Expression of the Extracellular Domain of the Rat Liver Prolactin Receptor and Its Interaction with Ovine Prolactin

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Kyle P. Hooper, R. Padmanabhan, and Kurt E. Ebner‡

From the Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66160-7421

A clone of the extracellular domain of the rat liver prolactin receptor was generated by the RNA-based polymerase chain reaction, and the NH2-terminal 210 amino acids were expressed in HEK cells using a vaccinia virus/ T7 hybrid expression system. The protein was isolated from serum-free culture medium directly by chromatography on an ovine prolactin affinity column and yielded ~1.5 mg of protein/liter of suspension culture. The extracellular domain of the rat prolactin receptor is well defined and is being subjected to mutational analysis (12, 13) to determine its interaction with ovine prolactin.

The extracellular domains of a number of membrane-bound receptors have been generated by mutagenesis and expressed in a soluble form in both eukaryotic and prokaryotic systems (14-21). These soluble binding proteins are readily isolated, bind their protein ligands with KD values comparable to those for the membrane-bound receptor, and are highly suitable for structural and biological studies. For example, studies with the extracellular domain of the human growth hormone receptor have shown that two molecules of the extracellular domain dimerize upon binding of one molecule of human growth hormone both in solution (22) and in the crystallized complex (23). Receptor dimerization has also been demonstrated with the extracellular domains of both the tumor necrosis factor a (24) and epidermal growth factor (15) receptors and may be a general requirement to initiate signal transduction in some systems.

In this study, a clone of the rat liver PRL receptor extracellular domain (PRED) was generated by RNA-based PCR and expressed in HEK cells using a vaccinia virus/ T7 hybrid expression system (25). The protein was readily isolated from serum-free culture medium by oPRL affinity chromatography and has a molecular mass of 38.5 kDa on SDS-PAGE. Purified PRED was very effective in blocking the oPRL-dependent mitogenesis of Nb2 cells and has an IC50 in the picomolar range. The binding of 125I-oPRL to immobilized PRED is of high affinity, and the binding of 125I-PRED to immobilized oPRL exhibited positive cooperativity with a Hill coefficient of 1.73. High pressure gel filtration chromatography was used to demonstrate the formation of a complex consisting of one molecule of ovine prolactin and two molecules of the extracellular domain of the rat prolactin receptor. Complex formation occurred with human growth hormone, not with ovine growth hormone, a non-lactogen.

Prolactin, a polypeptide hormone secreted by the anterior pituitary, exhibits a multitude of biological activities in a variety of species and requires initial interaction with a cell-surface receptor (1). Prolactin receptors have been cloned and sequenced from rat (2), mouse (3), rabbit (4), human (5), bovine (6), and chicken (7) tissues. The cDNA for the rat liver prolactin receptor predicted a protein with a 19-amino acid signal sequence, an NH2-terminal 210-amino acid extracellular domain, a 24-amino acid transmembrane domain, and a COOH-terminal 57-amino acid cytoplasmic domain, for a total of 291 amino acids. Two other forms of the rat prolactin receptor have been isolated, one from the rat Nb2 cell line that has a total of 393 amino acids (8) and the other from rat ovary and liver that has a total of 591 amino acids (9). The differences between these receptor forms occur in the COOH-terminal cytoplasmic region. The prolactin family of receptors is closely related to the growth hormone family of receptors (10), and both belong to a larger superfamily that includes the cytokine and hematopoietic family of receptors (11). The rat liver prolactin receptor is well defined and is being subjected to mutational analysis (12, 13) to determine its interaction with ovine prolactin.

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‡To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160-7421. Tel.: 913-588-7007; Fax: 913-588-7440.

↑The abbreviations used are: PRL, prolactin; oPRL, ovine prolactin; hGH, human growth hormone; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PRED, prolactin receptor extracellular domain; HPLC, high pressure liquid chromatography; SMEM, Joklik's modified minimal Eagle's medium for suspension cultures; FBS, fetal bovine serum; TBS, Tris-buffered saline.
mented with 10% fetal bovine serum. The cells were grown to confluency in 25-cm² flasks to a final concentration of 3 × 10⁹ cells/flask in a 5% CO₂, 95% air atmosphere at 37 °C incubator. Green monkey kidney BSC-1 cells (ATCC CCL26) and human TK- 143B cells (ATCC CRL8303) were grown under the same conditions. Human cervical epithelial carcinoma HeLa S3 cells (ATCC CCL2.2) were grown at 37 °C in DMEM medium supplemented with 10% fetal bovine serum. The cells were cultured in 100-mm dishes using standard methods to maintain a confluence of 70-80% culture. The cells were mutagenized by transfection of DNA, and clones were obtained by cloning into 96-well plates with 10% FBS. The cell cultures were maintained in Dulbecco's modified Eagle's medium (basal medium supplemented with 10% fetal bovine serum and 10% donor horse serum). The cells were made quiescent by transfer to basal medium containing only 1% FBS and 10% donor horse serum (slow-down medium) for 24 h. All assays were performed in stationary medium (basal medium supplemented with 10% donor horse serum).

Production of PrePRED cDNA by RNA-based PCR—Total RNA was isolated from the liver of a 19-day pregnant Sprague-Dawley rat by the method of Andrews et al. (27). RNA-based PCR was performed essentially by the method of Kawasaki et al. (28). One μg of total RNA was used as template for cDNA synthesis by reverse transcriptase in the presence of 100 μM of random hexamers as promoter, 200 units of Superscript reverse transcriptase (Life Technologies, Inc.) and 40 units of RNasin (Promega Biotec) in a total volume of 20 μl. The reverse transcriptase reaction was used for PCR with the addition of 100 ng of the upstream primer KE7 (CCATGTGCATCTTGCTGTC, 100 ng of the downstream primer KE2 (GTCCTCTAAGGGTGGATAAGC- GTAACGA), 100 ng of AmpliTaq DNA Polymerase (Perkin-Elmer Cetus Instruments) in a 100-μl total reaction volume. Both reactions were performed in 1 ml AmpliTaq PCR buffer. PCR was performed for 30 cycles on a Perkin-Elmer Cetus Instruments DNA Thermal Cycler using a profile that included denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, and DNA synthesis at 72 °C for 1 min.

Subcloning of PrePRED cDNA into pTM1—A stop codon was added to the 3' end of the prePRED cDNA by performing a second PCR with 1 μl of the RNA-based PCR product with 100 ng each of two primers, KE7 and KE6 (GCTTAAGCTA-KE2), using the same profile as described above for 20 cycles. This product was subcloned into the NcoI/SmaI-cut, linearized pTM1 vector by blunt-end ligation as described by Sam- brook et al. (29). The insert was sequenced by the method of Sanger et al. (30) as modified by Del Sal et al. (31) to verify the accuracy of the PCR.

Production of Recombinant Vaccinia Virus—The prePRED cDNA was incorporated into the genome of wild-type vaccinia strain WR (ATCC VR1354) by homologous recombination into the thymidine kinase gene. Recombinant virus plaques were picked, and the positive ones were identified by DNA hybridization (32). Primary recombinant vaccinia virus isolates encoding PRED and vTF7-3, encoding T7 RNA polymerase (ATCC VR1354), were used to coinfect CV-1 cells in 25-cm² flasks at a multiplicity of infection of 10 plaque-forming units/cell. The cells were infected at 50 °C for 1 min, and DNA synthesis at 72 °C for 1 min.

Recombinant vaccinia virus stock was collected after 72 h of culture at 37 °C and then subjected to a 50-ml centrifugation at 3000 g for 15 min in a clinical centrifuge. The pellet was washed twice with 50 ml of SMEM. The cells were then resuspended in Dulbecco's modified Eagle's medium containing only 10% FBS and 1% donor horse serum. The cells were washed with 100 ml of Dulbecco's modified Eagle's medium containing 10% FBS and 1% donor horse serum and were resuspended in Dulbecco's modified Eagle's medium containing 10% FBS and 1% donor horse serum (slow-down medium) for 24 h. The cells were centrifuged at 1500 rpm as described above before, and the conditioned medium was filtered through a 0.45-mm filter. The filtrate was stored at 4 °C for 1 week and used as conditioned medium.

Inhibition of Nb2 Cell Mitogenesis—Assays were performed in triplicate using 24-well cell culture plates as previously described (35). Cells were transferred to the slow-down medium for 24 h. Quiescent cells were trypsinized, suspended in sterility saline, and then centrifuged at 300 x g before, and the conditioned medium was added to the cells. The cells were suspended in 3 ml of SMEM at a final concentration of 3 × 10⁵ cells/ml with stationary medium. For the assay, 900 μl of cells were combined with 100 μl of the test sample. PRED samples (50 to 30 ng/ml) were prepared with 100 ng/ml oPRL in phosphate-buffered saline, 10 μg MgCl₂, and 0.1% bovine serum albumin and filter-sterilized. After an incubation period of 72 h at 37 °C, the samples were counted manually using a hemocytometer.

High Pressure Gel Filtration Chromatography—In a total volume of 20 ml, 100 μl of each sample was injected onto a TSK-500W column (2.6 × 75 cm) in 0.1 M sodium phosphate, pH 7.0, in a 1.5-ml microcentrifuge tube. After 5 min, 10 μg of PRED or oPRL were added, bringing the final volume to 30 ml, and the columns were equilibrated with 0.1% porcine gelatin and 0.05% NaN₃. The solutions were incubated for 7 h at ambient room temperature and then passed over a 0.75 × 60-cm TosohaAs G3000SW TSK-Gel column at 0.35 ml/min. Elution was effected with TBS containing 10 μg MgCl₂, and monitoring was at 220 nm. Protein standards were obtained from Bio-Rad.

Iodination of Proteins—One IODO-BEAD (Pierce Chemical Co.) was combined with 1 μl of carrier-free Na¹²⁵I (Amerham Corp.) and 200 μg sodium phosphate, pH 7.0, in a 1.5-ml microcentrifuge tube. After 5 min, 10 μg of PRED or oPRL were added, bringing the final volume to 30 ml, and the columns were equilibrated with 0.1% porcine gelatin and 0.05% NaN₃. The solutions were incubated for 7 h at ambient room temperature and then passed over a 0.75 × 60-cm TosohaAs G3000SW TSK-Gel column at 0.35 ml/min. Elution was effected with TBS containing 10 μg MgCl₂, and monitoring was at 220 nm. Protein standards were obtained from Bio-Rad.

Binding Assays—The binding assay of [¹²⁵I]oPRL to immobilized PRED was a modification of the method of Harris et al. (16). PRED was coupled to AminoLink affinity matrix as described by Pierce Chemical Co. with 96% efficiency. The column was equilibrated with 50 ml Tris-HCl, pH 7.5, 0.05% NaN₃, and then with 100 ml of 50 ml Tris-HCl, pH 7.5, 0.05% NaN₃. Bound oPRL was eluted with 200 ml of 0.5 M Tris-HCl, pH 2.5. One microgram of iodinated oPRL was injected into a column of affinity-purified PRED as described above for [¹²⁵I]-PRED using oPRL affinity chromatography with a 10-15% recovery.

AminoLink—Antisera were generated against the first 13 NH₂-terminal amino acids of PRED (QSFFPGFIREHKCC) in rabbits (34). PRED antisera were determined by enzyme-linked immunosorbent assay against 1 μg of peptide was 1:15,000. The antisera recognized prolactin receptors on Western blots of 19-day pregnant rat liver membranes as well as PRED and were most useful in evaluating the efficiency of transient infections.

Large-Scale Production of PRED—HeLa cells were grown in suspension culture (500 ml at a cell density of 5 × 10⁶ cells/ml) and then centrifuged for 5 min at 1500 rpm in an IEC PR-2 centrifuge. The cells were resuspended in 25 ml of SMEM containing 2.5% FBS and infected with a mixture of both vED-B2 and vTF7-3 at a multiplicity of infection of 2 plaque-forming units/cell. The viruses were allowed to infect the cells for 2 h with shaking at 37 °C. The cells were then transferred to a 50-ml polypropylene centrifuge tube, and SMEM was added to a final concentration of 50 ml. The cells were centrifuged at 500 x g at room temperature for 30 min. The supernatant was allowed to drip through a 0.45-μm filter. The filtrate was brought to 0.05% NaN₃ and 10 μg MgCl₂.

Purification of PRED—Ovine prolactin was coupled to AminoLink affinity matrix as described by Pierce Chemical Co. with 96% efficiency. The column was equilibrated with 50 ml Tris-HCl, pH 7.5, 0.05% NaN₃, and then with 100 ml of 50 ml Tris-HCl, pH 7.5, 0.05% NaN₃. Bound oPRL was eluted with 200 ml of 0.5 M Tris-HCl, pH 2.5. One microgram of iodinated oPRL was injected into a column of affinity-purified PRED as described above for [¹²⁵I]-PRED using oPRL affinity chromatography with a 10-15% recovery.
mg of oPRL/ml of gel) was diluted 1:150 into a 50:50 suspension of Tris-derivatized matrix. Twenty-five μl of diluted matrix were combined with 25 μl of 50 mM Tris-HCl, pH 7.5, 0.1% bovine serum albumin, 0.05% NaNR and with 50 μl of various concentrations of 125I-PRED in the same buffer containing 20 mM MgCl2. The binding reaction was incubated at room temperature for 4 h and was mixed as described before, but every 15 min. The beads were washed twice with TBS, 0.1% bovine serum albumin, 0.05% NaNR. Nonspecific binding was generally <25% of total binding.

RESULTS

Expression and Purification of Rat Liver PRED—The cDNA for rat liver PRED, which included its 19-residue signal sequence, was generated by RNA-based PCR of total liver RNA or poly(A⁺) RNA from a 19-day pregnant rat. The cDNA was successfully amplified by RNA-based PCR using both RNA preparations only when they were copied by reverse transcriptase using random hexamers. The use of specific oligodeoxynucleotide primers alone on the RNA templates was unsuccessful in giving rise to PCR products (data not shown). This PCR product, prePRED, was subcloned into vaccinia virus/T7 hybrid expression vector pTM1 (Fig. 1). This mammalian expression vector places the cloned cDNA under the control of the bacteriophage T7 promoter. The cDNA sequence was flanked by the 5' untranslated sequence of the encephalomyocarditis virus (37) upstream and the T7 transcription terminator downstream. The resulting plasmid, pTED-39, was sequenced in the coding region of PRED to verify the accuracy of the PCR, and the sequence was found to be identical to that reported by Boutin et al. (2). The prePRED cDNA from clone pTED-39 was incorporated into the genome of the wild-type vaccinia virus (vEd-B2), was chosen for amplification and purification based on the high level of PRED expression in infected monkey kidney (CV-1) cells used for screening the plaque isolates.

HeLa cells were infected with a mixture of vTF7-3, encoding the T7 RNA polymerase, and vEd-B2 at a multiplicity of infection of 2, and the infected cells were incubated for 24 h in serum-free SMEM. The conditioned medium (500 ml) was applied directly to an oPRL affinity column; and after the column was washed with high salt, PRED was eluted under acid conditions (38) as a single symmetrical protein peak. Purified PRED stained as a single protein and had an estimated molecular mass of 38.5 kDa as determined on SDS-polyacrylamide gels (Fig. 2). In addition, [35S]methionine-labeled PRED from the oPRL column subjected to SDS gel electrophoresis and fluorography was a single band with no other bands present. The ability to express PRED in serum-free medium greatly facilitated the purification of PRED. The yields of PRED obtained in serum-containing and serum-free media were comparable and ranged from 1.2 to 1.6 mg of PRED isolated per liter of HeLa cells over five isolations. The amount of PRED in serum-free medium as estimated from SDS gels was ~15% of the total protein, which was ~5 μg/ml. The amount of PRED isolated was ~80%. [35S]Methionine-labeled PRED analyzed by non-SDS-PAGE showed at least five isoforms, and Ferguson plots (39) on the relative mobility of the five major bands with respect to the dye front versus gel percent yielded parallel lines consistent with the possibility that they are charge isomers. PRED had a maximum absorbance at 276 nm and had a slight shoulder near 290 nm, which is characteristic of exposed tryptophan. PRED was readily detected by Western blots with antibody generated against the first 13 NH₂-terminal amino acids of PRED.

Inhibition of Nb2 Cell Mitogenesis by PRED—Nb2 cells require lactogenic hormones (26) for growth, and maximal stimulation occurs near 1 ng/ml oPRL. An opportunity was available to test if PRED was an effective inhibitor of the oPRL-dependent growth of Nb2 cells. As shown in Fig. 3, PRED was an excellent inhibitor of oPRL-dependent mitogenesis. The IC₅₀ was estimated to be 7.1 μM in the presence of 1 ng/ml oPRL. A parallel incubation of the Nb2 cells with varying concentrations of oPRL in the absence of PRED yielded an EC₅₀ of 5.0 μM, with maximal stimulation occurring between 0.63 and 1.3 ng/ml. Incubation of the Nb2 cells with PRED alone was no different than incubation of the cells in the absence of oPRL and PRED. These results showed that PRED was an effective inhibitor of oPRL function in this biological assay, which was over a 3-day

![Fig. 2. SDS-polyacrylamide gels of expressed and purified prolactin receptor extracellular domain. Lane 1, 1.5 μg of total protein from serum-free vEd-B2/vTF7-3-infected HeLa cell medium after expression; lane 2, 1 μg of oPRL affinity-purified PRED. Molecular mass markers are shown on the right.](image-url)
Interaction between PRED and Prolactin—Derivatization of PRED to beads was found to be the best method for reducing nonspecific binding in determining the $K_d$ for the interaction between $^{125}$I-oPRL and PRED. $^{125}$I-oPRL formed a saturable complex with PRED (Fig. 4, top), and Scatchard analysis (40) yielded a $K_d$ of 1.16 nM for the experiment shown (Fig. 4, bottom). The average $K_d$ from three separate experiments was $1.21 \pm 0.19$ nM. Previous studies (22) have shown that the extracellular domain of the hGH receptor formed a 2:1 complex with hGH, and complex formation was demonstrated by high pressure gel filtration chromatography experiments. Accordingly, different molar ratios of PRED to oPRL were reacted for 7 h at room temperature, and their products were analyzed by high pressure gel filtration chromatography (Fig. 5, top). A plot of log molecular mass versus elution time was linear. Using this standard curve, oPRL migrated at 26 kDa and PRED at 65 kDa, and the complex migrated at 160 kDa. Using these values, the calculated molecular mass of a (PRED)$_2$-oPRL complex was 156 kDa, which is consistent with the observed value of the complex. The apparent high molecular mass of PRED on this column may be due to its high carbohydrate content and/or an expanded structure. At a 1:1 ratio of PRED to oPRL, a high molecular mass peak is present; an oPRL peak is also apparent, but the PRED peak is absent. At a 2:1 ratio, the amount of the high molecular mass species is increased, whereas oPRL is almost at the base line, and again the PRED peak is absent. At a 3:1 ratio, the high molecular mass peak remains the same, but a PRED peak is present. The samples analyzed in Fig. 5 were incubated for 7 h before loading on the column, subsequent studies showed that complex formation between oPRL and PRED was complete within a 15-min incubation period and that MgCl$_2$ was not required for complex formation. Additional studies (Fig. 5, bottom) showed that a complex was generated with a 2:1 molar ratio of PRED to hGH, a known lactogen, but not with ovine growth hormone (oGH, ---) as it was with the hormone held at 0.5 µM.
paragene residues in the receptor and subsequent analysis suggested that carbohydrates could contribute ~8 kDa to the apparent molecular mass of the receptor. The vaccinia system is known to glycosylate expressed proteins (37), but the exact composition of the carbohydrates has not been examined to date. In a recent study, Werenskiold (42) used the vaccinia virus system to express the T1 gene product in three different cell types that generated the same series of higher molecular mass forms on SDS gels. Treatment of these forms with endoglycosidases and sialidase suggested the presence of a complex array of carbohydrate units perhaps due to overexpression. As a result, the system becomes overloaded, and aberrant glycosylation can occur. When PRED was expressed in CV-1 or HeLa cells under conditions of transient or low expression, the apparent molecular mass was ~32 kDa. This molecular mass is similar to that of a naturally occurring prolactin-binding protein present in rabbit milk (43) that presumably contains the normal N-linked carbohydrate units. High level expression of PRED generated a 38-kDa protein that may be the product of aberrant glycosylation as demonstrated with the T1 protein (42). The various charge forms observed on non-SDS gels may reflect differences in sialic acid residues and have been demonstrated with other glycoproteins (44). PRED generated in this study does contain neutral sugars and does bind to a concanavalin A-Sepharose column, indicating the presence of carbohydrate (data not shown).

In a number of cases, it has been demonstrated that the extracellular domain of a protein hormone receptor is highly effective in removing hormone from the system. For example, the soluble interleukin-1- and interleukin-4-binding proteins inhibited interleukin-1- and interleukin-4-mediated events, respectively, in vitro (45). The soluble form of complement receptor type 1, which binds C3b and C4b, blocked the activation of both the classical and the alternative complement pathways in vitro and suppressed complement-induced postischemic myocardial injury in vivo (46). PRED effectively blocked the PRL-stimulated growth of rat lymphoma Nb2 cells at concentrations in the low picomolar range, which is comparable to the concentration of oPRL required for growth (Fig. 3). It would appear that the complex of oPRL with PRED is quite stable (3 days at 37 °C) since no growth re-occurred, indicating no dissolution of the complex to generate free oPRL. Thus, PRED may be an effective inhibitor for both in vitro and in vivo studies of PRL-dependent biological functions. A concentration of 1 ng/ml of oPRL is ~40 pm and provides maximal growth for the Nb2 cells. PRED at 80 pm and in the presence of 40 pm oPRL, which represents a 2:1 ratio of PRED to oPRL, inhibited growth of the Nb2 cells by ~90%, which is near the stoichiometry of PRED to oPRL (2:1) determined by high pressure gel filtration chromatography.

DISCUSSION

The extracellular domain of the rat liver prolactin receptor was cloned by RNA-based PCR and expressed at high levels in HeLa cells using a vaccinia virus/T7 hybrid expression system. The soluble protein was secreted into the culture medium pre-
binding. In the converse study, $^{125}$I-PRED was bound to immobilized oPRL; the Scatchard plot (see Fig. 6) was indicative of positive cooperativity, and a Hill coefficient of 1.73 was obtained. These results, to our knowledge, are the first demonstration of positive cooperativity in a protein receptor/protein hormone system and are consistent with the binding of two molecules of PRED to a single prolactin molecule.

It is well established that divalent cations, $\text{Mg}^{2+}$ in particular, are required for the binding of prolactin to the isolated native receptor and to the receptor in isolated membranes and in whole cells possessing receptor (38). In this study, it was demonstrated that divalent cations are not required for complex formation, and they are not required for the binding of $^{125}$I-PRED to oPRL or of $^{125}$I-oPRL to PRED. Previously, similar results were obtained with the binding of hGH to hGH-binding proteins (extracellular domain), where it was shown that divalent cations are not required (22). It is possible that the divalent cation requirement for the whole receptor may be due to charge sequestering or that in membranes a metal-dependent accessory protein may be required for efficient binding. The resolution of the role of divalent cations will require additional experimentation.

The growth hormones and prolactins belong to the same gene family (48) and are structurally related to each other. The receptors of the growth hormones and prolactin belong to a larger gene family of single membrane-spanning receptor proteins that includes both cytokine and hematopoietic receptors (11). The extracellular domain of the human growth hormone receptor interacts with human growth hormone in a 2:1 complex to form a trimERIC structure, which has been subjected to x-ray crystallography (23). Site-directed mutagenesis of hGH (22, 49) and x-ray crystallography of the trimeric complex (23) has shown that receptor dimerization may be important for members of the ligand binding domain of the growth hormone receptor.

The recent studies by Fuh et al. (51) relating to the design of antagonists to the human growth hormone receptor suggest that receptor dimerization may be important for members of the PRL/growth hormone/cytokine family including rat prolactin (52).

REFERENCES


