Specific Endometrial Binding Sites for Bovine Placental Lactogen Are Antigenically Similar to the Growth Hormone Receptor (43919)

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Abstract. **Bovine liver growth hormone receptors bind both bovine growth hormone (bGH) and bovine placental lactogen (bPL) with high affinity (K_d = 1.4 × 10^{-11} *M* and 3.0 × 10^{-11} *M*, respectively). By contrast, the uterine endometrium of pregnant cattle has high-affinity (K_d = 8.0 × 10^{-11} *M*) binding sites for bPL but, displays negligable binding of bGH. A polyclonal antiserum raised against the extracellular domain of the bGH receptor, was used to determine if there was antigenic similarity between the liver bGH receptor and the endometrial bPL binding site(s). On Western blots, this antiserum displayed cross-reactivity with a 180,000-mol wt protein (nonreducing conditions) in detergent-solubilized extracts of microsomal membranes from both tissues. However, different detergents (Triton X-100 for endometrium and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate [CHAPS] for liver) were required to solubilize the cross-reacting protein in the two tissues. The purified immunoglobulin fraction from this same antiserum also blocked binding of [^{125}I]bPL to microsomal membrane preparations from both liver and endometrium. These results indicate that the endometrial binding site for bPL is antigenically similar to the bGH receptor and raise the possibility that it may be a modified GH receptor.** [P.S.E.B.M. 1995, Vol 210]

Bovine placental lactogen (bPL) is a member of the growth hormone (GH)/prolactin (PRL) gene family (1). This placental hormone binds both GH (2) and PRL receptors (3) and consequently has both somatogenic and lactogenic activities *in vitro* (4). In the dairy cow, bPL has some somatogenic properties. Similarly to bovine GH (bGH), it increases both nitrogen retention and the circulating concentration of IGF-I (5, 6). However, whereas bGH also decreases tissue sensitivity to insulin and increases mobilization of lipids, bPL has no effect on these processes (5, 6). Therefore, bPL acts as a partial bGH agonist because it does not mimic all of the somatogenic effects of bGH. This raises the possibility that bPL may mediate some, or all, of its actions through a specific receptor.

Specific receptors for ovine placental lactogen (oPL) have been identified in fetal sheep liver (7, 8) and more recently, specific bPL binding sites have also been discovered in endometrial tissue from pregnant cattle (9, 10). The structure of these ruminant PL receptors is presently unknown, although there is some evidence to suggest that they may be related to GH receptors. Antiserum directed against the extracellular domain of the human GH receptor recognized proteins in microsomal fractions of fetal sheep liver containing specific oPL receptors (11). This antiserum also recognized proteins of similar molecular weight in microsomal fractions of maternal liver that contain GH receptors. Furthermore, although GH displays low affinity for ovine or bovine PL receptors, it is able to compete with radioiodinated PL in competition binding assays (7, 9).

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We have used an antiserum generated against the extracellular domain of the bovine GH receptor to examine whether there is antigenic similarity between endometrial bPL binding sites and the liver GH receptor.

Materials and Methods

Radioreceptor Assays. Liver and uterine endometrium from 4- to 5-month pregnant heifers were used to prepare crude microsomal membranes by the method of Haro *et al.* (12). Membrane preparations were treated with acid-glycine as described by Kessler *et al.* (10) to remove bound endogenous ligand(s) and stored at −80°C. Liver and endometrial microsomal membranes were used at concentrations of 1 and 0.29 mg protein/tube, respectively. Bovine PL, bGH, and bovine prolactin (bPRL) were all recombinantly derived (Monsanto Company, St. Louis, MO). A lactoperoxidase procedure was used to radioiodinate bPL and bGH. The specific activities were 74–76 and 65–78 Ci/mg for bPL and bGH, respectively. The buffer used in the radioreceptor assays was 25 m*M* Tris, 10 m*M* magnesium chloride, 0.1% (w/v) bovine serum albumin, pH 7.6, and assays were carried out as described by Staten *et al.* (2). Solubilization of liver and endometrial microsomal membranes by 1% (v/v) Triton X-100 or 10 m*M* 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) and radioreceptor assays with these soluble fractions were carried out exactly as described by Breier *et al.* (13).

The immunoglobulin fraction of serum (rabbit 296, pool 3) raised against recombinant extracellular domain of the bovine GH receptor was purified using an Econo-Pac Serum IgG purification kit according to the manufacturers' instructions (Bio-Rad Laboratories, Richmond, CA). Control rabbit immunoglobulins were purified from preimmune rabbit serum. The concentrated immunoglobulin fractions were incubated either with the liver or endometrial membrane preparations for 2 hr at 25°C before addition of radiolabeled ligand. The maximum final concentration of immunoglobulins in the assay tube was 2 mg/ml.

Western Blotting. Microsomal membrane preparations from liver and endometrium were sedimented by centrifugation (1,000 *g* for 25 min) and the pellet (10–20 mg protein) was resuspended in either 1% (v/v) Triton X-100 or 10 m*M* CHAPS to give 40–80 mg protein/ml. After 16–20 hr at 4°C, insoluble material was sedimented by centrifugation and samples (35 μg protein per lane, 25–50 μl supernatant) were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 14) (7.5% acrylamide, 1.5 mm thick gel) under both reducing and nonreducing conditions. The protein concentration of the supernatants was estimated by a bicinchoninic acid assay according to the manufacturers' instructions (Pierce, Rockford, IL). Following electroblotting (TE series Transphor Electrophoresis unit; Hoefer, San Francisco, CA) onto nitrocellulose (0.45 μm pore size; Schleicher & Schuell, Keene, NH) the membrane proteins were incubated for 4 hr at 25°C with either the same polyclonal antiserum (296 pool 3, diluted 1/2000) as used above or a rat monoclonal antibody raised against the extracellular domain of the bGH receptor (R1-M1-B11, diluted 1/1000). After washing, the blot was incubated with [^{125}I]-labeled protein G (New England Nuclear, Boston, MA) for 4 hr at 25°C. Protein bands that cross-reacted with the antiserum were visualized by autoradiography (X-OMAT film; Eastman Kodak Co., Rochester, NY). The molecular weight of these bands was estimated from prestained protein standards (Diversified Biotech, Newton Center, MA) run on the same gels.

Results

As reported previously (2), maternal liver membranes bound both [^{125}I]bGH and [^{125}I]bPL with high affinity. The K_d (estimated from Scatchard analysis of saturation binding experiments) for bPL and bGH was 1.4×10^{-11} and 3.0×10^{-11} *M*, respectively (2). Competition experiments with unlabeled ligands were also carried out to determine the specificity of the bPL binding sites in the liver membrane preparation. With [^{125}I]bPL, the concentration of unlabeled ligand required to reduce specific binding by 50% (ED_{50}) was approximately 75-fold higher for bGH than for bPL (30 vs 0.4 n*M* for bGH and bPL, respectively, Fig. 1). Nonspecific binding was similar for both hormones and in addition, bPRL did not compete with [^{125}I]bPL (Fig. 1).

Saturation binding studies were carried out with

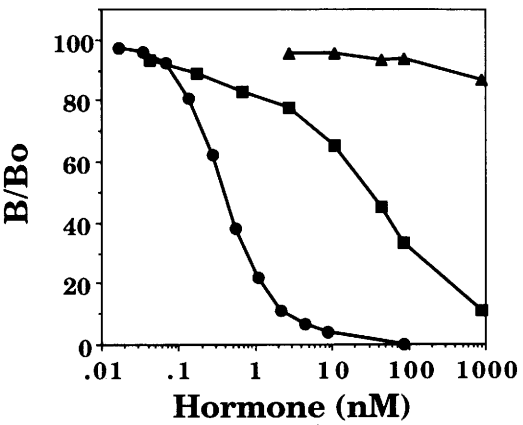

Figure 1. Competition binding between [^{125}I]bPL and increasing amounts of unlabeled bPL (closed circles), bGH (closed squares), or bPRL (closed triangles) for binding sites on bovine liver microsomal membranes. Points represent the means of three separate assays. Of the total radioactivity added, 36%–40% was bound specifically.

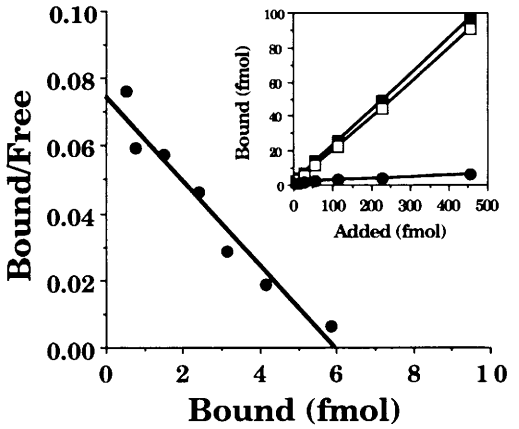

Figure 2. Scatchard analysis of saturation binding (inset) of [^{125}I]bPL to microsomal membranes of uterine endometrium from pregnant cows. Total (closed squares), nonspecific (open squares), and specific (closed circles) binding are shown. The K_d and B_{max} (mean ± SD, four assays) for bPL were 0.8 ± 0.08 pM and 21 ± 1.7 fmoles [^{125}I]bPL bound per milligram protein, respectively.

endometrial membranes. The K_d of binding sites for [^{125}I]bPL was estimated to be 8.0×10^{-11} M (Fig. 2) which was similar to the K_d for [^{125}I]bPL binding sites on the liver membranes (3.0×10^{-11} M). There was no measurable specific binding of [^{125}I]bGH in saturation binding studies (results not shown). In competition studies, using [^{125}I]bPL as the radioligand, the ED_{50} for bPL was 0.4 nM (Fig. 3). Both bGH and bPRL displayed some competition with [^{125}I]bPL, but the extrapolated ED_{50} of these hormones was more than 1000-fold greater than that for bPL (Fig. 3).

A number of different antisera raised against the bovine GHBP were tested for their ability to inhibit binding of [^{125}I]bPL to liver microsomal membranes. One of the sera (rabbit 296, pool 3) inhibited binding more than preimmune serum but, only at high concentrations (<1/50 final dilution). The high concentration of serum also decreased specific binding of radioligand to the membranes (not shown). The immunoglobulin (IgG) fraction was therefore partially purified from the antiserum and from preimmune serum, then concentrated (5.5 ml serum concentrated to 1 ml purified IgG). Preimmune IgG did not alter binding of [^{125}I]bPL to either liver or endometrial membranes whereas IgG raised against the extracellular domain of the bGH receptor inhibited binding of this radioligand to membrane preparations of both tissues (Fig. 4). Furthermore, the competition curves for the IgG derived from antiserum 296, pool 3 and for bPL were approximately parallel.

Initially, Western blotting of entire microsomal membrane preparations (both liver and endometrium) was attempted. Both the polyclonal antiserum (296, pool 3) and a monoclonal antibody (R1-M1-B11) raised against bovine GHBP failed to detect cross-reacting proteins using this approach (results not shown). This was probably due to the very low loading of receptor

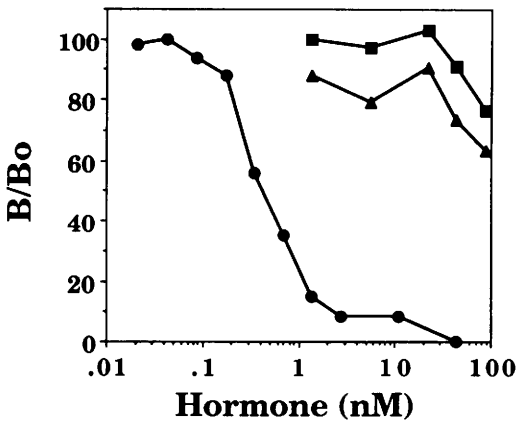

Figure 3. Competition binding between [^{125}I]bPL and increasing amounts of unlabeled bPL (closed circles), bGH (closed squares), or bPRL (closed triangles) for binding sites on uterine endometrium microsomal membranes. Points represent the mean of three separate assays. Of the total radioactivity added, approximately 4% was bound specifically.

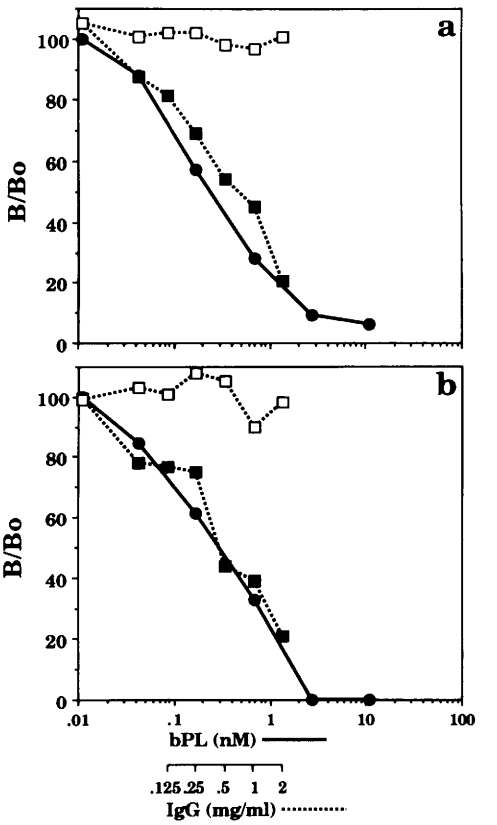

Figure 4. Competition binding assays using liver membranes (a) and endometrial membranes (b). Radioiodinated bPL (approximately 100,000 cpm) was incubated with the microsomal membrane preparations in the presence of increasing amounts of unlabeled bPL (closed circles, solid line), partially purified IgG from antiserum raised against the extracellular domain of the bGH receptor (closed squares, dotted line) or partially purified IgG from preimmune serum (open squares, dotted line). Points represent the mean of duplicate assays. Of the total radioactivity added, 11.4% and 4.1% was bound specifically by liver and endometrial membranes, respectively.

relative to total protein. Microsomal membrane preparations were therefore solubilized with either 1% Triton X-100 or 10 m*M* CHAPS in an attempt to increase the total quantity of receptor that could be loaded on a gel. The detergent soluble proteins were then fractionated by SDS-PAGE. Under nonreducing conditions, there was a cross-reacting protein (detected by both the polyclonal antiserum [Fig. 5] and the monoclonal antibody [not shown]) in liver membranes solubilized with CHAPS. The molecular weight of this protein was approximately 180,000 (Fig. 5). A protein of similar molecular weight was detected in endometrium solubilized with Triton X-100. The endometrial protein also cross-reacted with both the polyclonal antiserum (Fig. 5) and the monoclonal antibody (not shown). Under reducing conditions we were unable to detect cross-reaction of detergent solubilized proteins with either the polyclonal antiserum or the monoclonal antibodies (results not shown).

The detergent solubilized membranes were also tested for specific binding of [^{125}I]bPL. Liver membranes solubilized with CHAPS demonstrated specific binding (30% specific binding of [^{125}I]bPL per 0.1 mg protein) whereas there was no specific binding measurable in Triton X-100 solubilized extracts of liver membranes. However, there was no measurable specific binding of [^{125}I]bPL by either CHAPS or Triton X-100 extracts of endometrial membranes.

Discussion

Binding studies with maternal liver and [^{125}I]bGH indicate (as previously reported in Ref. 2) that bPL binds with high affinity to the bovine GH receptor. Competition studies with [^{125}I]bPL also indicated that bPL bound predominantly to the GH receptor in maternal liver. There appeared to be either very few, or no specific receptors for bPL in maternal liver because nonspecific binding was similar for both bPL and bGH (see Fig. 1). In addition, there were very few PRL receptors in the liver membrane preparations because there was virtually no competition for [^{125}I]bPL by high concentrations of bPRL. Nonetheless, even though [^{125}I]bPL appears to be binding predominantly to a single type of receptor, the competition curves for bPL and bGH are not parallel. We believe that this results from the fact that bPL only binds to a single site on the bGH receptor, whereas bGH can bind to two sites on its receptor (2). The ovine GH receptor behaves similarly: it also appears to form a homodimer when bound by ovine GH (oGH), whereas it binds to ovine PL (oPL) with 1:1 stoichiometry (13). Breier *et al.* (13) concluded that oGH and oPL both bind to a common receptor in sheep liver. Bovine GH and bPL also appear to bind to the same receptor in bovine liver.

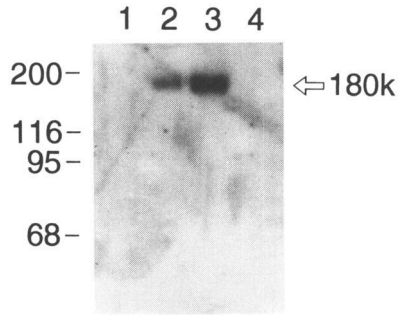

Figure 5. Western blotting of detergent solubilized extracts of maternal liver and endometrial microsomal membranes. Soluble membrane proteins were separated by SDS-PAGE (7.5% gel) run under nonreducing conditions. The antiserum used to detect the liver GH receptor and presumed endometrial bPL receptor was raised in a rabbit against the extracellular domain of the bovine GH receptor. Lanes 1 and 2 are liver extracts and Lanes 3 and 4 are endometrial extracts. Material in Lanes 1 and 3 was solubilized with 1% Triton X-100, and material in Lanes 2 and 4 was solubilized with 10 m*M* CHAPS. The molecular weight of the cross-reacting protein(s) in Lanes 2 and 3 was estimated to be 180,000.

Results from our receptor binding studies with membranes prepared from uterine endometrium were consistent with two previous reports (9, 10). The endometrial membranes displayed high affinity for bPL and very low affinity for either bGH or bPRL. Yet despite the virtual absence of binding sites for bGH, endometrial membranes contained a protein that was immunologically related to the extracellular domain of the GH receptor. By Western blotting, the soluble cross-reacting protein from endometrium was the same molecular weight as the liver GH receptor. The mature, nonglycosylated, monomeric GH receptor is predicted to have a molecular weight of approximately 70,000 (15). However, the molecular weight of the proteins identified by Western blotting were approximately 180,000. The putative GH/PL receptor was therefore probably associated with another protein. One possibility is that two receptor molecules are linked to form a homodimer. Another possibility is that the receptor(s) may be associated with another protein to form a heterodimer. This second alternative occurs with other members of the hematopoietin receptor superfamily (16). Unfortunately, we were unable to establish the molecular weight of the GH receptor under reducing conditions as the antiserum did not appear to cross-react with the reduced protein(s).

The second piece of evidence that the putative bovine PL receptor is structurally similar to the GH receptor is the inhibition of binding of [^{125}I]bPL to both GH and PL receptors by the same antiserum. The subclasses of antibodies in the serum that recognize the binding site of the GH receptor was apparently either low titer or low affinity because a high concentration of antiserum was required to inhibit binding. Nonetheless, partially purified IgG from antiserum raised against the extracellular domain of the bovine

GH receptor inhibited binding of bPL to the endometrial PL receptor in a dose-dependent manner.

Evidently, the cross-reacting protein from endometrium was not identical to the liver GH receptor because a different detergent was required for its solubilization. The bovine liver GH receptor was not solubilized by the nonionic detergent Triton X-100. Freemark *et al.* (17) also determined that the ovine GH receptor was insoluble in 1% Triton X-100, whereas binding sites for ovine PL were solubilized by this detergent. Conversely, the Zwitterionic detergent (CHAPS) only solubilized the bovine liver GH receptor. Binding studies with detergent solubilized extracts of liver and endometrium confirmed that the bovine liver GH receptor was only solubilized with CHAPS. Failure to detect binding of [^{125}I]bPL to either CHAPS or Triton X-100 extracts of endometrium also tends to support the idea that PL binding site in endometrium is not the same as the GH/PL binding site (GH receptor) in liver.

Overall, these results indicate that the endometrial PL binding site is different than the liver GH receptor; however, these binding sites (receptors) display antigenic similarity. We suggest that the endometrial PL receptor may even be an alternate or modified form of the GH receptor based on the following speculation. Bovine GH appears to form a stable ligand:receptor complex only when one molecule of bGH binds to two bGH receptors (2) because available evidence indicates that bGH does not form a stable interaction with a single GH receptor (2). On the other hand, bPL forms a very stable 1:1, ligand:receptor complex. Theoretically, the extracellular domain of the bovine GH receptor could be modified so that it is unable to form a homodimer in the presence of bGH. Potentially the ligand binding site(s) on the receptor could remain the same. This modified GH receptor would then retain high affinity for bPL but would have very low affinity for bGH by virtue of the fact that bGH only binds a dimeric GH receptor. However, it will probably be necessary to determine the structure of the bovine PL receptor to verify this speculation.

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