Identification of prolactin and growth hormone binding proteins in rabbit milk

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ABSTRACT Two distinct soluble proteins that specifically bind 125I-labeled human growth hormone (GH) are identified in the supernatant of ultracentrifuged rabbit milk, using HPLC gel filtration. The higher molecular weight protein is GH specific, whereas the other one is specific for prolactin (PRL). The PRL-binding protein has a very high affinity for the hormone, almost 10 times higher than the affinity of the mammary gland membrane receptor. The PRL-binding protein is immunoprecipitated by a monoclonal antibody against the PRL receptor; another monoclonal antibody, which inhibits the PRL binding to mammary gland membranes, is a poor competitor for the PRL binding to the milk protein. These findings suggest that the milk PRL-binding protein corresponds to the binding domain of the receptor, but also that the conformation of the receptor and of the binding protein might differ. The milk and the plasma GH-binding proteins have a similar binding affinity. In cross-linking experiments using 125I-labeled human GH, the M, of the GH-binding protein and of the PRL-binding protein were estimated to be 51,000 and 33,000, respectively. The binding proteins identified in the present work are probably responsible for the transport of their specific ligands in the milk. It is also conceivable that they have a role in the effects of GH and PRL in the mammary gland and/or the intestine of the young.

A specific binding protein (BP) for growth hormone (GH) has been identified in the serum of rabbit (1), man (2), as well as other species (3, 4). The identity of the amino acid sequence of the GH-BP and of the extracellular domain of the liver membrane GH receptor has been demonstrated for rabbit (5, 6), mouse (7), and rat (4). Such a serum BP has never been identified for prolactin (PRL). GH and PRL are present in milk (8); however, the mechanism of the transfer of these hormones from blood to milk is unknown. The possibility of a receptor-mediated process was examined by assessing the presence of soluble BPs in milk. Both a PRL-BP and a GH-BP are found in rabbit milk. We report here the identification and characterization of these two BPs. Antibodies specific to the PRL receptor also precipitate the milk PRL-BP, suggesting that the PRL-BP corresponds to the binding domain of the membrane receptor, as shown for the serum GH-BP (5).

MATERIALS AND METHODS

Preparation of Membrane Fractions and Milk Supernatant. Microsomal membranes were prepared from mammary gland of a bromocriptine-treated (three injections of 2 mg at 12-hr intervals) lactating rabbit, by differential centrifugation as described (9). Milk was collected from untreated rabbits at midlactation (12–15 days) and centrifuged at 100,000 × g for 30 min. The supernatant, which we called "milk," was used for all the experiments.

Measurement of BPs by HPLC. As previously described for plasma GH-BP (10), milk (100 or 200 μl) was incubated for 20 hr at 4°C in 100 or 200 μl of 0.1 M potassium phosphate (pH 7.0) containing 0.1% bovine serum albumin and 125I-labeled hormone (2 × 105 cpm). After filtration through a 0.45-μm Millipore minifilter, the incubation mixture was injected onto an HPLC Protein-Pack 300SW column (Waters). Elution was performed isocratically by using a degassed buffer (0.1 M Na2SO4/0.1 M potassium phosphate, pH 7.0) pumped at a rate of 0.5 ml/min. Radioactivity was recorded on-line by using a Berthold LB 504 γ detector connected to an Apple Ile computer.

Immunoprecipitation of PRL-BP. To analyze PRL binding by immunoprecipitation, milk samples (100 μl) were incubated with 125I-labeled ovine PRL (oPRL) (5 × 106 cpm) in the absence or presence of increasing concentrations of unlabeled oPRL (NIADDK-16) or monoclonal antibodies to the PRL receptor (M110 and A917) (11) in a total volume of 500 μl of Tris buffer (25 mM Tris, pH 7.5/10 mM MgCl2/0.1% bovine serum albumin). After 16 hr at room temperature, polyclonal antibodies (10 ml of serum 46) to the PRL receptor were added, and the incubation was continued for 2 additional hr (12). One milliliter of gamma globulin (0.1%) and 25% (vol/vol) polyethylene glycol (final concentration 12.5%) in phosphate buffer was added. The tubes were centrifuged, the supernatant was discarded, and the radioactivity of the pellet was recorded by an LKB γ counter.

Cross-Linking Experiments. Milk (500 μl) was incubated with 125I-labeled human GH (hGH) (2 × 105 cpm) in 50 mM phosphate buffer (pH 7.4). After 20 hr at 4°C, disuccinimidyl suberate in dimethyl sulfoxide was added at a final concentration of 0.5 mM. After 15 min, Tris-HCl (0.1 M, final concentration; pH 6.8) was added. The incubation mixture was desalted on a PD-10 column (Pharmacia) at 4°C, and the eluate was evaporated (Speed-Vac concentrator). After resuspension in H2O, injection onto a Protein-Pak 300SW column was performed, and the two peaks (GH-BP and PRL-BP) were collected separately. After desalting on PD-10 columns, lyophilization, and resuspension in H2O, the sample was analyzed by SDS/PAGE according to the method of Laemmli (13). Autoradiography was carried out by using Kodak X-Omat AR5 film (Eastman Kodak).

RESULTS

Identification of BPs in Milk. The elution profile of the gel filtration of 125I-labeled hGH (125I-hGH) incubated with rabbit...
milk is presented in Fig. 1; the profile shows two peaks that were both abolished in the presence of an excess of unlabeled hGH. Under the conditions used, almost all $^{125}$I-hGH incubated (91%) was specifically bound.

Since hGH is known to interact with both GH and PRL binding sites, the specificity of hGH binding to milk proteins was further investigated. As shown in Fig. 1, an excess of oPRL added to the incubation inhibited the lower molecular weight peak, whereas an excess of unlabeled bGH inhibited the first (higher molecular weight) peak.

Thus rabbit milk serum contains two distinct BPs: the higher molecular weight binding protein is GH specific and represents 32% of the hGH binding, whereas 68% of the bound hGH is associated with the other BP, which has lactogenic specificity. When $^{125}$I-labeled bovine GH (bGH) was used as a ligand, only the higher molecular weight protein was seen (Fig. 2 Upper). The specific binding of bGH was 14.3% of the radioactivity.

As shown in Fig. 2 Lower, when $^{125}$I-oPRL was used as a ligand, the lower molecular weight BP was detected. The elution time of the radioactive peak is that expected for the PRL-BP. The specific binding of $^{125}$I-oPRL was 33.3% of the radioactivity. To confirm the absence of a BP for PRL in the serum, $^{125}$I-oPRL was incubated with different concentrations of serum from a lactating rabbit under the same conditions as with milk, and no binding could be detected.

Characterization of the Milk PRL-BP. Fig. 3 shows a comparison of the specificity of the binding of $^{125}$I-oPRL to rabbit mammary gland membranes (Fig. 3A) and milk (Fig. 3B). For the latter, the immunoprecipitation technique described in Materials and Methods was used. As shown in this figure, 10-fold less oPRL (4.5 × 10$^{-10}$ M) is required to completely inhibit PRL binding to milk as compared to mammary membranes, suggesting that the affinity of the milk PRL-BP is much higher than that of the membrane PRL receptor. This was confirmed by Scatchard analysis. The affinities calculated from competition curves were 3 × 10$^{10}$ M$^{-1}$ and 5 × 10$^{8}$ M$^{-1}$ for the milk BP and for the membrane receptor, respectively. By contrast, the number of binding sites per milligram of protein was much higher in membranes (230 fmol) than in milk (10 fmol). However, mammary membranes were prepared from a lactating rabbit treated with bromocriptine, which has been shown to increase the concentration of membrane PRL receptors (9).

One monoclonal antibody (M110) previously developed against the membrane PRL receptor (11) also interacted with the PRL-BP present in milk, since $^{125}$I-oPRL binding was

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**Fig. 2.** Elution profile from an HPLC gel-filtration column of $^{125}$I-hGH and $^{125}$I-oPRL incubated with rabbit milk. (Upper) $^{125}$I-hGH (1 × 10$^5$ cpm) was incubated with 100 μl of milk without (●) or with (○) unlabeled bGH (100 ng). (Lower) $^{125}$I-oPRL (1.8 × 10$^5$ cpm) was incubated with 100 μl of milk without (●) or with (○) 100 ng of unlabeled oPRL.

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**Fig. 1.** Elution profile from an HPLC gel-filtration column of $^{125}$I-hGH incubated with rabbit milk. $^{125}$I-hGH (2 × 10$^5$ cpm) was incubated with 100 μl of milk without (●) and with (○) an excess (100 ng) of unlabeled hGH (Top), with an excess (100 ng) of unlabeled oPRL (Middle), or with an excess (100 ng) of unlabeled bGH (Bottom).
Fig. 3. Effect of oPRL and of two monoclonal anti-prolactin receptor antibodies (M110 and A917) on $^{125}$I-oPRL binding to mammary gland membranes (A) or to milk BP (B). Rabbit mammary gland microsomal membranes (200 µg of protein per tube) or rabbit milk (1 mg of protein per tube) were incubated with ~50,000 cpm of $^{125}$I-oPRL in the absence or in the presence of increasing concentrations of unlabeled oPRL or anti-prolactin receptor monoclonal antibodies (M110 and A917). Results are expressed as the percentage of specific binding of $^{125}$I-oPRL. Specific binding was 28.5% and 20.3% of the added radioactivity for mammary membranes and milk, respectively.

Fig. 4. Effect of bGH on the binding of $^{125}$I-bGH binding to milk (o) and plasma (c) BP. $^{125}$I-bGH (1 $\times$ 10$^3$ cpm) was incubated with 100 µl of milk or plasma and increasing concentrations of unlabeled bGH. Specific binding was 37.2% of added radioactivity for plasma BP and 16.6% for milk BP.

Fig. 5. Autoradiograph of a 7.5% polyacrylamide gel of $^{125}$I-hGH cross-linked to milk BPs. Cross-linking of $^{125}$I-hGH to milk and separation on HPLC of the two peaks were conducted as described in Materials and Methods. Lanes 1 and 2, $^{125}$I-hGH cross-linked to GH-BP in the absence and in the presence of 5 µg of unlabeled hGH, respectively; lanes 3 and 4, $^{125}$I-hGH cross-linked to PRL-BP in the absence and in the presence of unlabeled oPRL (5 µg). Arrows indicate the position of the molecular weight standards.

**DISCUSSION**

This study shows that a soluble PRL-BP is present in milk, whereas no such PRL-BP is found in plasma. The presence of a PRL-BP in human milk has been suggested, although its specificity was not studied (14). In milk, the PRL-BP coexists with a GH-BP. It would be of interest to look for the presence of such BPs in other biological fluids (amniotic fluid, cerebrospinal fluid, etc.). The PRL-BP probably corresponds to the binding domain of the membrane PRL receptor, since monoclonal antibodies to the receptor recognize both the receptor and the BP. However, our findings also suggest that the conformation of the PRL-BP and the receptor may differ: first, the binding affinity of the PRL-BP is 10 times higher than that of the receptor; second, one monoclonal antibody is able to discriminate between the two proteins. It is likely that the cytoplasmic domain of the PRL receptor affects the binding affinity for the hormone. In contrast, the milk and the plasma GH-BP have comparable hormone binding affinities. It is highly probable that the milk GH-BP also represents the
extracellular domain of the membrane GH receptor, as was demonstrated for the plasma BP (6).

Receptors for PRL are abundant in mammary gland membranes. Four different sized mRNAs for the PRL receptor have been identified in the mammary tissue (15); they all encode a long form of the receptor. The milk BP could result from a proteolytic cleavage of the membrane receptor. This mechanism has been proposed for the generation of the plasma GH-BP (5).

No specific binding sites for GH have been identified in mammary gland membranes though GH is a potent stimulator of milk production. However, the presence of GH receptor mRNA in the mammary tissue has recently been demonstrated in rabbit (16) and cow (17). These mRNA could encode the GH-BP found in milk and/or membrane GH receptors; these receptors, which are not detected in binding studies, could be rapidly cleaved to give rise to a soluble BP.

The potential biological role of these BPs in milk is still a matter of speculation. The BPs probably serve as transporters for their specific ligand. PRL, which is known to affect ion transport across epithelial cells, could act at the apical membrane of mammary epithelial cells or on intestinal cells of the suckling pup. It has been recently shown that PRL could be transported across the epithelium of the jejunum and ileum of the suckling rat (18) and it is documented that PRL may affect osmoregulation in the intestine (19). GH receptors have also been identified by immunochemistry in different parts of the intestine of the rat (20). Independent of the transport of their specific ligand, BPs may also have a specific biological function as has been demonstrated for IGF binding proteins (21). A recent paper (22) on the interleukin 6 receptor, a receptor that is classified in the same family as GH and PRL receptors (23), has introduced a new possible mechanism for signal transduction with the participation of the extracellular domain of the receptor. This work suggests that the interleukin 6 signal is mediated through a membrane glycoprotein (gp 130) that interacts with the extracellular part of the receptor. If a similar mechanism exists for the action of PRL and GH, the BPs that we have identified in milk and that correspond to the extracellular domain of the membrane receptors may play a crucial role not only in ligand transport but also in some biological effects of PRL and GH in the mammary gland and/or in the intestine of the young.