to a Scatchard plot, because the binding of the ligand is carried out at non-saturating concentrations of the latter. The apparent molecular mass of the purified monomeric fraction as determined by SDS-PAGE was 32 kDa, while the value obtained from gel filtration was only 27 kDa. Both values were higher than the 22.8 kDa deduced from the cDNA sequence, indicating extensive glycosylation. The affinity constant of the purified rbPRLR-ECD for oPRL was 5–10 times higher than the membrane-inserted, truncated or full-size receptor [12]. It was, however, similar to the non-purified ECD in insect cells conditioned medium, indicating that increased affinity is an intrinsic property of this protein.

Interaction of the purified rbPRLR-ECD with oPRL, [125 I]oPRL or [125 I]hGH clearly indicated that, even at a 20:1 rbPRLR-ECD:hormone molar ratio, only a 1:1 complex is formed. These results differ from the 2:1 (hGHR-ECD:hGH) stoichiometry of interaction reported recently for the ECD of hGHR (hGH binding protein) 1–238 [5,6] or 1–246 [7], both expressed and purified from *E. coli*. However, it should be emphasized

that the rbPRLR-ECD expressed in insect cells is truncated at its C-terminus by 12 amino acids and is heavily glycosylated [12]. Whether these differences could account for its inability to form 2:1 complexes is not presently known. It should be noted, however, that truncated of eight C-terminal amino acids in the non-glycosylated hGHR-ECD did not affect binding [17], and enabled hormone-induced receptor dimerization [5,6], although the sequence of dimerization was slightly different as compared to its non-truncated analogue ([7] and Fig. 4C). In addition, neither we (Sakal and Gertler, unpublished data) nor others (Postel-Vinay and Kelly, personal communication) have been successful in documenting the formation of 2:1 complexes between glycosylated hGH binding protein from human serum and hGH.

In view of these results, the role of glycosylation and

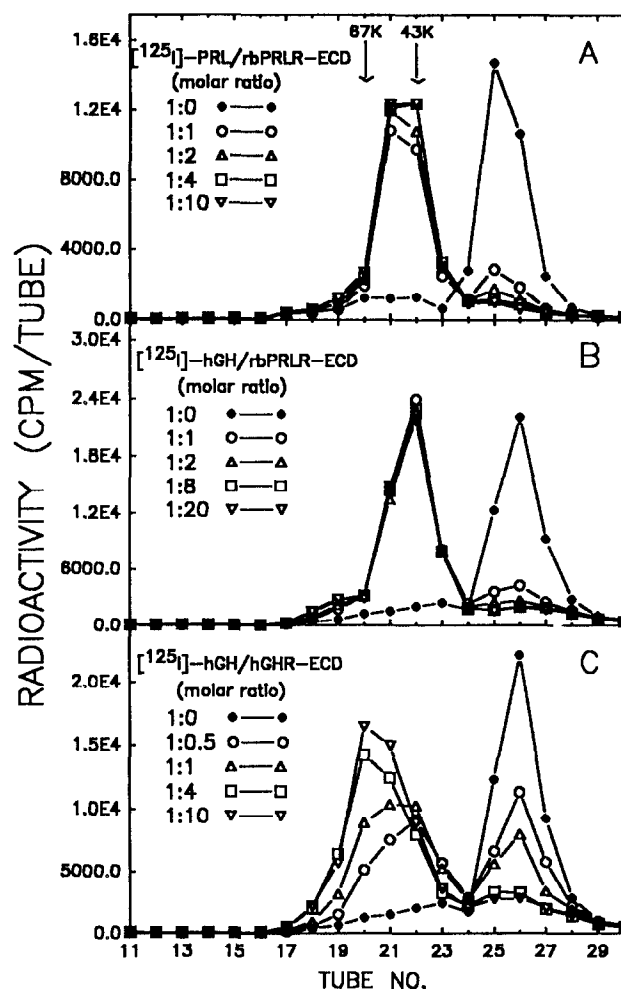


Fig. 4. Gel filtration of [125 I]oPRL:rbPRLR-ECD (A), [125 I]hGH:rbPRLR-ECD (B) or [125 I]hGH:hGHR-ECD (C) complexes on a Superdex-75HR 10/30 column. Complex formation was carried out during 16 h of incubation at room temperature, at various ECD:hormone ratios. Aliquots (200 μ l) of the incubation mixture were applied to the column and 0.5 ml fractions were collected and monitored for radioactivity. For more details see text.

of the 12 C-terminal amino acids of the rbPRLR-ECD in the putative formation of the 2:1 rbPRLR-ECD:hormone complex should be reexamined. Since low amounts of the purified rbPRLR-ECD do not allow efficient deglycosylation experiments, this aim could be achieved through inspection of *E. coli*-expressed, non-glycosylated, full-size and truncated rbPRLR-ECDs. Both constructs are currently being prepared in our lab.

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