Interaction of Lactogenic Hormones with the Soluble Extracellular Domain of Prolactin Receptors (43758)

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Abstract. **Two variants of rabbit prolactin receptor extracellular domain (rbPRLR-ECD) were prepared using insect/baculovirus (amino acids 1–198) and *E. coli* (amino acids 4–210) expression systems. Bovine PRLR-ECD (bPRPL-ECD amino acids 1–210) and human growth hormone receptor ECD (hGHR-ECD amino acids 1–246) were also prepared using *E. coli* expression system. All four proteins were purified as monomers with >95% homogeneity. Their affinity for various lactogenic and somatogenic hormones was determined by binding assays. The stoichiometry of complex formation with these hormones was studied by gel filtration on a Superdex 75 column, and bioactivity was determined by *in vitro* bioassays. The results summarized in this paper indicate that, in contrast to hGHR-ECD, in which the ability to form a 2:1 complex with hGH is indicative of the biological activity of the hormone, the ability or inability of prolactin and placental lactogen to form 2:1 complexes with rb or bPRLR-ECD cannot predict their biological activity. This conclusion does not preclude however, hormone- or antibody-induced dimerization of the membrane-embedded receptor.**

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Prolactin (PRL) initiates its biological action by binding to its specific receptor on the membranes of target cells. Nb_2 lymphoma cells, which are absolutely dependent on lactogenic hormones for their proliferation (1, 2), have proven to be a sensitive *in vitro* cell model for studying the mechanism of the mitogenic action of lactogenic hormones, such as PRL or human growth hormone (hGH) (3). We have found that monoclonal antibodies (mAbs) T6, U5 and U6 raised against PRL receptors in rat liver, exhibited mitogenic activity in the rat lymphoma lactogen-dependent Nb_2 cells and anti-mouse IgG enhanced their mitogenic effect. In contrast, the Fab fragment of T6 was not mitogenic, but its mitogenicity could be restored by anti-mouse IgG (4). At the conclusion of these studies, we suggested that the dimerization or oligomerization of the lactogen receptor in Nb_2-11C cells is an obligatory step in the transduction of the mitogenic signal (4).

Recently, hGH-induced dimerization of recombinant, nonglycosylated hGH receptor's extracellular domain (hGHR-ECD) containing amino acids (aa) 1–238 was documented (5). Crystallographic analysis of hGH and its complex with hGHR-ECD confirmed this finding, and two nonsymmetrical binding sites in hGH were identified (6). A 2:1 stoichiometry of hGHR-ECD:hGH interaction was also observed in our lab, using full-length (aa 1–246) hGHR-ECD (7). An antibody-induced receptor dimerization also initiated the mitogenic signal in a leukemia cell line expressing a hybrid receptor composed of the ECD of hGHR linked to the transmembrane and intracellular domains of murine granulocyte colony-stimulating factor receptor (8). Since this receptor also belongs to the family of cytokine/GH/PRL receptors (9), ligand-induced receptor dimerization was suggested to be common to this

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entire family (8). The finding that the hGH-induced mitogenic response of Nb_2 cells was attenuated at very high hGH concentrations was interpreted as indirect evidence for receptor dimerization by a single hormone molecule (10).

Since the ability of hGH-induced dimerization of the hGHR-ECD was suggested as evidence for dimerization of the full-size receptor and subsequent initiation of signal transduction (5–8, 10), we decided to study whether hormone-induced receptor dimerization also occurs in the PRL:PRLR-ECD interaction. To achieve this goal, the following recombinant ECDs were prepared, expressed, purified, and assayed: a construct encoding for rbPRLR-ECD (aa 1–198), prepared in an insect/baculovirus expression system and three constructs encoding for rbPRLR-ECD (aa 4–210), for bPRLR-ECD (aa 1–210) and for hGHR-ECD (aa 1–246), prepared in an *E. coli* expression system.

Studies with Recombinant rbPRLR-ECD Expressed in Insect Cells (11, 12)

The rbPRLR-ECD was expressed in an insect/baculovirus expression system (11) and then purified by affinity chromatography on immobilized PRL followed by gel filtration (12). The purified protein was over 90% homogeneous as indicated by SDS-PAGE and by gel filtration. Its molecular mass determined by SDS-PAGE was 32 kDa, and by gel filtration, 27 kDa. Both values were higher than the 22.8 kDa deduced from the cDNA sequence, indicating extensive glycosylation. The K_a value for interaction with ovine (o) PRL was 25.4 nM^{-1}. The stoichiometry of the interaction between purified rbPRLR-ECD and oPRL was studied by gel filtration on a Superdex™75 HR 10/30 column. At a 1:1 ratio of rbPRLR-ECD:oPRL, over 90% of the applied protein appeared as a complex with a molecular mass of ca 50 kDa, consistent with the predicted value. Raising the rbPRLR-ECD:oPRL molar ratios to 5:1 did not change the stoichiometry of the interaction. Similar stoichiometry was also observed when 10:1 and 20:1 rbPRLR-ECD:[^{125}I]oPRL or rbPRLR-ECD:[^{125}I]hGH ratios were applied. In control experiments carried out under identical experimental conditions, interaction of hGHR-ECD with hGH indicated formation of 2:1 complex. These results raised the question as to whether the inability of the rbPRLR-ECD to form 2:1 complexes with lactogenic hormones could be attributed to the fact that it has a C-terminal truncated domain, or if it was improperly glycosylated in the insect/baculovirus expression system? To answer this, variants of rbPRLR-ECD encoding for aa 4–210 and a full size bPRLR-ECD were prepared in *E. coli*. Both variants contained the full C-terminal sequence and were nonglycosylated.

Studies with Recombinant rbPRLR-ECD Expressed in *E. coli* (13)

The cDNA of the rbPRLR-ECD was obtained by PCR using rbPRLR cDNA (14) and inserted into the prokaryotic expression vector pTrc99A. This construct was transfected into *E. coli* strain W3110, which expressed the unfused protein after induction by isopropyl β-D-thiogalactopyranoside. The expressed rbPRLR-ECD protein, contained within the refractile body pellet, was solubilized, refolded and purified on a Q-Sepharose column (13). The yield of bioactive monomer was 40 to 50 mg/3 l of induced culture. The purified protein was over 98% homogeneous, as shown by SDS-PAGE and by chromatography on a Superdex column. Its molecular mass determined by SDS-PAGE in the absence of reducing agent was 25 kDa, and by gel filtration, 22 kDa.

Binding experiments, using the purified rbPRLR-ECD and [^{125}I]oPRL as a tracer, revealed that the abilities of oPRL, hGH, Des-7-hGH, and bovine placental lactogen (bPL) to compete with the radioligand gave IC_{50} values of 0.12–0.14 n*M*, whereas the IC_{50} values of Des-13 bPL, hPRL, and hPL were slightly higher. Scatchard analysis using the homologous hormone (oPRL) yielded a linear plot. The K_a value (25 nM^{-1}) was close to that of the purified rbPRLR-ECD prepared in the insect/baculovirus expression system (12). The K_a of the purified rbPRLR-ECD for oPRL was, however, an order of magnitude higher than those of truncated or full-size membrane-inserted receptors (11). Thus, whereas truncation at the ECD/membrane boundary had a major effect on K_a, neither truncation of the 12 carboxy-terminal amino acids, nor glycosylation, affected affinity. Binding experiments revealed that both rbPRL and porcine (p) PRL were 1000-fold less effective than oPRL. Since the biological activity of rbPRL in a rabbit mammary gland explant bioassay is only slightly lower than that of oPRL, its binding affinity for the soluble ECD does not serve as a predictive parameter of its biological activity.

The stoichiometry of the interaction between purified rbPRLR-ECD and oPRL (Fig. 1A), revealed that the oPRL:rbPRLR-ECD complex, which was eluted at a retention time (RT) of 11.41–11.44 min, was separated from the other components. Its molecular mass, calculated using several pure proteins as markers, indicated formation of a 1:1 complex with of ca 44 kDa, consistent with the predicted value. Raising the rbPRLR-ECD:oPRL ratio to 2:1 or 3:1 did not change the molecular mass of the complex, but the size of the 22-kDa peak (RT = 12.79–12.81 min), consisting of excess rbPRLR-ECD, gradually increased. In control experiments in which the interaction between purified hGHR-ECD and hGH was studied, formation of a 2:1

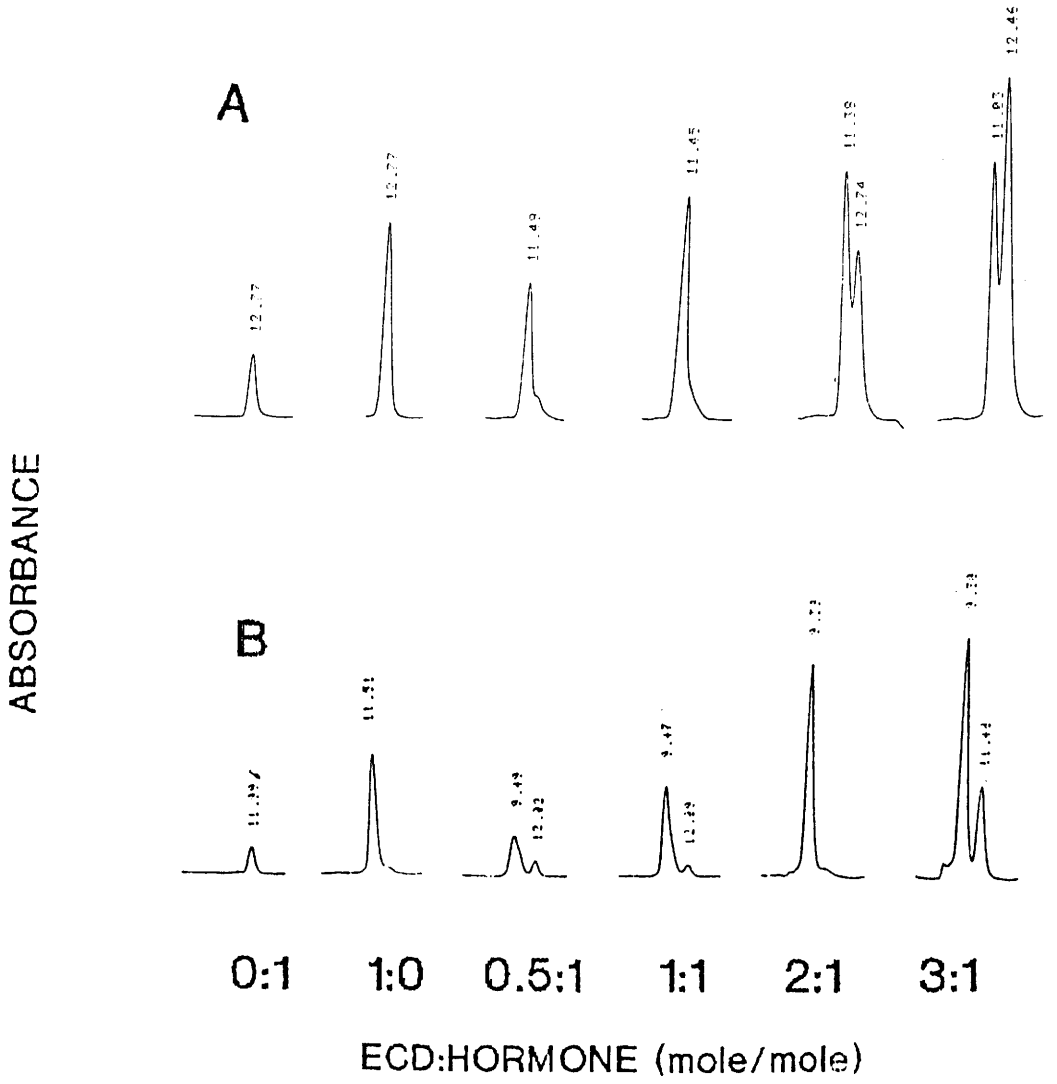

Figure 1. Gel filtration of rbPRLR-ECD:oPRL (A), hGHR-ECD:hGH (B) complexes on a Superdex™75 HR 10/30 column. Complex formation was carried out during 30–60 min of incubation at room temperature, using various rbPRLR-ECD:hormone ratios. Aliquots (200 μl) of the incubation mixture were applied to the column and complex formation was monitored by absorbance at 214 nm (A) and 280 nm (B). The column was developed at 1 ml/min. Each experiment was conducted at least three times.

complex was clearly indicated (Fig. 1B). Elution profiles and stoichiometries of a 1:1 interaction were also observed with rbPRL, pPRL, hPRL, hGH, Des-7 hGH, hPL and Des-13 bPL. A 1:1 stoichiometry of interaction in crystals of hPRLR-ECD with hGH has also been recently reported (15). The only exception was bPL, whereas a 2:1 rbPRLR-ECD:bPL stoichiometry was found. Similar stoichiometries of bPL (2:1) and all other hormones (1:1), were also found by interacting oPRL, hGH, bPL and Des-13 bPL with radiolabeled rbPRLR-ECD, an experiment in which higher ECD:hormone ratios could be achieved in the preincubation mixture. It seems therefore that truncation of bPL by 13 N-terminal amino acids changed the interaction stoichiometry from 2:1 to 1:1, indicating that the N-terminal portion of bPL participates in the second binding site. The N-terminal domain of bPL that extends beyond the putative first α-helix (6, 16) consists of 19 amino acids, as compared with five amino acids in hGH or pGH and 14 amino acids in oPRL (17). Therefore truncation of the 13 N-terminal amino acids of native bPL should not interfere with the integrity of the first α-helix as indicated by CD spectra bPL and Des-13 bPL (18). Despite the difference in the stoichiometry of interaction with rbPRLR-ECD, the *in vitro* lactogenic receptor-mediated activity of the Des-13-bPL was only slightly reduced in Nb_2 cells (18) and in rabbit mammary gland explants (13), whereas its somatogenic receptor-mediated activity was approximately 2-fold higher in 3T3-F422A rat preadipocytes (18), or rat hepatocytes (19). To verify whether the 1:1 rbPRLR-ECD:PRL complex also occurs in mammalian cells, an interaction of [^{125}I]oPRL with the glycosylated rbPRLR-ECD secreted into conditioned media

by COS 7 cells transiently transfected with plasmid pE, encoding for rbPRLR-ECD (20), was also tested. The molecular mass of the complex corresponded to 60–64 kDa. Since the glycosylated rbPRLR-ECD has a molecular mass of 38 kDa, this result is consistent with 1:1 complex formation.

The biological activity of the tested hormones mediated through membrane-embedded rbPRLR was determined by measuring β-casein synthesis in mammary gland explants from pseudopregnant rabbits. All hormones significantly ($P > 0.05$) stimulated β-casein synthesis above control levels. Ovine PRL, hGH, and bPL were more potent than the other hormones, whereas Des-7 hGH, Des-13 bPL, and rbPRL were slightly less active (13).

Studies with Recombinant bPRLR-ECD Expressed in *E. coli*

Recombinant bPRLR-ECD was subcloned from the full-size bPRLR cDNA and produced in *E. coli* using the same expression system as described for bPL (21). The expressed protein was refolded and purified to over 95% homogeneity by a procedure similar to that used for rbPRLR-ECD. The purified monomeric bPRLR-ECD interacted with either oPRL or hGH in a 1:1 stoichiometry yielding a stable complex, even in excess of bPRLR-ECD.

Binding experiments revealed that the purified bPRLR-ECD could also bind either [^{125}I]hGH or [^{125}I]oPRL with the same efficiency and hGH or oPRL could mutually compete with both tracers, exhibiting almost identical IC_{50} values (unpublished data). This result is of particular interest in view of our former findings, which documented that membrane-associated (22) or solubilized and partially purified (23) bPRLR exhibits 30- to 40-fold lower affinity for oPRL than for hGH. Thus, truncation of the ECD/membrane boundary increased the affinity toward oPRL, with almost no effect on the affinity toward hGH. Unlike the case of rbPRLR-ECD presented above, bPL exhibited a very low affinity toward bPRLR-ECD. These results explain the impaired ability of bPL to form a stable complex with bPRLR-ECD (unpublished data) and the observations that bPL is a selective GH but not a PRL agonist *in vivo* in dairy cattle (24).

Studies with the Plasma-Derived Extracellular Domain of hGHR

The stoichiometry of interaction between [^{125}I]hGH and plasma-derived hGHR-ECD was examined in plasma samples from two healthy subjects. The reaction was carried out in 10 m*M* Tris-HCl buffer, pH 7.4, containing 10 m*M* $MgCl_2$ and 125 m*M* NaCl. After a 30-min incubation at room temperature, 200-μl aliquots were applied to a Superose 12 10/30 column (Pharmacia, Uppsala, Sweden), which had been pre-equilibrated with the same buffer. As shown in Fig. 2, the peak of the specifically bound hormone was eluted in both cases between 23.0 and 23.5 min. Identical results were obtained with plasma from four other healthy subjects (not shown). In one of the subjects (Fig. 2A), a specifically bound shoulder (RT = 26 min) also appeared. The nature of this complex is not clear. The column was calibrated with IgG (RT = 21.04–21.12 min) and albumin (RT = 23.29–23.32 min).

To examine whether radioiodination of hGH may effect its ability to form a 2:1 hGHR-ECD:hGH complex, [^{125}I]hGH was preincubated with an excess of recombinant hGHR-ECD (in the absence or presence of excess unlabeled hGH) and chromatographed on a Superdex column under identical conditions. RTs of the complexes with iodinated and the noniodinated hGH were identical (12).

The RT of the hGHR-ECD:[^{125}I]hGH complex indicated a molecular mass corresponding to 75–80 kDa. Assuming that the glycosylated hGHR-ECD has a molecular mass of 56–61 kDa (25), this result indicates formation of a 1:1 hGHR-ECD:[^{125}I]hGH complex, in contrast to the 2:1 complex formed with the purified

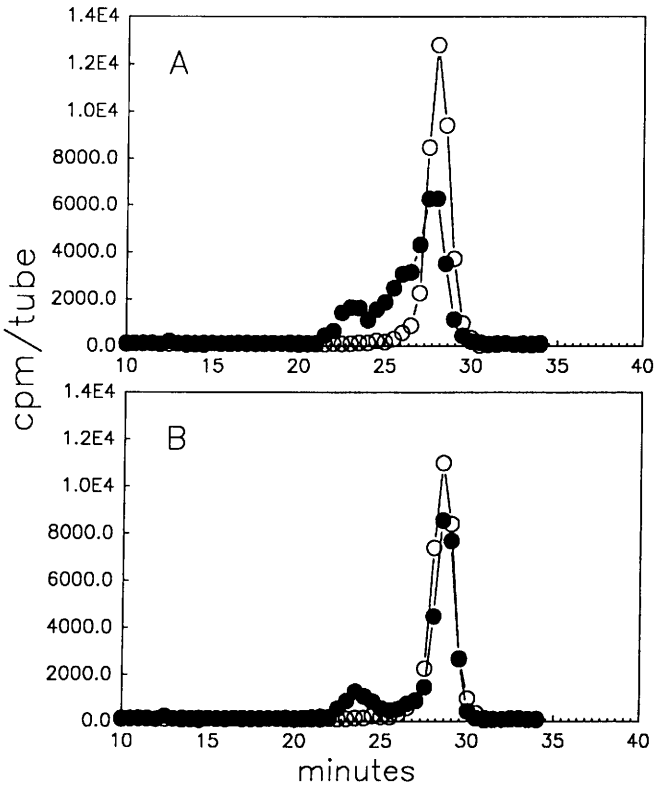

Figure 2. Separation of a complex between [^{125}I]hGH and the plasma-derived hGHR-ECD from two healthy subjects (A and B) on a Superose 12 column. Preincubation was carried out at room temperature for 30 min in the absence (●) or the presence (○) of the excess of nonlabeled hGH, prior to the application on the column. The column was developed at 0.5 ml/min at room temperature, and 0.25 ml fractions were collected. The radioactivity was determined using Kontron gamma counter. For other details see text.

recombinant nonglycosylated hGHR-ECD (5, 7, 12). This difference is not understood, but may be due to the following: (i) despite our finding that the radiolabeled hGH is capable of forming a 1:2 complex with the recombinant nonglycosylated hGHR-ECD, the glycosylated hGHR-ECD may nevertheless behave differently; (ii) a complex containing a heavily glycosylated hGHR-ECD could be retarded by the Superdex column. However, the fact that gel filtration, cross-linking, and Western blots all resulted in a similar molecular mass assessment (25) argues against the latter possibility. Therefore, the question of whether the naturally occurring glycosylated hGHR-ECD can form a 2:1 complex with hGH remains unanswered.

Conclusions

Results of our and other studies have been compiled to evaluate whether biological activity in the respective *in vitro* or *in vivo* models is related to the particular hormone's ability to dimerize the ECD of its corresponding receptor (Table I). Formation of 2:1 ECD:hormone complexes occurs mainly with hGH and bGH interacting with somatogenic receptor-derived ECDs, while PRLs and their respective R-ECDs form only 1:1 complexes, even when the PRL-dependent, PRLR-mediated biological response is well established. The only exception is the recent report, in which formation of 2:1 rat liver PRLR-ECD:oPRL was suggested (26). The interpretation of these results is, however, problematic due to the fact that in gel filtration experiments, the molecular mass of the purified ECD indicated existence of a dimer. In the case of bPL, both 1:1 and 2:1 complexes were found, irrespective of full biological activity in the related system. An unexplained observation is that naturally occurring serum-derived glycosylated hGHR-ECD forms only 1:1 complexes with hGH, in contrast to its recombinant, nonglycosylated analogue.

In view of these results, we postulate that the ability or inability of the tested PRLs or bPL to form 1:2 complexes with their respective soluble R-ECDs cannot predict their biological activity. This postulate does not preclude however, the occurrence of hormone- or anti-R Ab-induced dimerization of membrane-embedded PRLRs.

Although no conclusive explanation exists for the disparity in the results regarding the PRLR-ECDs presented in this review and the results reported for hGHR-ECD (5–7), two possible hypotheses may be proposed.

(A) formation of the 1:2 hGH:hGHR-ECD complex is facilitated by a double hydrogen bond between the hydroxyl moiety of Tyr^{200} in the first ECD molecule and between $Asp^{152}O\delta 2$ and $Ser^{201}O\gamma$ in the second. In the rb and bPRLR-ECDs, Tyr^{200} is substituted by Leu^{170} (14, 27, 28) which is incapable of forming these hydrogen bonds. Lack of these bonds could hamper formation of the 1:2 complex with the soluble ECD, whereas in the membrane-embedded receptor such a complex could occur because dimerization is stabilized by other interactions within the membrane or cytosolic domains.

(B) mAb A917 is capable of inhibiting binding of $[^{125}I]$oPRL to the membrane-embedded receptor, but not to the soluble rbPRLR-ECD expressed in insect/baculovirus (11, 12) or *E. coli* expression systems (13). This implies that the conformations of the soluble and membrane-embedded ECDs differ. This suggestion is

Table I. Compilation of Current Data on the Interaction of GHR-ECD and PRLR-ECDs with Lactogenic or Somatogenic Hormones

ECD	Hormone tested	Stoichiometry of the ECD:hormone complex	Biological activity of the hormone[a]	Reference
rbPRLR-ECD (1–198 gp)	oPRL, hGH	1:1	Yes (L)	(12)
rbPRLR-ECD (4–210)	oPRL, hGH, rbPRL, pPRL	1:1	Yes (L)	(13)
	Des-7 hGH, Des-13 bPL	1:1	Yes (L)	(13)
	bPL	2:1	Yes (L)	(13)
	hPRL,[b] hPL	1:1	ND	Present paper
bPRLR-ECD (1–210)	oPRL, hGH	1:1	Yes (L)	Present paper
hPRLR-ECD (1–210)	hGH	1:1	? (L)	(15)
rPRLR-ECD (1–210)	oPRL	2:1	? (L)	(26)
hGHR-ECD (1–246)	hGH, bPL	2:1	Yes (S), Yes (L)	(7, 13, 29)
	Des-7 hGH	1:1	Nil (S), Yes (L)	(7, 13, 29)
hGHR-gpECD	hGH	1:1	? (S)	Present paper
hGHR-ECD (1–238)	hGH	2:1	Yes (S)	(5)
	Des-7 hGH	1:1	ND	(5)
bGHR-ECD	bGH	2:1	Yes (S)	(16)
	bPL	1:1	Yes (S)	(16)

[a] Mediated through lactogenic (L) or somatogenic (S) receptor; gp, glycoprotein.
[b] (30).

further substantiated by the finding that soluble rbPRLR-ECD exhibits 10-fold higher affinity toward oPRL than the membrane-embedded receptor (11).

Although no documented model can be suggested at present to explain the interactions of PRL or other lactogenic hormones with PRLR, at least three possibilities explaining the present results can be proposed: (i) PRL (like GH with GHR-ECD) interacts simultaneously with two membrane-embedded PRLRs, but with only one PRLR-ECD, due to conformational changes in the latter; (ii) PRL (unlike GH with GHR-ECD) interacts with one PRLR, subsequently forming a 2:2 complex due to the interaction between two PRLR cytosolic or extracellular domains, thus leading to the formation of a dimerized receptor; (iii) PRL (unlike GH with GHR-ECD) interacts with only one PRLR, leading to a further reaction with another as yet unidentified membrane protein and formation of a tertiary complex with a second PRLR.

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