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Preparation of the Extracellular Domain of the Rabbit Prolactin Receptor Expressed in *Escherichia coli* and Its Interaction with Lactogenic Hormones*

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The cDNA of the extracellular domain of the rabbit prolactin receptor (rbPRLR-ECD) was cloned in the prokaryotic expression vector pTrc99A to enable its expression in *Escherichia coli* after induction with isopropyl-1-thio-β-D-galactopyranoside. The bacterially expressed rbPRLR-ECD protein, contained within the refractile body pellet, was solubilized in 4.5 M urea, refolded, and purified on a Q-Sepharose column by stepwise elution with NaCl. The bioactive monomeric fraction was eluted in 0.05 M NaCl, yielding 15–20 mg/8 liters of induced culture. The purified protein was >98% homogeneous, as shown by SDS-polyacrylamide gel electrophoresis in the presence or absence of reducing agent and by chromatography on a Superdex column. Its molecular mass was 25 kDa as determined by SDS-polyacrylamide gel electrophoresis in the absence of reducing agent and 22 kDa as determined by gel filtration. Binding experiments revealed remarkable differences between rabbit and porcine prolactins (PRLs) and the other tested lactogenic hormones. Gel filtration was used to determine the stoichiometry of the rbPRLR-ECD interaction with ovine, rabbit, and porcine PRLs, with human growth hormone and its truncated des-7 analogue, and with bovine placental lactogen (bPL) and des-13-bPL. The formation of only 1:1 complexes was indicated, except with bPL, for which a 2:1 complex was detected. Identical stoichiometry was also obtained using excess radiolabeled rbPRLR-ECD in gel filtration experiments. Interaction of ^{125}I-labeled ovine PRL with rbPRLR-ECD secreted into conditioned medium by rbPRLR-ECD cDNA-transfected COS 7 cells also indicated formation of 1:1 molar complexes. Despite the differences in binding potency and stoichiometries of the interaction with rbPRLR-ECD, all seven tested hormones were biologically active in inducing PRL receptor-mediated casein synthesis in explants of rabbit mammary gland. We therefore propose that the formation of the 1:2 complexes with soluble rbPRLR-ECD is not predictive of biological activity of the different lactogenic hormones.

Several studies using anti-prolactin receptor (PRLR)[1] antibodies and their Fab fragments have indirectly documented receptor dimerization as an initial step in PRL signal transduction subsequent to hormone binding (1–4). More recently, hGH-induced dimerization of the recombinant nonglycosylated human growth hormone receptor extracellular domain (hGHR-ECD) containing amino acids 1–238 has been documented in gel filtration and other studies (5). Crystallographic analysis of hGH and its complex with hGHR-ECD confirmed this finding, and two nonsymmetrical binding sites in hGH were identified (6). A similar 2:1 stoichiometry of the hGHR-ECD/hGH interaction was also observed in our laboratory using full-length (amino acids 1–246) hGHR-ECD (7). A recently published report has suggested that antibody-induced receptor dimerization initiates the mitogenic signal in a leukemia cell line expressing a hybrid receptor composed of hGHR-ECD linked to the transmembrane and intracellular domains of the murine granulocyte colony-stimulating factor receptor (8). The Fab fragments were, however, devoid of mitogenic activity (8).

Since the granulocyte colony-stimulating factor receptor also belongs to the family of cytokine/GH/PRL receptors (9), ligand-induced receptor dimerization was suggested to be common to this entire family (8). Another recent report showing that the hGH-induced mitogenic response of an Nb_2 rat lymphoma cell line that possesses PRLRs exhibits a bell-shaped curve and that, at very high hormone concentrations, the mitogenic response is attenuated was interpreted as indirect evidence that the lactogenic receptor is also dimerized by a single hormone molecule (10).

Since the ability of hormone-induced dimerization of hGHR-ECD was suggested as direct evidence for dimerization of the full-size receptor and subsequent initiation of signal transduction (5–8, 10), we elected to study whether hormone-induced receptor dimerization also occurs in the PRLR-ECD/PRL interaction. We have recently produced and expressed rbPRLR-ECD using an insect/baculovirus expression system (11). The expressed protein was purified by affinity chromatography on immobilized PRL, followed by gel filtration. The purified protein was >90% homogeneous as indicated by SDS-PAGE in the presence or absence of reducing agent and by gel filtration on a Superdex column under nondenaturing conditions (12). The

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[1] The abbreviations used are: PRLR, prolactin receptor; PRL, prolactin; hGH, human growth hormone; hGHR-ECD, human growth hormone receptor extracellular domain; rbPRLR-ECD, rabbit prolactin receptor extracellular domain; bPL, bovine placental lactogen; rbPRL, rabbit prolactin; oPRL, ovine prolactin: PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; IPTG, isopropyl-1-thio-β-D-galactopyranoside; HPLC, high pressure liquid chromatography; RT, retention time.

stoichiometry of its interaction with oPRL or hGH studied by gel filtration indicated formation of only 1:1 complexes, even at high rbPRLR-ECD:hormone molar ratios, in contrast to hGHR-ECD, which formed 2:1 complexes with hGH under identical experimental conditions (12). Since rbPRLR-ECD expressed in insect cells is truncated at its COOH terminus by 12 amino acids and is heavily glycosylated (11, 12), we wondered to what extent these differences account for its inability to form 2:1 complexes (12). To answer this question, another variant of rbPRLR-ECD expressed in *Escherichia coli* was prepared. This variant, composed of amino acids 4–210, contains the full COOH-terminal sequence and is nonglycosylated. Its preparation, purification, and characterization are the subject of this paper.

EXPERIMENTAL PROCEDURES

Materials—Recombinant bovine placental lactogen (bPL) and its truncated des-13 analogue were prepared as described previously (13). Recombinant hGH was obtained from Biotechnology General Inc., and its truncated analogue, des-7-hGH, was prepared as described previously (14). Ovine PRL (NIDKK AFP-8277E) and porcine PRL (USDA pPRL-B-1) were from the National Hormone and Pituitary Program (Bethesda, MD), and rabbit PRL (AFP-7730B) was from Dr. A. F. Parlow (Harbor-UCLA Medical Center). Monoclonal antibodies (mAbs) M110 and A917 were prepared as described previously (2). Carrier-free $Na^{125}I$ was purchased from DuPont NEN. Molecular weight markers for gel electrophoresis, RPMI 1640 medium, Dulbecco's modified Eagle's/Ham's F-12 medium, lysozyme, and bovine serum albumin (radioimmunoassay-grade) were obtained from Sigma. SDS-PAGE reagents and the protein assay kit were purchased from Bio-Rad. The Superdex™ 75 HR 10 column and Q-Sepharose (fast flow) were purchased from Pharmacia LKB Biotechnology AB (Uppsala). All other chemicals were of analytical grade.

Construction of rbPRLR-ECD Expression Vector—The cDNA of rbPRLR-ECD was obtained by polymerase chain reaction using 100 pg of the cloned rbPRLR cDNA (15) as template in a 25-cycle amplification. The cycling program was one cycle of 10 min at 94 °C, 0.5 min at 45 °C, and 1 min at 72 °C, followed by 24 cycles of 1 min at 94 °C, 0.5 min at 45 °C, and 1 min at 72 °C and by one cycle of 1 min at 94 °C, 0.5 min at 45 °C, and 10 min at 72 °C. The 5′-GGGGCCATGGGAAAACCTTTCATCTTCAAA and the 3′-GGGGTCTAGATCAATCTTTCATGGTAAAGT primers contained an *Nco*I site and a *Xba*I site, respectively. The 3′-primer also encodes an in-frame stop codon (TCA), just before the *Xba*I site. The fragment amplified between these primers encodes a polypeptide beginning at Gly^{29}, *i.e.* the fifth amino acid downstream from the putative signal peptide cleavage site, and extending to Asp^{235}, the last amino acid before the transmembrane domain. The polymerase chain reaction product was inserted between the *Nco*I and *Xba*I sites of prokaryotic expression vector pTrc99A (16). This construct was transfected into *E. coli* strain W3110, and isolated colonies expressed the unfused protein after induction by isopropyl-1-thio-β-D-galactopyranoside (IPTG). The bacterial clone expressing the highest level of rbPRLR-ECD was selected (clone W5) and used in all the expression experiments described in this paper. The W5 insert was sequenced and revealed no errors introduced by *Taq* polymerase.

Expression, Refolding, and Purification of rbPRLR-ECD—The transfected W5 *E. coli* cells (250 ml) were grown in LB medium (17) at 37 °C in 2-liter flasks to an A_{600} of 0.9. IPTG was then added to 1 mM; the cells were grown for an additional 4 h, pelleted for 10 min at 16,000 × *g*, and stored at −20 °C. IPTG did not interfere with cell growth, and the final yield was 6–7 g of wet precipitate/1 liter of culture. Frozen precipitate (50 g) was thawed and resuspended in cold 10 mM EDTA, 10 mM Tris-HCl, pH 8.0, in the presence of 0.5 mg/ml lysozyme. Following a 30-min incubation at 4 °C with occasional mixing, the cells were sonicated and pelleted for 30 min at 25,000 × *g*. The pellet containing the rbPRLR-ECD protein was sonicated in distilled water, pelleted twice, and solubilized in 1.5 liter of 4.5 M urea buffered with 40 mM Tris base. The pH was increased to 11.3 with NaOH, cysteine was added to 0.1 mM, and the clear supernatant was stirred at 4 °C. In cases where not all the material was solubilized, the suspension was centrifuged (10 min at 16,000 × *g*) prior to stirring. SDS-PAGE (data not shown) revealed that under these conditions, all rbPRLR-ECD was solubilized. After 1 h of stirring, the solution was diluted with 2 volumes of ice-cold water and dialyzed for 36 h against 4 × 20 liters of 10 mM Tris-HCl, pH 8.6. The solution was then loaded at 200 ml/h onto a Q-Sepharose column (2.6 × 7 cm) pre-equilibrated with 10 mM Tris-HCl, pH 8.6, at 4 °C. Elution was carried out using a discontinuous NaCl gradient in the same buffer at a rate of 100 ml/h, and 5-ml fractions were collected. Protein concentration was determined by absorbance at 280 nm, and monomer content was determined by gel filtration chromatography on a Superdex 75 column.

SDS-Polyacrylamide Gel Electrophoresis—SDS-PAGE was carried out according to Laemmli (18) using 12 or 15% gels. Gels were stained with Coomassie Blue R-250.

Binding Assays—The binding assays were carried out in 25 mM Tris-HCl, pH 8.0, supplemented with 10 mM $MgCl_2$ and 0.1% (w/v) bovine serum albumin (TMBA buffer) as described previously using goat anti-rbPRLR polyclonal antibody 46 for immunoprecipitation of the ECD-hormone complex (11). ^{125}I-oPRL was added at 0.05 pmol/tube. Iodination of oPRL and rbPRLR-ECD was performed as described previously (19).

Determination of Monomer Content and Complex Formation—High pressure liquid gel filtration chromatography was performed on 200-μl aliquots of Q-Sepharose column-eluted fractions, freeze-dried samples dissolved in H_2O (0.4–0.5 mg/ml), or complexes between purified rbPRLR-ECD and various hormones in a Merck Hitachi HPLC apparatus equipped with a D-2000 integrator and an L-6200 controller. A Superdex 75 HR 10/30 column pre-equilibrated with 25 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 10 mM $MgCl_2$ (TNM buffer) was developed with the same buffer at a rate of 1.0 ml/min at room temperature. Protein content was monitored by absorbance at 280 or 214 nm and quantified using the D-2000 integrator. Absorbance at 214 nm was preferred for monitoring complex formation to minimize the difference in molar absorbance at 280 nm between the tested hormones and purified rbPRLR-ECD resulting from different Trp contents. The molar extinction coefficient of rbPRLR-ECD at 280 nm was calculated using the molar extinction coefficients of tyrosine (1197) and tryptophan (5559), yielding a value of 63,200. The interaction between ^{125}I-rbPRLR-ECD and various hormones was determined in TMBA buffer. The concentration of each of the hormones was 0.05 pmol/ml, and the concentration of ^{125}I-rbPRLR-ECD was 0.0–0.3 pmol/ml. After a 16-h incubation at room temperature, 200-μl aliquots were applied to the Superdex column, which had been pre-equilibrated with the incubation buffer and connected to the HPLC apparatus. The column was eluted at 1.0 ml/min at room temperature, and 0.5-ml fractions were collected and monitored for radioactivity in a Kontron γ-counter.

Determination of Amino-terminal Sequence—Automated Edman degradation was used to determine the amino-terminal protein sequence. Degradations were performed on an Applied Biosystems Model 470A gas-phase sequenator (Foster City, CA) using the standard sequencing cycle (20). The respective phenylthiohydantoin-derivatives were identified by reversed-phase HPLC analyses using an Applied Biosystems Model 120A phenylthiohydantoin analyzer fitted with a Brownlee 2.1-mm inner diameter phenylthiohydantoin C_{18} column.

Casein Synthesis in Rabbit Mammary Gland Explants—Explant cultures of mammary tissue from 12–14-day pseudopregnant rabbits and measurements of casein content were performed as previously described (21).

Preparation of Conditioned Medium from COS 7 Cells—COS 7 cells were transiently transfected with the plasmid pE encoding for rbPRLR-ECD inserted in the PECE expression vector as described previously.[2] The cells were then cultured in serum-free medium for 3 days, after which the medium was concentrated by removing proteins with M_r values <10,000 and used for binding studies.

Statistical Analysis—One-way analyses of variance were performed. When the F values were significant ($p < 0.05$), means of the various treatments were compared using Duncan's multiple range test. All parametric data are expressed as mean ± S.E.

RESULTS

Expression of rbPRLR-ECD in E. coli—The production of rbPRLR-ECD from plasmid pTrc99A after induction of *E. coli* W3110 culture by IPTG is shown in Fig. 1. rbPRLR-ECD accumulated for up to 4–8 h post-induction, at which stage the recombinant protein constituted ~70% of the total insoluble cellular protein as determined by densitometric scanning of the Coomassie Blue-stained gel. The recombinant rbPRLR-ECD protein, detected by SDS-PAGE, was found only in the non-soluble refractile bodies (Fig. 1, *lanes 3–5*) and not in the cell

[2] Lesueur, L., Edery, M., Paly, J., Kelly, P. A., and Djiane, J. (1994) *J. Mol. Endocrinol.*, in press.

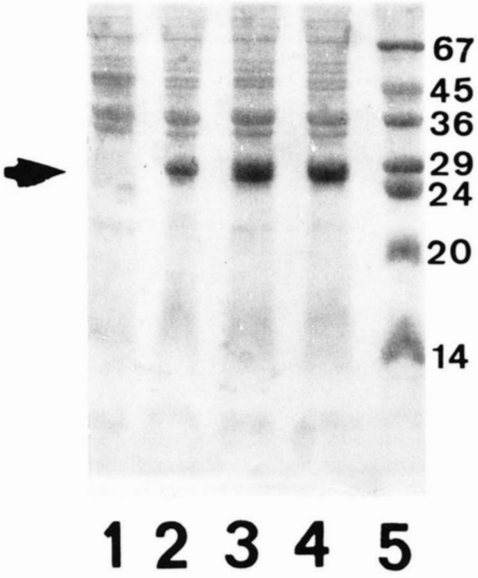

FIG. 1. **Electrophoretic analysis of insoluble fraction prepared from *E. coli* before (*lane 1*) and 2, 4, and 8 h after (*lanes 2–4*, respectively) induction with IPTG on 15% SDS-polyacrylamide gel in presence of β-mercaptoethanol.** *Lane 6*, molecular weight markers. The gel was stained with Coomassie Brilliant Blue R-250.

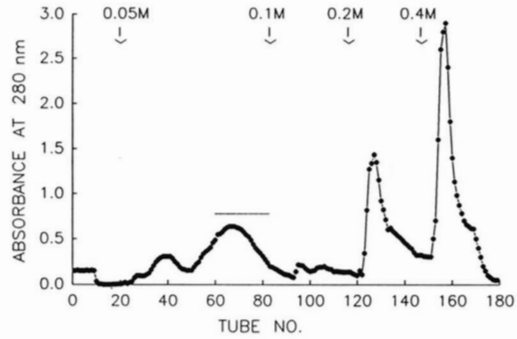

FIG. 2. **Purification of proteins extracted from refractile bodies on Q-Sepharose column.** The column (2.5 × 7 cm) was equilibrated with 10 mM Tris-HCl, pH 8.6, at 4 °C. The fraction containing refractile body proteins solubilized in 4.5 M urea in 40 mM Tris-HCl at pH 11.3 (4500 ml) was applied to the column at a rate of 200 ml/h. The column was then washed with 80 ml of 10 mM Tris-HCl, pH 8.6. The eluate was not collected. Elution was carried out using a discontinuous NaCl gradient in the same buffer at 100 ml/h, and 5-ml fractions were collected. The protein concentration was determined by absorbance at 280 nm. The purification procedure was performed four times, yielding almost identical results.

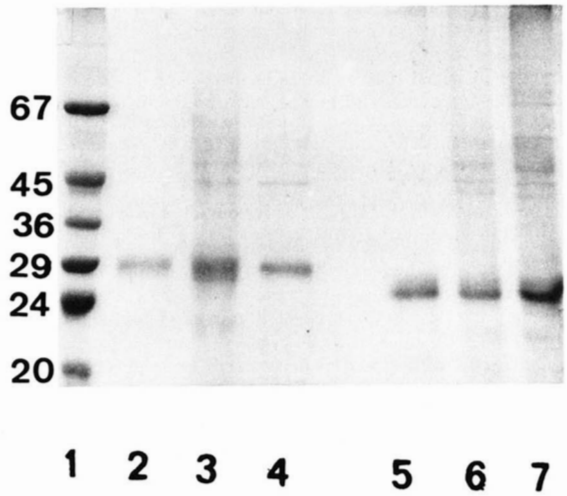

FIG. 3. **Electrophoretic analysis of fractions eluted from Q-Sepharose column on 12% SDS-polyacrylamide gel with (*lanes 2–4*) or without (*lanes 5–7*) β-mercaptoethanol.** *Lane 1*, protein molecular weight markers; *lanes 2* and *5*, 0.05 M NaCl eluate; *lanes 4* and *6*, 0.2 M NaCl eluate; *lanes 3* and *7*, 0.4 M NaCl eluate. The gel was stained with Coomassie Brilliant Blue R-250.

extract supernatant (data not shown). However, binding assays revealed small amounts of active rbPRLR-ECD in the supernatant as well.

Purification and Characterization of E. coli-expressed rbPRLR-ECD—rbPRLR-ECD within the refractile body pellet was solubilized in 4.5 M urea, refolded for 1 h at pH 11.3 in the presence of catalytic amounts of cysteine, and purified on a Q-Sepharose column by stepwise NaCl elution (Fig. 2). In preliminary experiments (data not shown), longer refolding periods (5–24 h) gradually diminished the amount of the monomeric ECD, yielding oligomers formed through disulfide bonds instead. The main fraction, which eluted at 0.05 M NaCl and contained monomeric rbPRLR-ECD (Fig. 2, tubes 60–80), was pooled, dialyzed against 0.05% $NaHCO_3$, and freeze-dried. The yield from ~8 liters of *E. coli* culture varied between 15 and 20 mg of pure monomeric protein. SDS-PAGE under both reducing and nonreducing conditions (Fig. 3, *lanes 2* and *5*, respectively) yielded a single band with a molecular mass of 25 kDa. The molecular mass of purified rbPRLR-ECD determined under nondenaturing conditions by gel filtration on a Superdex column was 22 kDa (see Fig. 5).

Amino-terminal sequence analysis of 5 amino acids resulted in the expected sequence (15), namely Gly-Lys-Pro-Phe-Ile, with respective yields of 30, 11, 19, 9, and 10 pmol/cycle. A trace of Met (1.2 pmol) was found in the first cycle, indicating that >97% of rbPRLR-ECD had been processed in the *E. coli* cells following its translation.

Fractions eluted from the Q-Sepharose column at 0.2 and 0.4 M NaCl consisted of >75% of the total protein loaded onto the column and were composed of mainly dimers and oligomers, respectively. They were composed of rbPRLR-ECD as documented by SDS-PAGE: both fractions yielded almost pure monomers under both reducing and nonreducing conditions (Fig. 3, *lanes 3* and *4* and *lanes 6* and *7*, respectively). After dialysis and freeze-drying, both fractions produced yellowish powder, in contrast to the 0.05 M eluate, which was white. Thus, oligomerization seems to originate probably from interaction with an unknown *E. coli*-derived pigment.

Binding Experiments—Displacement curves for binding of ^{125}I-oPRL to purified rbPRLR-ECD are presented in Fig. 4*A*. The abilities of oPRL, hGH, des-7-hGH, and bPL to compete with the radioligand were almost identical, yielding IC_{50} values of 0.12–0.14 nM, whereas the IC_{50} for des-13-bPL was slightly higher. Scatchard analysis based on the data presented in Fig. 4*A* with the homologous hormone (oPRL) yielded a linear plot (data not shown). The derived K_a value (25 ± 6 nM^{-1}; mean ± S.E., n = 2) was close to that for purified rbPRLR-ECD prepared in the insect/baculovirus expression system (11, 12). However, rabbit and porcine PRLs exhibited remarkably decreased abilities to compete with the ligand, with IC_{50} values 1000-fold higher than that for oPRL. Binding of ^{125}I-oPRL to rbPRLR-ECD was efficiently inhibited by mAb M110, but not by mAb A917 (Fig. 4*B*).

Interaction with Lactogenic Hormones—The stoichiometry of the interaction between purified rbPRLR-ECD and oPRL was studied by gel filtration using noniodinated hormones (Fig. 5). The rbPRLR-ECD·oPRL complex (Fig. 5*A*), which was eluted at a retention time (RT) of 11.41–11.44 min, was clearly separated from the other components. Its molecular mass was calculated using bovine serum albumin (67 kDa; RT = 10.52 min), ovalbumin (43 kDa; RT = 11.41 min), oPRL (23 kDa; RT = 12.62

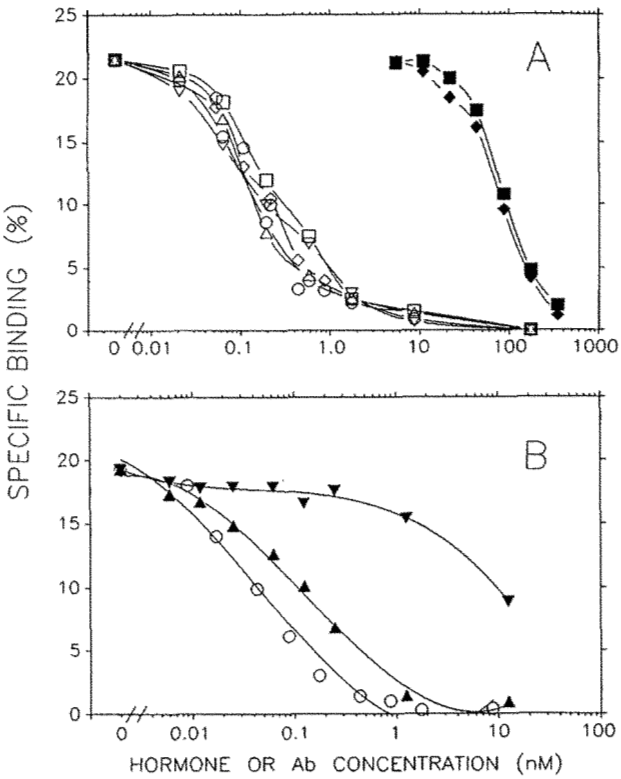

FIG. 4. *A*, competition of unlabeled oPRL (○), hGH (△), bPL (▽), des-13-bPL (□), des-7-hGH (◇), rbPRL (■), and porcine PRL (◆) with the binding of ^{125}I-oPRL to rbPRLR-ECD; *B*, competition of unlabeled oPRL (○), mAb M110 (▲), and mAb A917 (▼) with the binding of ^{125}I-oPRL to rbPRLR-ECD. The total binding was 30% in *A* and 28.5% in *B*. The nonspecific binding was 9% in both cases. Full lines were calculated using the Sigma plot curve-fitting program, and the symbols represent actual results. Shown are representative results of three (*A*) or two (*B*) experiments.

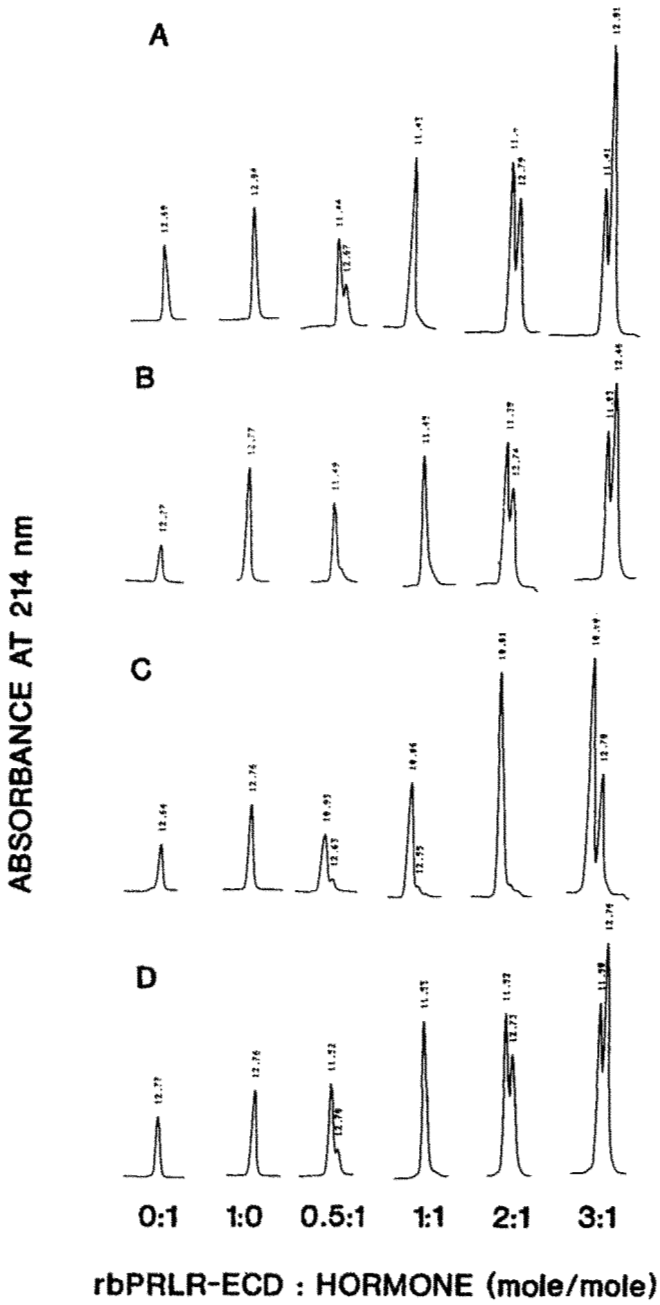

FIG. 5. **Gel filtration of rbPRLR-ECD·oPRL (*A*), rbPRLR-ECD·hGH (*B*), rbPRLR-ECD·bPL (*C*), and rbPRLR-ECD·des-13-bPL (*D*) complexes on Superdex 75 HR 10/30 column.** Complex formation was carried out during 30–60 min of incubation at room temperature in TNM buffer using the various rbPRLR-ECD:hormone ratios. Aliquots (200 μl) of the incubation mixture were applied to the column pre-equilibrated with the same buffer. Complex formation was monitored by absorbance at 214 nm. The column was developed at 1 ml/min. Each experiment was conducted at least three times. For further details, see text.

min), and hGH (22 kDa; RT = 12.77 min) as markers. At a 1:1 ratio of rbPRLR-ECD to oPRL, >90% of the applied protein appeared as a complex with a molecular mass of ~44 kDa, consistent with the predicted value. Raising the rbPRLR-ECD: oPRL ratio to 2:1 or 3:1 did not change the molecular mass of the complex, but the size of the 22-kDa peak (RT = 12.79–12.81 min), consisting of excess rbPRLR-ECD, gradually increased. Similar elution profiles and stoichiometries of interaction were also observed with hGH (Fig. 5*B*), des-13-bPL (Fig. 5*D*), rbPRL (Fig. 6), and des-7-hGH and porcine PRL (data not shown). The stoichiometry of interaction of rbPRLR-ECD with bPL was, however, quite different (Fig. 5*C*). At a molar ratio of 1:1, the main peak appeared at RT = 10.86 min, and a small peak at RT = 12.55 min was also seen. At a molar ratio of 2:1, only one peak (RT = 10.81 min) was found. Excess rbPRLR-ECD was only seen at a molar ratio of 3:1. The RT of 10.81 corresponds to a molecular mass of 56 kDa.

In view of the possible effect of Zn^{2+} on the binding of certain hormones to PRLR (23), the interaction between oPRL and rbPRLR-ECD was also studied in TNM buffer supplemented with 200 μM $ZnCl_2$. The addition of $ZnCl_2$ changed neither the RT nor the stoichiometry of the ECD-hormone complex (data not shown). However, prolonged incubation in the presence of 200 μM $ZnCl_2$ resulted in dimerization of rbPRLR-ECD in the absence of hormone. The stoichiometry of complex formation between oPRL and rbPRLR-ECD was also not influenced by the removal of $MgCl_2$, as documented by gel filtration in sodium phosphate buffer (data not shown).

To validate the differences between bPL and the other hormones, purified rbPRLR-ECD was iodinated, and complex formation between ^{125}I-rbPRLR-ECD and various hormones was monitored by gel filtration on a Superdex column following overnight preincubation in TMBA buffer. The ^{125}I-rbPRLR-ECD: hormone ratios were chosen to ensure at least 4–6 molar excess of the latter. As shown in Fig. 7, the peaks of the complexes between the ligand and oPRL, hGH, and des-13-bPL appeared in tube 23, corresponding to a molecular mass of 45 kDa, whereas the peak of the ^{125}I-rbPRLR-ECD·bPL complex was shifted to tube 22, corresponding to a molecular mass of 57 kDa. Integration of the total counts eluted in tubes 21–24 re-

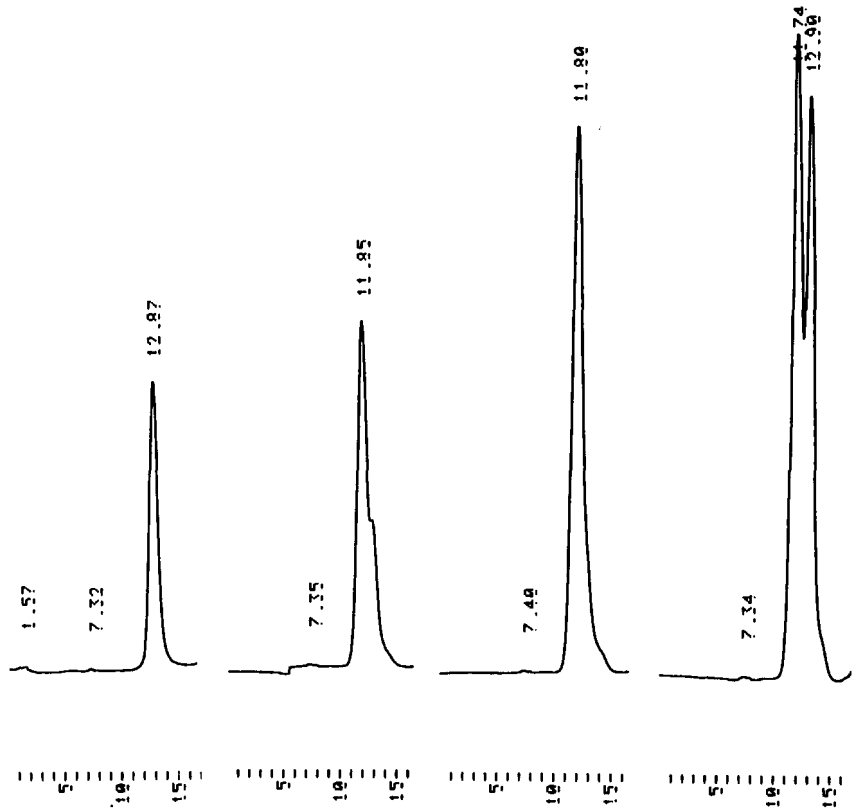

FIG. 6. **Gel filtration of rbPRLR-ECD·rbPRL complexes on Superdex 75 HR 10/30 column.** The rbPRLR-ECD:rbPRL ratios were (from *left* to *right*) 0:1, 0.5:1, 1:1, and 2:1. The retention times for bovine albumin (67 kDa) and ovalbumin (43 kDa) in this experiment were 10.61 and 11.49 min, respectively. The experiment was repeated twice. For further details, see the legend to Fig. 5.

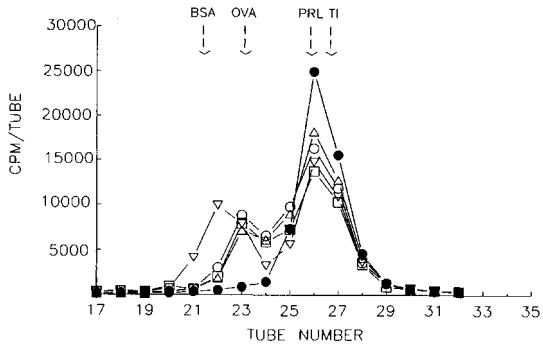

FIG. 7. **Gel filtration of ^{125}I-rbPRLR-ECD·oPRL (○), ^{125}I-rbPRLR-ECD·hGH (△), ^{125}I-rbPRLR-ECD·bPL (▽), and ^{125}I-rbPRLR-ECD·des-13-bPL (□) complexes or ^{125}I-rbPRLR-ECD (●) on Superdex 75 HR 10/30 column.** Complex formation was carried out during 16 h of incubation in TMBA buffer at room temperature with excess of rbPRLR-ECD. Aliquots (200 µl) of the incubation mixture were applied to the column pre-equilibrated with TNM buffer. The column was developed at 1 ml/min and 0.5-ml fractions were collected and monitored for radioactivity. The experiment was repeated two times, yielding almost identical results. For further details, see text. *BSA*, bovine serum albumin; *OVA*, ovalbumin; *TI*, trypsin inhibitor.

vealed that the net value (calculated as the difference between a sample of the complex and a sample containing the labeled ligand only) of the ^{125}I-rbPRLR-ECD·bPL complex was 60–70% higher (22,500 cpm) than that obtained with oPRL (14,000 cpm) hGH (13,500 cpm), or des-7-hGH (13,200 cpm).

Biological Activity of Lactogenic Hormones—The biological activity of the tested hormones mediated through membrane-embedded rbPRLR was determined by measuring α-casein synthesis in mammary gland explants from pseudopregnant rabbits. All hormones significantly stimulated α-casein synthesis above control levels ($p > 0.05$) (Fig. 8). Ovine PRL was more potent than the other hormones. Des-7-hGH, des-13-bPL, and rbPRL were slightly less active, but the differences at each hormone level were not statistically significant ($p > 0.05$).

Interaction of ^{125}I-oPRL with rbPRLR-ECD Secreted into Conditioned Medium by Transfected COS 7 Cells—To establish the molecular mass of the glycosylated rbPRLR-ECD·oPRL complex obtained from the conditioned medium of COS 7 cell cultures, fixed amounts of ^{125}I-oPRL and increasing amounts of conditioned medium in TMBA buffer were incubated overnight at room temperature. The extent of specific binding and the molecular mass of the complex were then monitored by gel filtration on a Superdex column (Fig. 9). Specific binding was calculated by dividing the sum of the counts/minute in tubes 21–24 by the total amount of counts in tubes 21–28. Maximal specific binding (79%) was obtained at 80–120 µl of conditioned medium, and half-maximal specific binding at 12 µl of conditioned medium (Fig. 9, *inset*). The elution profile was, however, similar over all concentration ranges, and the molecular mass of the complex corresponded to 60–64 kDa. Glycosylated rbPRLR-ECD has a molecular mass of 38 kDa as determined by SDS-PAGE followed by Western blotting,[3] indicating 1:1 complex formation.

DISCUSSION

Rabbit, rat, and bovine PRLR-ECDs possess 5 cysteine residues that form two S-S bridges (positions 12–22 and 51–62), leaving 1 cysteine (position 184) free (15, 24, 25). An unpaired number of cysteine residues is well-known to have an adverse effect on proper refolding, leading to formation of oligomers (26). To overcome this difficulty, we have utilized a short-time exposure to urea at high pH, which resulted in an optimal refolding of rbPRLR-ECD. It should be noted that in most of the procedures used to refold insoluble recombinant proteins, longer periods of refolding, up to several days, are recommended (27).

[3] C. Bignon, E. Sakal, L. Belair, N. Chapnik-Cohen, J. Djiane, and A. Gertler, unpublished data.

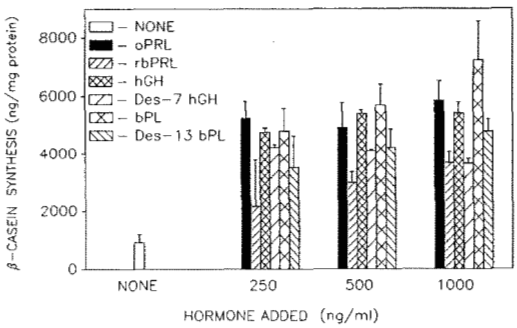

FIG. 8. **Synthesis of β-casein in explants of mammary gland obtained from pseudopregnant rabbits.** Results are mean ± S.E. of two or three experiments, each carried out in duplicate.

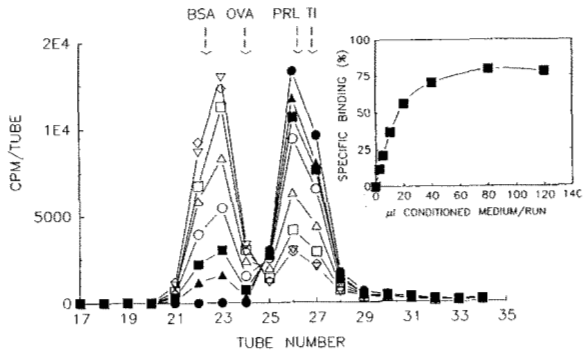

FIG. 9. **Gel filtration of complexes formed between ^{125}I-oPRL and rbPRLR-ECD from conditioned medium of COS 7 cells transfected with rbPRLR-ECD cDNA.** Complex formation was achieved during 16 h of incubation in TMBA buffer at room temperature using a constant amount of ^{125}I-oPRL (32,000 cpm/200 μl) and increasing amounts of conditioned medium (per 200 μl): none (●), 2.5 μl (▲), 5 μl (■), 10 μl (○), 20 μl (△), 40 μl (□), 80 μl (◇), and 120 μl (▽). Aliquots (200 μl) of the incubation mixture were applied to the Superdex 75 HR 10/30 column preequilibrated with TNM buffer. The column was developed at 1 ml/min, and 0.5-ml fractions were collected and monitored for radioactivity. *Inset*, total maximal binding was 80%, and nonspecific binding was 3%; specific binding was calculated from the data. The experiment was repeated two times. For further details, see text. *BSA*, bovine serum albumin; *OVA*, ovalbumin; *TI*, trypsin inhibitor.

The identity of purified rbPRLR-ECD was further verified by amino-terminal sequence analysis. The apparent molecular mass of the purified monomeric fraction as determined by SDS-PAGE was 25 kDa, whereas the value obtained by gel filtration was 22 kDa. Both values are close to the theoretical value of 23,972 Da, and the lower value obtained by gel filtration may result from the unique nonglobular shape of rbPRLR-ECD, assuming its structural similarity to hGHR-ECD (6).

The affinity constant of purified rbPRLR-ECD for oPRL was an order of magnitude higher than those of truncated or full-size membrane-inserted receptors (11) and was similar to that of glycosylated rbPRLR-ECD (amino acids 1–198) expressed in an insect/baculovirus expression system (11, 12). Thus, whereas truncation at the extracellular/membrane boundary has a major effect on the K_a, neither truncation of the 12 carboxyl-terminal amino acids nor glycosylation affects affinity. In binding experiments using ^{125}I-rbPRLR-ECD, both rabbit and porcine PRLs were 1000-fold less effective than oPRL. Since the biological activity of rbPRL in a rabbit mammary gland explant bioassay is only slightly lower (Fig. 8), its binding affinity for the soluble ECD does not serve as a predictive parameter of biological activity. The discrepancy between the binding and bioassays was not related to the experimental conditions of the binding experiment, which included precipitation of the hormone-receptor complex by polyclonal antibody, since gel filtration experiments using rbPRL as a competitor for ^{125}I-oPRL binding to the ECD yielded similar results (data not shown).

Direct interaction studies (Fig. 5) clearly suggested a 2:1 rbPRLR-ECD:bPL stoichiometry, whereas the stoichiometry of the former's interaction with other hormones was 1:1. This conclusion was further substantiated by interacting oPRL, hGH, bPL, and des-13-bPL with radiolabeled rbPRLR-ECD, an experiment in which higher ECD:hormone ratios could be achieved in the preincubation mixture. Since, in both gel filtration experiments, truncation of bPL by 13 NH_2-terminal amino acids changed the interaction stoichiometry from 2:1 to 1:1, we conclude that the NH_2-terminal portion of bPL participates in the second binding site. This conclusion is consistent with results reported for the interaction of hGH with hGHR-ECD (5, 6). It should be remembered that the NH_2-terminal domain of bPL extending beyond the putative first α-helix (6, 28) consists of 19 amino acids, as compared to 5 amino acids in hGH or porcine growth hormone and 14 amino acids in oPRL (29). Therefore, truncation of the 13 NH_2-terminal amino acids in native bPL should not interfere with the integrity of the first α-helix, and indeed, we have found the CD spectra of the truncated analogue and the full-size hormone to be identical (13). It is surprising, however, that the bioactivity of des-13-bPL was only partially affected: it exhibits ~50% less lactogenic receptor-mediated activity in Nb_2 cells (13) and in rabbit mammary gland explants (Fig. 8), whereas its somatogenic receptor-mediated activity is ~2-fold higher in 3T3-F422A rat preadipocytes (13) and in rat hepatocytes.[4]

Our suggestion that, unlike other tested hormones, one molecule of bPL binds two molecules of rbPRLR-ECD was derived not only from the fact that the single peak was obtained at a 2:1 molar ratio (Fig. 5*C*), but also on its elution at an RT of 10.8 min. Other ECD-hormone complexes had RTs of 11.43–11.53 min. Calculation of the molecular mass of the ECD·bPL complex according to a standard curve based on RTs for bovine serum albumin, ovalbumin, oPRL, and hGH yielded a value of 55.6 kDa, rather than the expected value of 65 kDa assumed from the 2:1 stoichiometry. This discrepancy could result from formation of a more compact structure in the complex, as suggested by Fritz *et al.* (30), who found that the trypsin-trypsin inhibitor complex is eluted from a Sephadex G-50 column in a position corresponding to a molecular weight of 26,000 rather than the expected value of 30,500. The 1:1 stoichiometry found in this work is not restricted to rbPRLR-ECD. Recently, we prepared recombinant PRLR-ECD derived from bovine mammary gland (amino acids 1–210) and have found that, like its rabbit counterpart, it also forms 1:1 complexes with either oPRL or hGH.[5]

The ability of hGH to form 1:2 complexes with hGHR-ECD has been suggested to be predictive of the former's biological activity (5, 10). Results presented in this paper lead one to question the extent to which this predictive parameter is applicable to other related hormones. We have therefore compiled some of our results and those of other researchers to evaluate whether biological activity in the respective *in vitro* or *in vivo* model is related to the particular hormone's ability to dimerize the ECD of its corresponding receptor (Table I). Formation of 2:1 ECD-hormone complexes occurs mainly with hGH and bovine GH interacting with somatogenic receptor-derived ECDs. Several PRLs and rabbit or bovine PRLR-ECD form only 1:1 complexes, even when the PRL-dependent PRLR-mediated biological response is well-established. In the case of bPL, occurrence of either 1:1 or 2:1 complexes was found, irrespective of

[4] D. Vashdi and A. Gertler, unpublished data.

[5] A. Gertler, N. R. Staten, A. Tchelet, N. Chapnik-Cohen, and G. G. Krivi, unpublished data.

TABLE I
Compilation of current data on the interaction of hGHR-ECD and PRLR-ECD or binding proteins with lactogenic or somatogenic hormones

ECD or BP[a]	Hormone tested	Stoichiometry of ECD-hormone complex	Biological activity of hormone[b]	Ref.
rbPRLR-ECD (1–198 gp)	oPRL, hGH,	1:1	Yes (L)	12
rbPRLR-ECD (4–210)	oPRL, hGH, rbPRL, pPRL	1:1	Yes (L)	This work
	Des-7-hGH, des-13-bPL	1:1	Yes (L)	This work
	bPL	2:1	Yes (L)	This work
bPRLR-ECD (1–210)	oPRL, hGH,	1:1	Yes (L)	Gertler *et al.*[c]
hGH-BP (1–246)	hGH, bPL	2:1	Yes (S)	7, 32
	Des-7-hGH	1:1	Nil (S)	7, 32
hGH-gpBP	hGH	1:1	? (S)	Sakal and Gertler[d]
hGH-BP (1–238)	hGH	2:1	Yes (S)	5, 22
	Des-7-hGH	1:1	ND	5, 22
bGH-BP	bGH	2:1	Yes (S)	Staten *et al.*[e]
	bPL	1:1	Yes (S)	

[a] BP, binding protein; gp, glycoprotein; pPRL, porcine prolactin; ND, not determined.
[b] Mediated through the actogenic (L) or somatogenic (S) receptor.
[c] A. Gertler, N. R. Staten, A. Tchelet, N. Chapnik-Cohen, and G. G. Krivi, unpublished data.
[d] E. Sakal and A. Gertler, unpublished data.
[e] N. R. Staten, J. C. Byatt, D. M. Laird, and G. G. Krivi, submitted for publication.

full biological activity in the related system. An unexplained observation is that naturally occurring glycosylated hGH-binding protein forms only 1:1 complexes with hGH, in contrast to its recombinant nonglycosylated analogue.

In view of these results, we postulate that the ability or inability of the tested PRLs or bPL to form 1:2 complexes with their respective soluble R-ECDs does not predict their biological activity. This postulate does not preclude, however, the occurrence of hormone- or anti-receptor antibody-induced dimerization of membrane-embedded PRLRs.

Although no conclusive explanation exists for the disparity in the results regarding PRLR-ECD presented here and in a previous paper (12) and in the results reported for hGHR-ECD (5–7), two possible hypotheses may be proposed. (*a*) Formation of the 2:1 hGHR-ECD·hGH complex is facilitated by a double hydrogen bond between the hydroxyl moiety of Tyr^{200} in the first ECD molecule and between Asp^{152} O-δ2 and Ser^{201} O-γ in the second. In rabbit and bovine PRLR-ECDs, Tyr^{200} is substituted by Leu^{170} (15, 25, 31), which is incapable of forming these hydrogen bonds. Lack of these bonds could hamper formation of the 1:2 complex with the soluble ECD, whereas in the membrane-embedded receptor, such a complex could occur because dimerization is stabilized by other interactions within the membrane or cytosolic domains. (*b*) mAb A917 is capable of inhibiting binding of ^{125}I-oPRL to the membrane-embedded receptor, but not soluble rbPRLR-ECD expressed in the insect/baculovirus (11) and *E. coli* (Fig. 4*B*) expression systems, implying that the conformations of the soluble and membrane-embedded ECDs differ. This suggestion is further substantiated by the finding that the soluble ECD exhibits 10-fold higher affinity for oPRL than the membrane-embedded receptor (11). Thus, because of the conformational constraints imposed by the cytosolic domain, the conformation of the free ECD may be such as to prevent heterotrimer formation and thus to be valueless in predicting biological activity based on stoichiometry.

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