Ruminant Placental Lactogens Act as Antagonists to Homologous Growth Hormone Receptors and as Agonists to Human or Rabbit Growth Hormone Receptors

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Growth hormone receptor (GHR)-mediated activity of ruminant placental lactogens (PLs) and ovine (o) GH was compared, using cells transfected with full size human (h), rabbit (rb), and oGHRs. All three PLs acted as agonists in heterologous bioassays, whereas in homologous bioassays in cells transfected with oGHRs they antagonized the oGH activity. Despite these differences, oGH and PLs bound with similar affinity to the oGHR extracellular domain (oGHR-ECD), indicating that the binding occurs through hormone site I. Gel filtration of complexes between oPL and oGHR-ECD showed a 1:1 stoichiometry, confirming this conclusion. The oPL T185D and bPL T188D, which exhibited weak biological activity mediated through GHRs, behaved as site I antagonists, whereas oPL G130R and bPL G133R formed a 1:1 complex with GHR-ECDs and bound to h/rb/oGHR-ECDs with affinity similar to that of wild-type oPL. They had no agonistic activity in all models transfected with h/rb and oGHRs, but were antagonistic to all of them. In conclusion, ruminant PLs antagonize the activity of oGH in homologous systems, because they cannot homodimerize oGHRs, whereas in heterologous systems they act as agonists. The structural analysis hints that minor differences in the sequence of the GHR-ECDs may account for this difference. Since the initial step in the activity transduced through cytokine/hemapoietic receptors family is receptor homodimerization or heterodimerization, we suggest that the question of homologous versus heterologous interactions should be reexamined.

Ruminant and other species’ placentas synthesize and secrete unique proteins belonging to the growth hormone/prolactin (GH/PRL) family and are termed placental lactogens (PLs). Ovine (o) (1, 2), bovine (b) (3) and caprine (c) (4) PLs were isolated from placentas and found to be 22–23-kDa proteins (5). Recombinant oPL (6, 7) and bPL (8), and recently cPL as well (9), have been prepared, and the recombinant proteins can now be produced in amounts that allow in vivo studies. Cloning of cPL enabled us to compare its primary structure to that of oPL and bPL. The similarity between cPL and oPL exceeds the one between bPL and oPL or cPL. In contrast to these, the similarity between the corresponding GHs and PRLs in the three ruminant species is much greater (5). It has been proposed that this finding suggests into possible different physiological roles that PLs may play in the three species, but this point has not yet been proven (5). Recently, we have determined the three-dimensional structure of the 1.2 complex between oPL and the rPRLR-ECD, and have been able to identify the 25 residues of oPL that participate in site I of the hormone and 24 residues that participate in site II (10). This finding, along with our former direct and indirect experiments using recombinant extracellular domains (ECDs) of GH and PRL receptors, suggests that the initial step in PL signal transduction consists of dimerization of the respective receptor, as is well documented for GHs (11).

One early observed, unique property of ruminant PLs is their ability to bind to both PRL and GH receptors, including receptors of hGH (for review, see Refs. 12–14). Comparative binding studies of oPL and oGH to fetal liver microsomes, along with demonstration of oGHR mRNA in fetal liver, prompted several research groups to suggest that oGH and oPL bind to identical or at least related proteins (15–17). Using a similar approach, we have previously studied the biological activity of the three ruminant PLs in several in vitro bioassays, in which the signal was transduced through heterologous (mouse, rabbit, and human) GHRs (7, 9, 18–22). In all cases the activity of bPL, oPL, or cPL was equal to that of oGH, bGH, or hGH despite some differences in affinity. Furthermore, mutagenesis of bPL allowed us to prepare several bPL analogues with the selectively reduced or abolished somatogenic activity, whereas the lactogenic activity (as judged by the Nb2 rat lymphoma cell proliferation bioassay) was not changed (20–22). These experiments were paralleled by protein-interaction studies that showed that bPL, similarly to hGH, is capable of forming a 1:2 complex with h- and rbGHR-ECDs. In contrast to these results, Staten et al. (23) reported that bPL interacts with bGHR-ECD in a 1:1 stoichiometry, whereas bGH forms a 1:2 complex. More recently, the same group briefly reported that bPL antagonized bGH action in BaF3 cells stably transfected with bGHRs and was devoid of proliferative activity (24). These reports prompted us to reexamine whether the somatogenic activity of ruminant PLs is relevant in homologous species. To answer this question, we developed an oGHR-mediated bioassay in 293 cells, prepared recombinant oGHR-ECD, and used them in the present study.
EXPERIMENTAL PROCEDURES

Materials—Recombinant bPL, bPL G133R, bPL K73D, bPL T188D, oPL, oGHR, and non-glycosylated human GHR-ECD were prepared as described previously (7, 8, 18–22, 25). Recombinant caprine (c) PL was recently prepared in our lab (9). Rabbit (rb) non-glycosylated GHR-ECD was prepared in our laboratory, and its preparation will be described elsewhere. Carrier-free Na^2222^ was purchased from NB Scientific Laboratories. Secrecy and eucaryotic weight markers were purchased from SDS-PAGE, RPMI 1640 medium, lysosyme, naldixic acid, Triton X-100, bovine serum albumin (radioimmunossay grade) were obtained from Sigma. SDS-PAGE reagents and protein assay kit were purchased from Bio-Rad. Fetal calf serum and horse serum were purchased from Labotal Co. Science Products. Molecular weight markers for SDS-PAGE, RPMI were prepared as described previously for bPL (18). The inclusion body pellet expressed protein was found in the inclusion bodies, which were pre-

Construction of oPL Analogue T185D Expression Vector—Synthetic gene fragment for preparation of oPL analogue T185D was constructed using polymerase chain reaction (PCR) technology. Oligonucleotides (primers) were used to generate a double-stranded DNA from a template, pET-8-oPL (7), for subcloning. An NcoI site (underlined) was created with a forward primer at the 5’ end of the gene, which also added an initiation methionine codon immediately upstream to the first mature codon (alanine) (5’-GGGATATATACCAAGCCAGCTACTAC-3’) and a III site (underlined), was included close to mutation area at the 3’ end of the gene, with a reverse mutant primer (5’-GAATATACTCGAGTACCGGAAAATTAC-3’). The reverse mutant primer encoded the mutation of interest. The PCR reaction was conducted using Taq polymerase in a capillary PCR apparatus (Idaho Technology, FL), with the following program: 15 cycles of (0 s × 94 °C, 0 s × 55 °C, 25 s × 72 °C), 15 a × 72 °C. The PCR product was gel-purified, subcloned into pGEM-T vector (Promega, Madison, WI), and transfected to JM-109 Escherichia coli cells. The NcoI/III insert was isolated and ligated into the parental vector (pET8) encoding for the wild-type oPL (7), from which the respective NcoI/III insert was removed. Subsequently, the pET8/oPL T185D clone was sequenced and used for transformation of BL21 E. coli cells. One of the isolated colonies that expressed the protein after induction by isopropyl-β-D-galactopyranoside was selected for large scale preparation. Automatic DNA sequencing was performed to confirm the proper sequence. 

Construction of oPL Analogue G130R Expression Vector—The oPL analogue expression vector was modified with the Quickchange™ mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions, using two complementary primers: (5’-GGGCAAAATACGTTCGCTGAAAGGACTGCAC-3’) and (5’-GTATCCTAGTCAGAAGTTGAC-3’). These primers were designed to contain a specific restriction site (AIII), still conserving the same amino acid sequence, for colony screening. The screening included 12 PCR cycles and the use of Pfu polymerase enzyme for the reaction. The temperature mutant construct was wild-type oPL (7) and pMON3922 (26). The mutated construct was then digested with DpnI restriction enzyme, which is specific to methylated hemihemidihexyllic DNA (target sequence: 5’-G-MATC-3’), in order to digest the template and to select for mutation-containing synthesized DNA. The vector was then transfected into XL1-competent cells. Ten colonies were then screened for mutation, using the specific restriction site designed, and revealed 80% efficiency. Two colonies were sequenced and confirmed to contain the mutation and no undesired misincorporation of nucleotides.

Expression, Refolding, and Purification of oPL Analogues—E. coli MON105 cells transformed with the expression plasmids containing the oPL G130R were incubated in 500 ml of Terrific Broth (TB) medium (27) by shaking at 200 rpm at 37 °C in 2-liter flasks to an optical density of 0.8 at 600 nm, 30 °C × 72 °C. The PCR product was chosen for large scale expression, which was prepared as described above for oPL G130R analogue. Preparation of inclusion bodies and the refolding procedure was identical to that of oPL analogue G130R, except that after the solubilization in 4.5 mM urea, the solution was stirred at 4 °C for 48 h, prior to dialysis against 10 mM Tris-HCl buffer pH 8.0 and subsequent purification on a Q-Sepharose column (2.6 × 7 cm), preequilibrated with the same buffer. The monomeric fraction was eluted with 150 mM NaCl at the same buffer, dialyzed, and lyophilized.

Binding Experiments—Binding to soluble ovine, rabbit, and human GHR-ECDs was carried out as described previously (7, 28). The ligand was [125I]oGHR or [125I]oPL, and the competitors were oPL, bPL, oPL, and oPL analogues. Iodination of oGHR and oPL was performed according to the protocol described previously (28). Determination of Monomer Content and Complex Formation—High performance liquid chromatography gel-filtration chromatography on a Superdex™ 75 HR 10/30 column was performed with 200-μl aliquots of Q-Sepharose-column-eluted fractions, freeze-dried samples dissolved in H2O, or complexes between the soluble recombinant GHR-ECDs and oGHR, oPL, or oPL analogues, using methods described previously (7, 28).

In Vitro Bioassays in Stably Transfected FDC Cells—Two in vitro bioassays, in which the signal was transduced through somatogenic receptors, were based on the proliferation of FDC-P1 cells transfected with rabbit (clone FDC-P1-3B9) or human (clone FDC-P1-9D11) GHRs (31, 32) as described before (21). Cell growth was determined by counting the cells with a Coulter counter (Coulter Electronics Inc., Hialeah, FL). The number of doublings was calculated as described previously (33).

In Vitro Bioassays in Transiently Transfected 293 Cells—Two additional bioassays were carried out in a 293 cell line transiently transfected with hGHR or oGHR and co-transfected with a plasmid that carries the luciferase reporter gene under the control of a six-repeat sequence of LHRE (lactogenic hormone response element with a Stat5 binding sequence) fused to a minimal thymidine kinase promoter. The transfection and the bioassay were carried out as described previously (34). The vector encoding for full-size oGHR in SPT2 vector was obtained from Dr. Tim Adams (35). It was first digested with XbaI at 37 °C for 1 h and then with EcoRI at room temperature for 5 min. The reaction products were then separated on a 1.0% agarose gel, and the insert corresponding to ~3200 bases was purified and ligated to pcDNA3 mammalian expression vector, linearized with XbaI and EcoRI. The ligated plasmid was propagated, isolated, and sequenced to ensure the proper ligation.

In Vitro Bioassays in Nb2 Cells—An in vitro bioassay, in which the signal was transduced through lactogenic receptors, was performed in rat Nb2-11C lymphoma cell proliferation bioassay, in which the original protocol was slightly modified (33).

RESULTS

Purification of oGHR-ECD and oPL Analogues—The profile of oGHR-ECD elution from a Q-Sepharose column shows that over 60% of the protein was eluted with 0.15 M NaCl (data not
shown). Every fifth tube was analyzed for monomer content, and fractions containing >98% pure monomer were pooled, dialyzed against NaHCO₃ (1:5 salt:protein ratio), and lyophilized. The overall yield was 110 mg of monomeric protein obtained from a 5-liter fermentation culture. This fraction was further used for binding and biological studies. Fractions eluted with 0.4M NaCl consisted mainly of oligomers (data not shown). SDS-PAGE of the pooled monomer fraction, performed with and without β-mercaptoethanol according to Laemmli (36), revealed only one band with a molecular mass of 28 kDa (data not shown). The oligomeric fraction eluted with 0.4M NaCl has also yielded a main 28-kDa band, indicating that the oligomers were formed by non-covalent interactions. The preparation of oPL analogues (oPL G130R and T185D) was carried out according to the protocol described for the wild-type recombinant oPL (7). The monomeric fractions were eluted from the Q-Sepharose column developed with 10 mM Tris-HCl buffer at pH 9.0 by 0.05 M NaCl, dialyzed against NaHCO₃ at a 4:1 protein:salt ratio and freeze-dried. The homogeneity of the purified proteins was also verified by SDS-PAGE under reducing and non-reducing conditions (data not shown). The biological activity of both analogues resulting from proper renaturation was further evidenced by their ability to stimulate the proliferation of the lactogenic receptor-mediated Nb₂ bioassay (data not shown) and to bind to human, rabbit, and ovine GHR-ECDs (Fig. 2). The relative activity of the G130R and T185D analogues in Nb₂ bioassay as compared with wild-type oPL was, respectively, 2.5% and 87%.

Gel-filtration Experiments—The stoichiometry of the interactions between soluble human, rabbit, and ovine GHR-ECDs and oPL, oPL T185D, oPL G130R and, in the latter case, also with oGH was studied by gel filtration. The complexes were prepared at a constant concentration (1.75 μM) of the hormones and variable concentrations (1.75–5.25 μM) of the respective receptor ECDs. Ovine PL formed a 1:2 complex with hGHR-ECDs, which was eluted at the retention time of 11.22 min (Fig. 1A), confirming the previous results (7). At the oPL:hGH-ECD ratio of 1:3, an excess of hGHR-ECDs was observed. In contrast, both oPL T185D and G130R formed only 1:1 apparent complexes in both cases (Fig. 1A). This conclusion was based on both a comparison of peak sizes, their retention times (11.94 and 12.44 min, respectively), and the fact that, at 1:2 analogue:GHR-ECD ratios, an excess of the latter could be seen. The shape of the peaks of the oPL T185D:hGHR-ECD complexes and the fact that the retention time values shift forward by increasing the ECD:analogue ratio indicate that a very weak
The oPL analogue T185D exhibited agonistic activity (Fig. 1C). The oPL analogue G130R acted similarly to oPL. The results of the interaction of oPL T185D with oGHR-ECD do not, however, indicate formation of either 1:1 or 1:2 complexes, which likely results from the loss of binding capacity, as shown in the next section.

**Binding Experiments**—As oPL is capable of binding to both homologous and heterologous somatogenic receptors, several binding assays were performed. The results of a comparative binding assay in which the ability of oPL, oPL G130K, and oPL T185D to compete with 125I-oPL or 125I-oGH for binding to recombinant human, rabbit, and ovine GHR-ECDs is shown in Fig. 2. The results (for binding of bPL and cPL to human and rabbit GHR-ECD, see our previous articles Refs. 7, 9, and 20–22) clearly demonstrate that all tested hormones (except oPL T185D) have an almost identical capacity to compete with the ligand for binding to h-, rb-, and oGHR-ECDs, respectively. The oPL analogue T185D exhibited 90 and >500 times lower competitive capacity in the binding to h- and rbGHR-ECDs, respectively, and no capacity at all in binding to oGHR-ECD.

In all cases, results were calculated using both one-site and two-site analysis (37). In the two-site analysis, calculations were based upon an assumption that both sites contribute equally to the binding. In all analyses, the quality of fit for the nonlinear correlation was very high ($R^2 > 0.97$), making the choice of an appropriate model difficult. Therefore, additional arguments were taken into consideration. (a) In all bioassays (see below), oPL G130R exhibited antagonistic activity, strongly suggesting that its ability to bind the receptor through site II was severely or completely damaged. (b) The same argument holds for the activity of oPL in 293 cells transiently transfected with oGHR. (c) In the previously described gel-filtration experiments, oPL G130R formed only a 1:1 complex with all three GHR-ECDs, whereas a 1:2 stoichiometry was observed for the interaction of oPL with human and rabbit GHR-ECDs but only a 1:1 stoichiometry with oGHR-ECD. (d) The displacement curves of oPL, oPL G130R, bPL, cPL, and oGH in the binding to oGHR-ECD were quite similar (the respective IC_{50} values were 3.2, 1.9, 1.5, 4.0, and 1.1 x 10^{-6} M, despite the fact that only the latter is an agonist in 293 cells transfected with oGHR. Taken together, it seems that under the conditions in which the binding experiments were performed, the radiolabeled ligand binds to site I only, giving the one-site model an advantage.

**Proliferative Activity of oPL and oPL Analogues G130R and T185D in FDC-P1 Cells**—Ovine PL was an agonist in FDC-P1 cells, transfected with either rabbit (Fig. 3A) or human (Fig. 3C) receptors. The respective EC_{50} values of 2.5 x 10^{-11} M and 8.3 x 10^{-12} M were similar to those previously reported for oPL (7) bPL, hGH (21) and cPL (9), and the maximal activity of those three hormones was equal. The oPL analogue T185D acted as a partial agonist in both types, and the respective EC_{50} values were 1000-fold and 60-fold higher as compared with oPL. In contrast, the oPL G130R was devoid of any agonistic activity (Fig. 3, A and C). In both cell lines, stimulated for proliferation with 1.8 x 10^{-10} M oPL, the oPL G130R analogue acted as a weak antagonist with an IC_{50} of 4.3 x 10^{-8} M in the case of FDC-P1–3B9 cells (Fig. 3B) and 3.0 x 10^{-8} M in the case of FDC-P1–9D11 cells (Fig. 3D).

**Diverse Activity of oGH, oPL, cPL, bPL, T185D, oPL G130R, bPL T188D, bPL K73F, and oPL G133R in 293 Cells Transiently Transfected with hGH and oGH Receptors**—Ovine PL stimulated the expression of LHRE promoter-linked luciferase activity in cells transfected with hGHR. The respective EC_{50} values for oPL and hGH were 5.3 x 10^{-10} M and 2.7 x 10^{-10} M, and the maximal activities of both hormones were equal (Fig. 4A). The analogue oPL T185D exhibited agonistic
activity 18 times weaker, and oPL G130R was not active at all (Fig. 4A). The activity of oGH had in this model was very low (EC\textsubscript{50} = 6.6 \times 10^{-3} M; data not shown), consistent with the inability of ruminant GHS to recognize primate GHR (38). In cells stimulated for growth with 4.35 \times 10^{-2} M oPL, the oPL G130R analogue acted as an antagonist with an IC\textsubscript{50} value of 3.7 \times 10^{-8} M (Fig. 4B), similarly to its action in FDC-P1 cells stably transfected with hGHR (Fig. 3D). On the other hand, in cells transfected with oGHR, only oGH acted as an agonist (EC\textsubscript{50} = 1.3 \times 10^{-9} M), whereas oPL, oPL T185D, and oPL G130R were not active at all (Fig. 4C). The 5-fold difference in the maximal induction of cells transfected with hGHR and
oGHR does not likely indicate a difference in signaling activity, but rather results from the fact that in the transfection of the latter, 25-fold higher amount of cDNA (2.5 mg versus 0.1 mg) was used. In 293 cells stimulated with 4.35 x 10^-9 M oGH, both oPL and oPL G130R (but not oPL T185D) exhibited antagonistic activity, and the respective IC50 values were 6.1 x 10^-8 M and 3.5 x 10^-8 M. The activity of bPL and cPL was similar to that of oPL. In 293 cells transiently transfected with hGHR, they acted as agonists (Fig. 5A) with the respective EC50 values of 4.9 x 10^-10 M and 3.2 x 10^-10 M, whereas in 293 cells transfected with oGHR, they were not active (Fig. 5B) and acted as antagonists (Fig. 5C). Interestingly, the antagonistic activity of bPL in this experiment (IC50 = of 5.3 x 10^-9 M), was higher than that of cPL (IC50 = of 1.2 x 10^-7 M), and even than that of oPL (IC50 = of 1.9 x 10^-8 M; data not shown). Bovine PL T188D, K73F, and G133R analogues were not active in 293 cells transfected with oGHR, similarly to bPL (data not shown). However, bPL G133R analogue was a potent antagonist, K73F was a weak antagonist, and T188D analogue, similarly to oPL T185D, has no antagonistic activity (Fig. 5D).

DISCUSSION

The main results of the present study that were summarized in Table I show that all three ruminant PLs acted as antagonists in 293 cells transiently transfected with full-size oGHR. The antagonistic activity cannot be attributed to an improper refolding, as all three molecules were active in the lactogenic receptor-mediated Nb2 rat lymphoma cell bioassay and in FDC-P1 cells stably transfected with h- or rbGHRs (see above and Refs. 7, 9, and 18–22). The finding that all three PLs were agonists in both FDC-P1 and 293 cells transfected with hGHRs further emphasizes that the activity is dependent upon the chosen type of receptor and not upon the cell model. We also found that recombinant or native oPLs lack the ability of oGH to inhibit the insulin stimulation of lipogenesis in homologous adipose tissue. Furthermore, oPL could not inhibit this oGH activity. In contrast, all three ruminant PLs exhibited PRL-like activity not only in a heterologous Nb2 cell proliferation bioassay (7, 9, 18), but also in ovine mammary gland explants or ovine acini culture bioassays based on stimulation of β-casein synthesis (9) and in 293 cells transiently transfected with ovine and bovine full-size PRLR. Taken together, these observations along with our previously presented findings provide a new paradigm. In heterologous systems ruminant PLs act as GHRs' agonists, whereas in homologous systems they are either inactive or they act as antagonists.

The proposed notion is fully supported by the present and former binding and interaction studies. Whereas oPL, bPL, and cPL were capable of forming 1:2 complexes with hGHR-ECD (see Fig. 1, and Refs. 7, 9, 19, and 21) or rbGHR (Fig. 1), oPL (Fig. 1) and bPL (23) formed only 1:1 complexes with homologous GHR-ECDs. Since GH-induced homodimerizations of
GHRs are sequential (39), we suggest that the ability of oPL’s site II for homologous interaction is compromised and they act as site II antagonists (40), thus interacting with the receptors exclusively through site I. Binding studies (Fig. 2) along with the results of Gluckman’s group (16) and ours (7, 9, 21, 22) suggest that oPL, cPL, and bPL bind with high affinity to membrane-embedded somatogenic receptors and to GHR-ECDs. We conclude that this binding occurs through oPL’s site I only. More detailed arguments for this notion were presented under “Results” and in our recent paper describing the activity of hGH des(1–6,14) analogue (34).

The results obtained with oPL analogues also support our hypothesis. The oPL analogues T185D, which exhibited weak mitogenic activity in FDC-P1 cells transfected hGHR or rbGHR, also had a reduced capacity (Fig. 2, A and B) or no ability at all (Fig. 2C), to compete with the ligand for the binding to human, rabbit, or ovine GHR-ECD (Fig. 2). This analogue was a weak antagonist in 293 cells transfected with human GHRs (Fig. 4A), but was inactive and did not antagonize oGH activity in 293 cells transfected with oGHR (Fig. 4, C and D). In contrast, the oPL analogue G130R and bPL analogue G133R that form a 1:1 complex with GHR-ECDs (Fig. 1, and data not shown) and bind to human, rabbit, or ovine GHR-ECD with affinity similar to that of wild-type oPL (Fig. 2 and Ref. 22), were not active in cells transfected with human, rabbit, and bovine GHRs, but were antagonistic in all of them (Figs. 3 and 5, and Ref. 22).

The key event that leads to biological response for GHR and PRLR is receptor homodimerization (41), which initially occurs via binding of the ligand to two extracellular domains of these receptors. The extracellular domains of these receptors (42, 43) consist of two FBN-III type domains (D1 and D2) with a short helical region connecting them. The interdomain flexibility, as well as the variation of binding loop conformation, contributes to the structural plasticity of the receptor to form either the cognate or coincidental hormone-receptor complexes. Receptor interdomain flexibility has already been observed in the structures of hGH:hGHR (42), hGH:PRLR (43), and oPL:rPRLR (6).

The structural and biochemical aspects of the hGH:hGHR complex have been extensively studied (42, 44, 45), and the hGHR is activated via sequential dimerization (11, 40), as was also shown for erythropoietin receptor (46). The initial GH:GHR 1:1 complex must have the correct orientation to allow binding of receptor 2 to both the GH site II and receptor 1, otherwise, dimerization would not occur, and signaling will be abolished. Mutations on GH site II (40) or in the receptor-receptor interface (47) lead to non-productive complexes. In the structure of the active oPL-rPRLR 1:2 complex, a similar homodimerization has been observed (6), and we assume that a similar mode of hormone-receptor assembly will occur in the PL, GH active receptor complexes. The results that ruminant PLs act as agonists toward both hGHR and rbGHR, and as antagonists to oGHR (Table I) can clearly be attributed to their capability of induce either 2:1 active or 1:1 inactive complexes. The sequence alignment (Fig. 6) shows that there are several notable differences, especially in the binding loops, between human or rabbit, and ovine or bovine GHRs. The main differences are as follows: a four-residue deletion can be observed at the L2 loop (residues 77–80), two substitutions at the L3 loop (I103V, I105T), and a single substitution at L4 (E127D). The L2 binding loop in the hGHR contributes very little to the binding of hGH, and a substantial segment of it does not have clear electron density maps, which may indicate high flexibility. The other three residues, Ile-103, Ile-105, and Glu-127, are part of the GHR functional epitope (48) and interact with both sites of the hGH. This may lead to the assumption that these differences in primary structures, especially in conserved binding determinants, may attribute the different biological activities. It is most probable that the overall orientation, in which the coincidental 1:1 PLs-oGHR complexes exist, does not permist the correct recognition of receptor 2 that leads to the formation of active 2:1 complexes.

Our present results raise again the question about the physiological role of oPL and bPL in vivo and the way ruminant PL signal is transduced. Several studies aimed at the elucidation of oPL and bPL binding sites in homologous maternal and fetal liver have been extensively reviewed (13, 14). In fetal liver, specific PL binding sites were detected (16, 17, 49, 50), but their molecular nature has not yet been identified and an attempt to purify a unique oPL receptor was also not successful. We (51) and others (52) have demonstrated that bovine endometrium microsomes contain high affinity binding sites for bPL, which have low affinity for either bGH or bPRL. However, the interaction of homologous PRLs with their receptors is very transient and may be overlooked in classical binding studies (53). Therefore, the physiological relevance of the findings that suggested that oGH and oPL bind to a common receptor (16) and the demonstration of oGHR mRNA in fetal liver (15, 17, 49) is not yet clear. The in vivo activity of ruminant PLs in homologous species was also extensively reviewed (13, 14). It was suggested that PLs are involved in the mammotropic action and in maternal and fetal metabolism, but the receptors that transduce this effect have not yet been identified. Recent studies (54–57) indicate that oPL in vivo exhibits oGH-like mam-

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**TABLE I**

Biological activity of oPL, bPL, cPL, oGH, and o/bPL analogues in vitro and stoichiometry of their complexes with oGHR-ECD or bGHR-ECD

| Hormone | Bioassays in cell lines | Complex with oGHR-ECD |
|---------|------------------------|-----------------------|
|         | FDC-P1                 | FDC-P1                | 293 | 293 | Nb2 |       |
| oGH     | ++ ++ + + ++ ++ ++     | None                  |     |     |     |       |
| oPL     | ++ ++ + + + + ++ ++    | NT                    |     |     |     |       |
| oPL T185D | +  +  +  +  +  + +     | ANTG                  |     |     |     |       |
| oPL G130R | ANTG   ANTG ANTG       | ANTG                  |     |     |     |       |
| bPL     | ++ ++ + + + + + + + +  | None                  |     |     |     |       |
| bPL T185D | +  +  +  +  +  + +     | ANTG (w)              |     |     |     |       |
| bPL G133R | ANTG   ANTG NT         | ANTG                  |     |     |     |       |
| cPL     | ++ ++ + + + + + + + +  | NT                    |     |     |     |       |

| Hormone | Bioassays in cell lines | Complex with oGHR-ECD |
|---------|------------------------|-----------------------|
|         |                        |                       |

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*6 A. M. de Vos, P. A. Elkins, and H. W. Christinger, unpublished results.

*7 H. Leibovitch, E. Gootwine, and A. Gertler, unpublished results.*
mogenic and growth-promoting activity in pseudo-pregnant ewes and lambs, but completely lacks the GH-like galactopoietic properties. Similar result were obtained in cows, although in that case weak galactopoietic activity was reported (58).

Staten et al. (25) reported that bPL forms only a 1:1 complex with the bGHR-ECD, raising the question of whether bPL activates homologous somatogenic receptors. The same group has recently reported that bPL was not active and antagonized homologous GHR, unless they act as antagonists, but the signal is transduced through homologous PRLRs; (hGHR-ECD)2 contacts (hGHR-ECD)2; and (hGHR-ECD)1 contacts: site II; $\alpha$, site II only. ECD-ECD contacts: $++, +, ECD 1; xx ** ++ +$.

The findings showing that ruminant PLs are capable of signaling through oPRLR show that alternative educated choice. The findings showing that ruminant PLs are suggested (14). Our present state of art does not allow an specific tissues or under unique physiological conditions as ECD in a way that allows dimerization of GHR is expressed in heterodimerizing bGHR- and bPRLR-ECDs,8 but obviously do face plasmon resonance hint that bPL and oPL are capable of feasible. Preliminary results based on kinetic analysis by sur-

Fig. 6. Comparison of the primary structures of human, rabbit, ovine, and bovine GHR-ECDs. $\beta$ strands in domain I are marked $\alpha$–$g$, and in domain 2, $\alpha$–$g$, disordered x-tal structure. hGH-(bGHR-ECD)$_2$ structures (L1–L6) were marked according to Refs. 52 and 52: $\rho$, sites I and II; $\delta$, site I only. ECD-ECD contacts: $++, +, ECD 1; xx ** ++ +$, both ECDs. Amino acids that are identical in ovine and bovine GHR-ECDs but differ-

tant in human and rabbit GHR-ECDs are marked with bold letters. Missing amino acids are marked by dots.
