Identification and Characterization of the Prolactin-binding Protein in Human Serum and Milk*

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The actions of prolactin (PRL) are mediated by its receptor, a member of the superfamily of single transmembrane cytokine receptors. High affinity binding proteins for the closely related growth hormone have been found in the sera of several species including humans and are generated by alternative splicing or proteolysis of the growth hormone receptor extracellular domain (ECD). In contrast, no conclusive evidence has been presented that an analogous prolactin-binding protein (PRLBP) is expressed in human serum. Using both monoclonal and polyclonal antibodies generated against hPRL and the ECD of the human prolactin receptor, co-immunoprecipitation analyses of human serum identified a 32-kDa hPRLBP capable of binding both hPRL and human growth hormone. A measurable fraction of circulating PRL (36%) was associated with the hPRLBP. Despite well documented sex differences in serum hPRL levels, there were no significant differences in the levels of hPRLBP found in the sera of normal adult males and females (15.3 ± 1.3 ng/ml versus 13.4 ± 0.8 ng/ml, respectively (mean ± S.E.)). Immunoprecipitation studies also detected the PRLBP in human milk albeit at lower concentrations than found in sera. Deglycosylation did not alter its electrophoretic mobility, indicating an absence of carbohydrate moieties and suggesting that the hPRLBP spans most of the PRLR ECD, a result confirmed by limited proteolysis and mass spectrometry. The potential function of this serum chaperone was assessed in vitro by the addition of recombinant hPRLBP to the culture medium of the PRL-dependent Nb2 T-cell line. These studies revealed that the hPRLBP antagonizes PRL action, inhibiting PRL-driven growth in a dose-dependent manner.

Prolactin and growth hormone receptors are members of the cytokine receptor superfamily. Both the prolactin receptor (PRLR)¹ and growth hormone receptor (GHR) are single-chain transmembrane proteins composed of an extracellular, transmembrane, and intracellular domain. The hormones prolactin (PRL) and growth hormone (GH) exert their effects at the molecular level by inducing the homodimerization of their respective receptors, initiating the activation of receptor-associated kinases and signaling cascades.

Until the last few decades, it was believed that peptide hormones circulated freely, but experimental evidence has proven otherwise. Although PRL was found to associate with serum IgG (1), a GH-binding factor was initially identified in the serum of pregnant mice (2) and subsequently discovered in rabbit (3) and human sera (4, 5). With the subsequent cloning of the GHR cDNA, a GH-binding protein (GHBP) was found to have amino acid sequence identity with the extracellular domain of the membrane-bound GHR (6). The GHBP is a 246-residue glycoprotein with a molecular mass of 50–60 kDa. The generation of GHBP occurs via two separate mechanisms in mammals. In the mouse and rat, alternative splicing of a primary RNA transcript generates a truncated receptor in which the transmembrane domain is replaced by a short hydrophilic sequence (7–9). In other species, such as humans and rabbits, a full-length GHR is expressed, but GHBP is generated by proteolytic cleavage of the extracellular domain (6, 10, 11).

To date, although proteins in milk and serum have been found to interact with PRL, the existence of a free PRL extracellular domain (ECD), i.e. a PRLBP, has not been established. Evidence for PRL-binding proteins in milk has been shown within humans (12, 13) as well as pigs, sheep, and rabbits (13, 14). Although the identity of two serum PRL-binding proteins has been recently established, namely IgG (1, 15) and cyclophilin B (16), no clear evidence exists for a PRLBP in mammalian serum (14, 17).

In this study, we characterize a PRLBP in human serum with identity to the ECD of the PRLR. The serum PRLBP was immunoreactive with both poly- and monoclonal antibodies generated against the extracellular domain of the PRLR and shows a proteolytic profile similar to a recombinant PRLR extracellular domain. Tryptic digest mass spectrometry (MS) further confirmed identity with the PRLR ECD. Coimmunoprecipitations revealed the association of the PRLBP with both serum PRL and GH. Furthermore, unlike the GHBP, the serum PRLBP is not glycosylated. Based on its PRL binding ability, it was also shown to inhibit the hormone-induced proliferation of Nb2 cells.

EXPERIMENTAL PROCEDURES

Sera and Milk Samples—Human sera were obtained from healthy male and female adult donors. Aliquots were stored at −80 °C and clarified by centrifugation at 12,000 × g for 30 min before use. Human serum and milk samples were obtained from healthy male and female donors.

PBS, phosphate-buffered saline; BSA, bovine serum albumin; mAb, monoclonal antibody.
milk was obtained from a lactating mother and defatted by centrifugation. Infarstrant was collected by avoiding the upper lipid layer as well as any sediment and stored at 80 °C.

Expression of Recombinant Human Prolactin-binding Protein (rh-PRLB)—A CDNA fragment of the human long PRLR was amplified by polymerase chain reaction (PCR) using primers homologous to the mature form of the extracellular domain. The primers PRLR-1 (5′-CAGATTCCAGTTACCTCGTGGA-3′) and PRLR-211′ (5′-GCTCGAGCTATCTGGGTCGTAC-3′) were used in the 50-μl amplification reaction with 50 ng of DNA template, 5 μl of 10× polymerase chain reaction buffer, 3 μl of 25 mM MgCl2, 1 μl of 10 mM dNTP mix, and 5 units of Taq polymerase (Life Technologies, Inc.). After a 2-min incubation at 94 °C; the reaction was amplified for 30 cycles of 94 °C for 30 s, at 47 °C for 30 s, and at 72 °C for 30 s. It was then extended at 72 °C for 2 min. The amplified fragment was purified and fractionated by phenol/chloroform extraction followed by ethanol precipitation. The pellet was resuspended in 40 μl of H2O, and 10 μl of the sample was digested with EcoRI and XhoI restriction enzymes and ligated into the corresponding restriction sites of pGEX4T-1 (Amersham Pharmacia Biotech). The clone was subsequently checked for amplification errors by dideoxynucleotide sequencing. The resulting glutathione S-transferase (GST)-ECD was expressed as per kit instructions. Briefly, a 1-liter culture of Escherichia coli transformant was grown to mid-log phase and induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h. Pelleted cells were suspended in 5 ml of PBS containing 10 ml of EDTA and 10 ml of Tris-HCl, pH 8.0, in the presence of 0.5 mg/ml lysozyme. Purification and solubilization of refolded GST-ECD was then carried out as described previously (18). For purification of bioreactive recombinant human PRLBP (rhPRLBP), 1 ml of refolded GST-ECD preparation was incubated with 300 μl of glutathione beads for 30 min at 4 °C. After washing the beads three times with PBS, protein was eluted in 300 μl of PBS containing 10 mM reduced glutathione. Thrombin protease (10 units) (Amersham Pharmacia Biotech) was added to the elution at room temperature for 18 h. The digested protein solution was dialyzed overnight in 4 liters of PBS and cleared of GST protein by three incubations with fresh glutathione beads. The purified rhPRLBP was electrophoresed on a 12% SDS-polyacrylamide gel and stained with Coomassie Blue to ensure the complete removal of GST protein as well as undigested fusion protein. The sample was incubated for 1 h with polyvinylpyrrolidone beads to remove residual lipopolysaccharide and then filter-sterilized, aliquoted, and flash frozen.

Immunoprecipitation of PRLBP and PRL from Serum and Milk—Sera and milk samples (1 ml) were thoroughly preclarified of endogenous immunoglobulin by repeated overnight incubation with 300 μl of a protein A–G bead mixture. Precleared sera were then incubated overnight at 4 °C with 5 μl of rabbit anti-PRL antisera se developed by our laboratory and characterized elsewhere (19). As a negative control, an equal aliquot of serum was incubated with 5 μl of normal rabbit serum. Immune complexes were then precipitated by the addition of 50 μl of protein A beads for 30 min at 4 °C. After three washes with lysis buffer (20 ml Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS)), the samples were washed once more with lysis buffer and boiled for 2 min. The samples were then centrifuged, and the supernatant was used for immunoblot analysis. The gel slices were subjected to in-gel tryptic digestion by rehydrating with 200 ng of sequencing grade trypsin (Promega, Madison, WI). Extractable tryptic peptides were subjected to MALDI-MS analysis using a paracrystalline film matrix method for desalting the extracted tryptic peptides for mass spectrometry. Data were collected and submitted for mass data base searching using the Profound algorithm (Rockefeller University, New York, NY).

Inhibition of Nb2 Lymphoma Cell Proliferation with Recombinant PRLBP—Nb2–11C cells were maintained in Fisher's medium supplemented with 10% FCS, 10% horse serum, 1 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM β-mercaptoethanol. To assess the ability of recombinant hPRLBP to inhibit PRL-induced cellular proliferation, 105 Ñb2 cells were incubated in triplicate wells in medium consisting of Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with sodium selenide, linoleic acid, insulin, and transferrin (T6+; Calbiochem) in the presence or absence of 50 μM hPRL, hPL, or hGH. Increasing concentrations (0–100 nM) of recombinant hPRLBP or 100 nM hPL, hGH, or BSA were simultaneously added to the wells. After overnight culture, cells were pulsed with 1 μCi of [3H]thymidine at 37 °C for 4 h. The incorporation of radiolabel was determined by scintillography of the harvested, washed cells.
RESULTS

Expression of a PRLBP in Human Serum—To date, no protein with homology to the PRLR ECD has been found in sera, and the existence of such a protein in milk has been only indirectly implicated (12–14). This may in part be attributable to the absence of high avidity anti-PRLR antibody commercially available. Using a recently characterized anti-PRLR antiserum (19) in conjunction with a monoclonal antibody, we revisited the existence of a bona fide PRLBP (i.e., the free PRLR ECD) in human serum. To that end, human serum extensively precleared of immunoglobulin was immunoprecipitated with antiserum raised against the extracellular domain of the PRLR. Immunoblot analysis of this precipitate with a specific anti-PRLR mAb shows that the immunoprecipitate contained an immunoreactive protein corresponding to the approximate molecular weight of the extracellular domain of the human PRLR (Fig. 1A). This band was absent in the control lane using normal rabbit serum (NRS) or anti-PRLR antiserum (NRS-PRLR). Immunoprecipitates were electrophoresed in parallel with known quantities of recombinant PRLBP. Amounts of PRLBP in serum were calculated based on densitometry quantitation of standards. These results are representative of one of three experiments.

The Serum PRLBP Binds Serum PRL and GH—Unlike any other species, hGH has the capacity to bind both the PRLR and GHR. Indeed, previous studies have shown that a milk PRLR-interacting protein and GHBP are both capable of binding radiolabeled hGH (14). To determine whether there is an association between hPRL or hGH and PRLBP in human serum, coimmunoprecipitations were performed. As shown in the top panel of Fig. 2A, anti-PRLR immunoblot analysis of anti-PRL immunoprecipitates revealed the association of the PRLBP with serum PRL. Stripping and reprobing the blot with anti-PRL antibody shows that the immunoprecipitating antiserum was specific for PRL (Fig. 2A, bottom panel). As shown in Fig. 2B, anti-GH immunoblot analysis of anti-PRLR immunoprecipitates revealed that hGH was also bound to the PRLBP. To quantify the amount of serum PRL complexed with PRLBP, immunoprecipitations were performed using anti-PRLR and anti-PRL antisera followed by immunoblotting with anti-PRL antisera in conjunction with known concentrations of a PRL standard. As shown in Fig. 2C, 28 ng of PRL was found in 1 ml of a donor's serum, of which 10 ng or 36% coimmunoprecipitated with PRLBP as determined by known quantities of PRL electrophoresed in parallel. By similar methods, the amount of serum GH complexed with PRLBP was analyzed. In this donor's serum, 2.6 ng of the 5 ng of GH present in 1 ml of serum associated with PRLBP (data not shown). To further investigate the PRL binding properties of the PRLBP, a recombinant form of the PRLBP was generated in E. coli for subsequent analysis by surface plasmon resonance. Using various concentrations of rPRLBP, the dissociation constant (Kd) of binding site I was measured at 13.4 nM, an observation similar to those reported in depth elsewhere2 and in good agreement with existing biosensor data (21).

Both Sexes Have Comparable Levels of Serum PRLBP—Because the previous experiments utilized the serum of a single, nonlactating female donor, we wished to examine the variation

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in the levels of PRLBP observed between the sexes. This was accomplished by anti-PRLR immunoprecipitation analysis of donor serum and milk. As shown in Fig. 3, there was no significant difference in the amounts of PRLBP found in the sera of nonpregnant, nonlactating females (lanes 1-5) and males (lanes 6-9) with respective levels averaging $15.3 \pm 1.3$ ng/ml versus $13.4 \pm 0.8$ ng/ml PRLBP. We also observed no significant difference in the amounts of PRLBP found in the sera of these women as compared with sera from 24-h postpartum mothers (data not shown). Additionally, PRLBP was found in the breast milk of a lactating mother (Fig. 3A, lane 10) although at somewhat lower levels than in serum, as was similarly shown for other mammals (13, 14).

**Biochemical Identification of the PRLBP**—The PRLR is known to be glycosylated on the asparagine residues of its extracellular domain (22). To determine whether the PRLBP was glycosylated to a similar degree, immunoprecipitated PRLBP was treated with the general deglycosidase glycosidase F and then electrophoresed along with recombinant PRLBP expressed in *E. coli* (Fig. 4A). No shift in electrophoretic mobility was observed upon glycosidase F treatment, suggesting that serum PRLBP was not glycosylated and had the same apparent molecular mass as the recombinant form of the PRL ECD (32 kDa). Although two antibodies generated against the extracellular domain of the PRLR were immunoreactive against serum and milk PRLBP (Figs. 1 and 2), we could not entirely rule out that this was attributable to cross-reactivity to a highly homologous protein. Attempts at N-terminal amino acid sequencing, however, were noninformative, possibly because of a blocked N terminus. Therefore, serum PRLBP was excised from an SDS-polyacrylamide gel and subjected to protease digestion with Asp-N or Lys-C (Fig. 4B). As a positive control, recombinant PRLR ECD was digested in parallel. GST digestion served as a negative control. The peptide fragments resulting from the digestion of serum PRLBP showed an electrophoretic profile highly similar to the PRLR ECD (Fig. 4B, row 1 versus row 2). In contrast, the pattern differed from that of GST (Fig. 4B, row 2 versus row 3), suggesting that the PRLBP was indeed homologous to the extracellular domain of the PRLR. To confirm this observation, MALDI-MS was performed on PRLBP immunoprecipitated from serum as compared with recombinant human PRLBP. Four tryptic peptides with molecular masses of 971, 1399, 1772, and 1893 correlated between the serum PRLBP and the positive control of rPRLR ECD (Fig. 4C, middle versus bottom panel). Mass data base searching revealed homology to the PRLR ECD, and two peaks corresponded to tryptic fragments of this region (Fig. 4C, arrows in the bottom panel).

**Recombinant Human PRLBP Inhibits the Proliferation of Nb2 cells Stimulated with Human PRL**—As the GHBP can modulate the activity of GH, we wished to determine the effect of purified recombinant PRLBP on the biological activity of hPRL using the rat Nb2–11C lymphoma cell proliferation assay (Fig. 5). As increasing concentrations of PRLBP were added, a 3-fold reduction in cell proliferation was noted (Fig. 5A). Importantly, the PRLBP by itself was not toxic to cells. Therefore, the overall decrease in proliferation could be attributed to the PRLBP competing with cell surface receptors for ligand. This competition also extended to other members of the somatolactogenic family of ligands. As the control protein BSA was observed to have no positive or negative effect on PRL-induced proliferation (data not shown), we compared the effects of coinucipitation of BSA or PRLBP with other somatolactogenic hormones on Nb2 cell proliferation. The inclusion of PRLBP into the medium of either PL- or GH-stimulated Nb2 cells was found to decrease ligand-induced cell proliferation, an effect not observed for BSA (Fig. 5B).

**DISCUSSION**

Previous searches for the presence of a soluble form of the PRLR in mammalian biological fluids only resulted in the discovery of a putative PRLBP in milk samples (12–14). However, attempts to find a PRLBP in sera were unsuccessful, resulting from the general lack of robust anti-PRLR antibodies. In this study, we show evidence for a soluble PRLBP in human plasma. Using a high avidity anti-PRLR antiserum (19) generated by our laboratory, it was possible to immunoprecipitate a protein from human plasma, that was subsequently recognized by an anti-PRLR monoclonal antibody during immunoblot analysis (Fig. 1A). This serum PRLBP was observed to have the same molecular mass (32 kDa) as the extracellular domain of the human PRLR expressed in *E. coli* and was not glycosylated based on the inability of a glycosidase to affect its electrophoretic mobility (Fig. 4A). Our findings extend an observation by Mercado and Baumann (12) in which a PRL-binding protein in milk bound to hGH affinity columns, but unlike the GHB in human plasma, did not bind to wheat germ lectin columns, suggesting an absence of glycosylation. However, this study was unable to confirm the immunological identity of this species as that of the PRLR ECD. Interestingly, the predicted molecular mass of rhPRLBP expressed in *E. coli* is 24.5 kDa, yet both native and recombinant forms migrate as 32-kDa proteins. This difference in the predicted and observed masses may be attributable to the high content of acidic and proline residues comprising the PRLBP (21%). Previous studies have reported this same discrepancy because of the retarded mobility of a variety of proteins based on proline and acidic residue content (23–25). To confirm the identity of the PRLBP with the PRLR ECD, the immunoprecipitated PRLBP was subjected to limited proteolysis and SDS-polyacrylamide gel electrophoresis analysis alongside similarly digested rhPRLR ECD (Fig. 4B). Both proteins exhibited similar peptide fingerprints from both digestions, further supporting the correlation between the
**Fig. 4. Biochemical characterization of the PRLBP.**

**A**, PRLBP is not glycosylated. Precleared serum was immunoprecipitated with anti-PRLR antiserum, and the immunoprecipitate was washed and incubated with or without 10 units of glycopeptidase F (PNGase F) in buffer for 8 h at 37°C. Samples were immunoblotted in parallel with recombinant PRLBP and probed with a 1:1000 dilution of anti-PRLR mAb. 

**B**, proteolytic analysis of serum PRLBP versus PRLR ECD. Serum was immunoprecipitated with anti-PRLR antiserum as described above, electrophoresed, and stained with Coomassie Brilliant Blue. Excised bands were digested with Asp-N and Lys-C, and peptide fragments were separated on a 20% Tris-Tricine polyacrylamide gel. Bands were visualized using the Silver Stain Plus kit (Bio-Rad) and scanned for densitometric analysis. Recombinant PRLR ECD served as a positive control, and GST served as a negative control. These results are representative of one of two experiments.

**C**, MALDI-MS identification of the hPRLBP. Serum was immunoprecipitated with protein A beads covalently linked to anti-PRLR antibodies. Eluted hPRLBP was electrophoresed and stained with Silver Stain Plus (Bio-Rad). Excised bands were digested with trypsin and subjected to MALDI-MS. Two peptides corresponding to the ECD of the human PRLR are indicated. *rECD*, recombinant ECD; *pT*, trypsin autolysis product; *TOF*, time of flight; *LD*, limit of detection.
The mechanism for PRLBP generation as well as its tissue source is unknown. The generation of GHBP varies by species because rodents produce GHBP by alternative splicing mechanisms (7, 8, 26), whereas humans (6, 10) and rabbits (11) produce GHBP by the specific proteolysis of the membrane receptor. Indeed, Saito et al. (27) observed the constitutive release of GHBP from IM-9 cells. This was believed to be the result of surface GHR being cleaved by a metalloprotease because EDTA inhibited the production of GHBP. Membrane-bound PRLR is known to be N-link glycosylated (28), an event crucial for its cell surface targeting (22). The fact that a soluble, nonglycosylated PRLBP is present in milk and serum in human females therefore suggests that the binding protein may be produced by a combination of deglycosylation and proteolysis of the full-length PRLR. Indeed, although we observed the association of glycosylated full-length PRLR from T47D cell lysates with concanavalin A beads, neither rhPRLBP nor serum PRLBP was observed to bind to the beads (data not shown). This suggests that only the deglycosylated form of the PRLBP is found in serum. Interestingly, it has been shown that certain plasma membrane glycoproteins of hepatocytes undergo rapid deglycosylation (29, 30). As the liver abundantly expresses the PRLR (31), proteolytic cleavage of deglycosylated hepatic receptors could be one possible source of PRLBP in human serum. Current studies are underway to address this hypothesis.

The PRLBP was present in the sera of both males and females at comparable levels. This absence of sexual dimorphism is reminiscent of the PRLR itself (32). Like previous reports, which indicated that a protein capable of binding PRL was present in human milk (12, 14), our data would now indicate that this species represents the PRLBP. Additionally, the milk PRLBP was observed to be at a lower concentration than serum PRLBP, which is consistent with the observed levels of GHBP in human milk versus serum (12). Preliminary data from our laboratory suggest that there is no significant increase in serum PRLBP levels from mothers 24 h postpartum (data not shown). Interestingly, this is in direct contrast with the levels of GHBP observed during rodent gestation. In the mouse, increases in the levels of serum GHBP are observable on Day 9 of gestation, and by late gestation serum GHBP levels increase 30-fold (33). This may also be regulated by GH itself because continuous exposure to elevated GH in nonpregnant rats results in the up-regulation of GHBP (9, 34). In contrast, however, it is interesting to note that a pregnancy-associated rise in GHBP is not driven by GH (35).

Because the membrane-bound PRLR is capable of binding both PRL and GH, we wished to determine the ligand binding characteristics of the PRLBP. Surface plasmon resonance experiments for the hPRLBP were consistent with previously reported hormone-receptor interactions such as those observed by Gertler et al. (21). The PRLBP was found associated with both PRL and GH in human serum, and ~36% of plasma PRL was found associated with PRLBP in a single donor (Fig. 2). In a separate single donor, ~53% of plasma GH was found to be associated with the PRLBP. This result is similar to that observed with GHBP because GHBP is complex 45–55% of circulating GH under basal conditions (36–38). This has been shown to slow the renal clearance of GH, thereby providing a longerlasting reservoir of hormone (39, 40). Quantitative analysis of the molar concentration of the PRLBP, GH, and PRL indicated that the PRLBP is nearly saturated with somatolactogenic hormone, suggesting that the ligand-bound PRLBP may serve as a significant buffer to hormone levels.

Although a decrease in the clearance of PRL by the PRLBP could enhance the activity of PRL in vivo, in vitro studies using recombinant hGHBP have shown that GHBP inhibits GH activity (41). Similarly, the bovine PRLR extracellular domain has been shown to inhibit the bioactivity of ovine PRL on Nb2 cells (42). Because the hPRLBP was capable of binding PRL, we wished to determine whether a recombinant form of the human PRLBP could inhibit the hPRL-induced proliferation of Nb2 cells (Fig. 5A). A dose-dependent decrease in Nb2 cell proliferation was observed when recombinant hPRLBP was added to cells. This was also observed in the presence of hPRL and hGH (Fig. 5B), suggesting that the PRLBP is capable of binding these hormones as well. Although PRLBP was capable of inhibiting PRL-mediated cell proliferation in vitro, the effects of PRLBP in vivo may differ greatly. In a rat model, human
GHB complexed with hGH was cleared at a significantly slower rate, exhibited limited extravascular availability, and degraded at a slower rate than free hGH (39). Similarly, hypophysectomized rats given GH in conjunction with GHB showed significantly greater growth and weight gain compared with animals given GH alone (20). Based on these findings, the in vivo presence of PRLBP may serve to maintain a reservoir of PRL in the circulation, providing a releasable pool of free hormone in times of diminishing supply.

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