Cloning, preparation and characterization of biologically active recombinant caprine placental lactogen

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Abstract

Caprine placental lactogen (cPL) cDNA was cloned by reverse transcription (RT)-PCR from total RNA of goat placenta. The PCR product encoding for the mature protein was gel purified, ligated to pGEM-T and finally subcloned into a pET8c prokaryotic expression vector. E. coli cells (BL-21) transformed with this vector over-expressed large amounts of cPL upon induction with Isopropyl-1-thio-β-D-galactopyranoside. The expressed protein, found in the inclusion bodies, was refolded and purified to homogeneity on Q-Sepharose and SP-Sepharose columns, yielding two electrophoretically pure fractions (cPL-Q and cPL-S), composed of over 98% of monomeric protein of the expected molecular mass of ~23 kDa. Binding of cPL to the extracellular domain (ECD) of prolactin receptors (PRLR) from rat (r), rabbit (rb), and bovine (b), growth hormone receptors (GHR) from human (h) and rabbit, and binding to rabbit mammary gland membranes revealed similar binding profiles for cPL-Q, cPL-S and ovine (o)PL. Caprine PL was capable of forming 1:2 complexes with hGHR-ECD, rbGHR-ECD, rPRLR-ECD and rbPRLR-ECD whereas with bPRLR-ECD only a 1:1 complex was detected. The biological activity of both cPL fractions resulting from proper renaturation was further evidenced by their ability to stimulate proliferation of Nb2 cells, FDC-P1 cells transfected with rabbit or human GHRs and by stimulation of β-casein synthesis in rabbit and ovine mammary gland acini cultures.


Introduction

Placental lactogens (PLs) are secreted from placenta of primates, rodents and ruminants into the maternal and fetal circulations. Those polypeptide hormones, sharing various degrees of homology, are structurally related to the growth hormone (GH)/prolactin (PRL)/cytokine family of hormones (Wallis 1992). They are usually 22–23 kDa proteins, and some of them are glycosylated yielding higher molecular masses (Forsyth 1986). All members of this family exhibit a similar mechanism of action and receptor activation via homo- or heterodimerization of the receptor extracellular domain and subsequent transphosphorylation of receptor-associated JAK2 or other related kinases (Goffin & Kelly 1997).

Although the specific biological functions of PLs are not understood, they probably have multiple biological effects, similar to GH and PRL. The level of PL in maternal and fetal blood is highly species dependent, e.g. the concentration in ewes is relatively high, whereas in cows it is very low (Byatt et al. 1987). It has been suggested that PL may function as a unique fetal growth hormone, based on findings that the ovine (o) fetus responds to oPL. This response includes stimulation of glycogen synthesis, amino acid transport, cellular proliferation and insulin-like growth factor (IGF-I) synthesis. These biological effects in the fetus are only slightly, or not at all, affected by ovine or human (h) GHs or oPRL, suggesting that oPL may have specific effects (for review see Anthony et al. 1995). We have found that bovine (b) PL and oPL are capable of acting through prolactin receptors (PRLRs) in bovine (Shamay et al. 1990) and ovine (Sakal et al. 1997) mammary glands as well as in the rat Nb2 lymphoma cell line (Byatt et al. 1990, Sakal et al. 1997). We have also shown by binding experiments that unique bovine placental lactogen receptors (PRLRs) are present in the endometrium of pregnant cows (Galosi et al. 1991). Thus, bPL and most likely also oPL (Anthony et al. 1995) are unusual in that they are capable of recognizing and subsequently exhibiting their biological activity through three different types of receptors: (i) specific PLRs which it has been suggested exist mainly in fetal tissues (Freemark & Comer 1989), (ii) growth hormone receptors (GHRs) and (iii) PRLRs.

Caprine placental lactogen (cPL), similar to oPL, was increased during pregnancy, and its level during weeks 16–20 of pregnancy correlated with postpartum milk yield (Forsyth 1986). The endocrine profile during caprine
Materials and Methods

Materials

Recombinant hGH was obtained from Biotechnology General Inc. (Ness-Ziona, Israel). Recombinant non-glycosylated rabbit (rb), rat (r) and bovine (b) PRLR extracellular domains (ECDs), hGHR–ECD and oPL were prepared as described previously (Bignon et al. 1994, Sandowski et al. 1995, Tchelet et al. 1995, Sakal et al. 1997). Rabbit GHR–ECD was also prepared in our laboratory and its preparation will be described elsewhere. In order to clone cPL we used the known sequence of oPL and have found that cPL shows 85% homology to oPL. It enabled us to clone, express and purify cPL to homogeneity and thus to compare its biological activity with that of oPL, which we have studied before (Sakal et al. 1997).

Expression, refolding and purification of cPL

Transformed E. coli BL21 cells were grown in Terrific Broth medium to OD_{600}=0.9 and IPTG was added to a final concentration of 0.4 mM. Cells were grown for an additional period of 4 to 6 h, and then harvested by 10 min centrifugation at 6000 g and frozen. Over 95% of the cPL protein was found in the inclusion bodies which were prepared according to the previously described protocol (Gertler et al. 1992). The inclusion body pellet obtained from 2.5 l fermentation culture was solubilized in 600 ml 4.5 M urea buffered with 40 mM Tris base. The pH was increased to 11.3 with NaOH, cysteine was added to 0.1 M, and the clear solution was stirred at 4 °C for 1 h. It was then diluted with two volumes of water and dialyzed for 48 h against 4 × 10^{-1}·10 mM Tris–HCl, pH 9.0. The resultant solution was loaded at 120 ml/h onto a Q–Sepharose column (2.6 × 7 cm), pre-equilibrated with 10 mM Tris–HCl, pH 9.0 at 4 °C. Elution was carried out using a discontinuous NaCl gradient in the same buffer at a rate of 120 ml/h, and 10 ml fractions were collected. Protein concentration was determined by absorbance at 280 nm, and monomer content by gel-filtration chromatography on a Superdex 75 HR 10/30 column. The monomeric cPL was designated cPL–Q. As cPL was only partially absorbed to Q–Sepharose, the unabsorbed solution was brought to pH 5.0 with 10% HAc and reloaded on SP–Sepharose column (1.6 × 8 cm) pre-equilibrated with 25 mM NaAc, pH 5.0 at 4 °C. The
cPL was eluted from the column using a discontinuous NaCl gradient as described for the Q-Sepharose column, and was designated cPL-S.

**Determination of the amino-terminal sequence**

Automated Edman degradation technique was used to determine the amino-terminal protein sequence. Degradation was performed on an ABI Model 470A gas-phase sequencer (Foster City, CA, USA) using the standard sequencing cycle. The respective phenylthiohydantoin (PTH)-amino acid derivatives were identified by RP-HPLC analyses, using an ABI Model 120A PTH analyzer fitted with a Brownlee 2·1 mm i.d. PTH-C18 column.

**Determination of purity, monomer content and complex formation**

SDS-PAGE was carried out according to Laemmli (1970) using 12·5% gel. Gels were stained with Coomassie Brilliant Blue R. HPLC gel-filtration chromatography on a Superdex 75 HR 10/30 column was performed using 25 mM Tris–HCl buffer, pH 8·0, containing 150 mM NaCl (TN buffer). Two hundred microliter aliquots of either Q-Sepharose or SP-Sepharose column-eluted fractions, freeze-dried samples dissolved in H2O or complexes between the soluble recombinant GHR- or PRLR-ECD and cPL, were analyzed using methods detailed previously (Bignon et al. 1994).

**Binding assays**

Hormones binding to the different ECDs and to rabbit mammary gland (MG) microsomal fraction were assayed as described previously (Sandowski et al. 1995, Sakal et al. 1997) using radiolabeled125I-oPL as a ligand. Iodination of oPL was performed according to the protocol described by Gertler et al. (1984).
In vivo bioassays

Three in vivo bioassays in which the signal was transduced through lactogenic receptors were performed: Nb2-11C lymphoma cell proliferation bioassay (Gertler et al. 1985) and β-casein production in rabbit or ovine mammary gland acini culture (see below). Two additional in vivo 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-RGHRB9) or human (clone FDC-P1-HGHRD11) GHRs (Rowlinson et al. 1995, 1996). Cells cultured in RPMI-1640 medium supplemented with 5% horse serum and hGH (100 ng/ml) were washed and resuspended in RPMI-1640 medium supplemented with 5% horse serum at a concentration of 150 000 cells/ml in 24-well plates. Hormones were then added and the cells were grown for an additional period of 48 h. Cell growth was determined by counting the cells with a Coulter counter (Coulter Electronics Inc., Hialeah, FL, USA) and the number of doublings was calculated as described previously (Gertler et al. 1985).

Rabbit and sheep mammary gland acini culture

Rabbit mammary gland was quickly recovered and kept on ice. The mammary gland was then cut and kept in Hank’s balanced salt solution (HBSS) medium. Connective tissue and muscle were removed from each cut, and the remaining epithelial mammary tissue was then cut into small fragments with a scalpel and incubated for 2 h in HBSS medium containing 200 UI/ml collagenase IV, and 200 UI/ml hyaluronidase (Sigma Chemical Co.) and 2% BSA at 37 °C. After digestion, the cellular suspension was filtered to remove the remaining connective and the undigested tissue. The acini suspension was decanted rapidly to eliminate the less heavy fibroblastic cells. The latter procedure was repeated five times. The acini suspension was then plated in 24-well plates in Dulbecco’s modified Eagle’s medium (DMEM)/HAM-F12 medium (Gibco BRL, Paisley, Strathclyde, UK) containing 2% Ultroser SF (Biosepra, S.A., Villeneuve-la-Garenne, France), 2 mM l-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 50 µg/ml gentamycin. After 4 days culture, the acini were incubated with different concentrations of oPRL or cPRL for 48 h. The β-casein secreted into the medium was determined by radioimmunoassay as described before (Jahn et al. 1987). The procedure for ovine mammary gland acini was similar to that described above, except that tissue digestion was performed in Earle’s BSS medium with collagenase III (Gibco BRL, Paisley, Strathclyde, UK) containing 2% Ultraser SF (Biosepra, S.A., Villeneuve-la-Garenne, France), 2 mM l-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 50 µg/ml gentamycin. After 4 days culture, the acini were incubated with different concentrations of oPRL or cPRL for 48 h. The β-casein secreted into the medium was determined by radioimmunoassay as described before (Jahn et al. 1987). The procedure for ovine mammary gland acini was similar to that described above, except that tissue digestion was performed in Earle’s BSS medium with collagenase III (Gibco BRL) rather than collagenase IV. Two washings were performed for 20 min at 37 °C in the presence of 0·01% DNAse I, grade II (Boehringer, Mannheim, Germany) and, after a final wash to remove the enzyme, the cells were seeded and subsequently treated as described above.

Figure 2 Comparison of the predicted full amino acid sequences (including signal peptides) of ovine, caprine and bovine placental lactogens. The differences between cPL and oPL are marked by *, and those between cPL and bPL are marked by #. The & sign indicates missing amino acids. The sequence of the mature protein starts at position 38 (oPL numbering).
Results

Sequence analysis of the proteins encoded by the four cDNA clones

As described in Materials and Methods, the cPL cDNA was obtained by RT-PCR of goat placenta RNA. Sequence analysis revealed two kinds of sequences illustrated by clones 1 and 4 on the one hand, and by clones 2 and 3 on the other (Fig. 1). The four mismatches between these two sequences (G/A206, G/T208, G/A269 and A/G574) resulted in only two amino acid substitutions: S63 in clones 1 and 4 to G63; and V84 in clones 1 and 4 to I84 in clones 2 and 3. These two sequences could represent two alleles of the same gene with similar expression levels. After signal peptide removal, the cDNA encoded a 197 amino acid protein, which is two amino acids shorter than oPL. When aligned with oPL (Fig. 2), cPL exhibited 86% identity and 92% similarity at the protein level, with a shortened signal peptide of 11 amino acids for cPL (Fig. 2). The identity and similarity to bPL were 67% and 79% respectively, close to the values of 69% and 81% obtained in a respective comparison of oPL and bPL.

Purification and characterization of E. coli-expressed cPL

The protein contained in the inclusion bodies prepared from 2·5 l induced cells was refolded in 4·5 M urea as described in Materials and Methods. The refolded cPL was purified on a Q-Sepharose column by stepwise elution with increasing concentrations of NaCl (Fig. 3A). Aliquots from every sixth tube were analyzed for monomer content by gel filtration through a Superdex column. Fractions eluted with 0·05 M NaCl (see Fig. 3A, tubes 16–19) that contained monomeric cPL were pooled, dialyzed against NaHCO3 (at a 4:1 protein/salt ratio) and freeze-dried. This fraction (designated cPL-Q) contained pure monomer (Figs 3A and 4B), whereas fractions eluted in 0·1 M and 0·3 M NaCl contained increasing amounts of dimers and oligomers (not shown). Over 40% of the monomeric cPL was not absorbed by the Q-Sepharose column and was fully recovered in the breakthrough fraction (not shown). Therefore, the breakthrough fraction from the Q-Sepharose column was adjusted to pH 5·0 with 10% HAc and applied to a SP-Sepharose column (1·2 × 8 cm) pre-equilibrated with 25 mM NaAc buffer, pH 5·0. Elution was carried out using a discontinuous NaCl gradient in the same buffer. The flow rate was 120 ml/h in both columns and 10 ml fractions were collected.

Figure 3 Purification of proteins extracted from inclusion bodies on Q-Sepharose and SP-Sepharose columns. (A) The Q-Sepharose column (2·5 × 7 cm) was equilibrated with 10 mM Tris–HCl, pH 9·0, at 4 °C. The refolded proteins were applied to the column at a rate of 120 ml/h and eluted with a discontinuous gradient of NaCl in the same buffer. (B) The non-absorbed fraction from the Q-Sepharose column was adjusted to pH 5·0 by 10% HAc and applied to a SP-Sepharose column (1·6 × 8 cm) pre-equilibrated with 0·25 M NaAc buffer, pH 5·0. The column was then washed with a discontinuous gradient of NaCl in the same buffer. The flow rate was 120 ml/h in both columns and 10 ml fractions were collected.

Obtained from the Q-Sepharose column (cPL-Q) and ~25 mg protein obtained from the SP-Sepharose column (cPL-S). The freeze-dried hormone was stored at −70 °C for at least 6 months, during which time neither dimers nor oligomers were formed. The purity of the monomeric cPL under non-denaturing conditions (Fig. 4B) was further confirmed by SDS-PAGE analysis in the presence or absence of reducing agents (Fig. 4A). Under reducing conditions both fractions yielded a single band with an apparent molecular mass of ~23 kDa, close to the theoretical value of 22 599 Da (that includes an additional N-terminal Met) and similar to that of oPL. Under non-reducing conditions a single band was also observed. The mobility of this band was faster indicating globular
structure. Amino-terminal analysis of the first 7 amino acids yielded the expected sequence, namely Met-Glu-Asn-Tyr-Pro-Pro-Tyr for both cPL-Q and cPL-S. The respective yields (in picomols) were for cPL-Q: 163, 135, 115, 153, 141, 85, 99, and for cPL-S: 157, 145, 118, 147, 112, 98 and 116.

Binding experiments

Radiolabeled oPL was chosen as a ligand for the binding and competition experiments that were conducted with oPL, cPL-Q and cPL-S (Fig. 5 and Table 1). The results were analyzed by PRIZMA software (GraphPad Prism)

Figure 4 (A) SDS-PAGE of oPL, cPL-Q and cPL-S on 12.5% SDS-polyacrylamide gel in the presence (lanes 1, 2, and 3) and in the absence (lanes 4, 5 and 6) of β-mercaptoethanol. The gel was stained with Coomassie Brilliant Blue R. Molecular mass markers are shown on the left. (B) Determination of the cPL-Q and cPL-S monomer content by gel-filtration chromatography on a Superdex 75 HR 10/30 column. Protein content was monitored by absorbance at 280 nm using a Merck-Hitachi D-2000 integrator. The column was developed at 1 ml/min with 25 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl. The numbers above the peak show the retention time. The retention times of bovine albumin (67 kDa) and oPL (23 kDa) were 9.45 and 11.88 min respectively.
Version 2.0 (1994) according to a non-linear regression one-site competition dose–response curve. In all analyses the degree of the non-linear correlation was very high (R² > 0.97) and higher than for the two-site competition curves. Though IC₅₀ values for oPL, cPL-Q and cPL-S (as compared with oPL) were slightly less effective competitors for rbPRLR-ECD, rPRLR-ECD and rbMG microsomal fraction and slightly more effective for rbGHR-ECD and hGHR-ECD. As shown before for oPL (Sakal et al. 1997) and for bPL (Gertler et al. 1996), the IC₅₀ values for binding to bPRLR-ECD were higher than for other receptors and the specific binding of the ¹²⁵I-oPL was lower. This was a result of lower affinity and subsequently the need to use a larger amount of bPRLR-ECD to achieve specific binding.

**Gel-filtration experiments**

The stoichiometry of the interactions between purified rbPRLR-ECD, rPRLR-ECD, bPRLR-ECD, hGHR-ECD, rbGHR-ECD and cPL-Q was studied by preparing the respective complexes at increasing ECD:hormone ratios, while maintaining a constant concentration (1.6 μM) of the latter. Caprine PL-Q formed a detectable 1:2 complex with rbGHR-ECD and hGHR-ECD, whereas with bPRLR-ECD only a 1:1 complex was observed (Fig. 6). These conclusions were based on a
comparison of the sizes of the complex peaks and their retention times. The complexes with rbPRLR-ECD and rPRLR-ECD showed 1:2 stoichiometry as indicated by the retention times of 9.89–9.95 and 10.02–10.07 min respectively compared with a retention time of 10.88 min for the complex with bPRLR-ECD. The appearance of the excess of ECDs when the hormone was preincubated with two equivalents of PRLR-ECD indicated, however, that the complex was not stable and dissociated in the course of gel filtration. Similar experiments performed with cPL-S showed the same results (not shown).

**Biological activity in vitro**

Lactogenic activity of cPL-Q and cPL-S in a Nb₂ cell proliferation bioassay was as potent as that of oPL (Fig. 7A) and the respective EC₅₀ values were 1.4 × 10⁻¹² M,

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<th>Receptor</th>
<th>IC₅₀ values (nM)</th>
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<tr>
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<td>rbGHR-ECD</td>
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**Table 1** The IC₅₀ values of oPL, cPL-Q and cPL-S calculated from the binding experiments presented in Fig. 5.

Figure 6 Gel filtration of cPL-Q complexes with (A) hGHR-ECD, (B) rbGHR-ECD, (C) rbPRLR-ECD, (D) rPRLR-ECD and (E) bPRLR-ECD on a Superdex 75 HR 10/30 column. Complex formation was carried out during 20 to 30 min incubation at room temperature in TN buffer using various ECD:cPL ratios. The initial hormone concentration was constant (1.6 µM). Aliquots (200 µl) of the incubation mixture were applied to the column, pre-equilibrated with the same buffer. Complex formation was monitored by absorbance at 280 nm. The column was developed at 1 ml/min. Each experiment was conducted at least three times.

Figure 7 Effect of oPL (□), cPL-Q (△) and cPL-S (▽) on the proliferation of (A) Nb₂-11C lymphoma cells, (B) FDC-P1-hGHR9D11 cells, and (C) FDC-P1-rbGHR3B9 cells. Number of doublings was calculated as described previously (Gertler et al. 1985).
1.2 × 10^{-12} M and 1.3 × 10^{-12} M. Caprine PL stimulated β-casein synthesis in rabbit (Fig. 8A) and in sheep acini culture (Fig. 8B). In rabbit culture, the activity of cPL-Q was slightly higher than that of oPRL, (EC50 = 2 × 10^{-9} vs 6 × 10^{-9} M), whereas in sheep culture it was slightly lower (EC50 ~ 30 × 10^{-9} vs 10 × 10^{-9} M). In both cases the concentration of cPL required for half-maximal activity were two to three orders of magnitude higher than in a Nb2 cell proliferation assay. Somatogenic activity of cPL-Q and cPL-S was almost identical to that of oPL in two heterologous bioassays in FDC-P1 cells transfected with either hGHRs (Fig. 7B) or rbGHRs (Fig. 7C). The respective EC50 values for cPL-Q, cPL-S and oPL calculated from Fig. 7B were 4.7 × 10^{-11} M, 8.7 × 10^{-11} M and 6.4 × 10^{-11} M and from Fig. 7C 2.5 × 10^{-11} M, 2.1 × 10^{-11} M and 5.5 × 10^{-11} M. These values were 20- to 50-fold higher compared with those observed in Nb2 cells.

**Discussion**

Caprine PL was cloned in and expressed in *E. coli* BL21 cells. Two electrophoretically pure cPL fractions (cPL-Q and cPL-S) composed of over 98% of monomer and a small quantity of dimers were obtained. Since the N-terminal analysis of both fractions was identical we assume that they differ by a small charge change. The more acidic fraction, cPL-Q, was probably created by deamination of one or more glutamine residues during the refolding procedure at a high pH. As shown above, this difference had no effect on the biological activity, which was equal to that of oPL, the biological activity of which has been documented previously (Sakal et al. 1997). These results prompt us to conclude that both cPL fractions were properly refolded. There was no difference in the ability of the cPL fractions to bind to several types of soluble or membrane-embedded lactogenic and somatogenic receptors. Their ability to form complexes with different soluble GHR- or PRLR-ECDs was also identical and in general very similar to that of oPL.

Cloning of cPL enabled us to compare its primary structure to that of other ruminant placental lactogens. The data clearly show that 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 (Byatt et al. 1992). It has been suggested that this finding may be indicative of different physiological roles that PLs may play in the three species (Byatt et al. 1992). So far, this point has not been proven. We have at present elucidated 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 the 24 residues that participate in site II (Christinger et al. 1998). Interestingly, the extent of identity (and similarity) between oPL and cPL in these residues (72% and 80% for site I and 79% and 89% for site II) was not higher than the overall identity. The equal potency of both hormones to induce a biological effect mediated through rPRLR (see Fig. 7A) suggests that differences in the primary structure may not necessarily be indicative of different biological activities, although the final conclusion requires results using homologous models.

A particular feature observed previously for bPL and oPL (Tchelet et al. 1995, Gertler et al. 1996, Sakal et al. 1997), namely the low affinity for binding for the soluble bPRLR-ECD and the inability to show an apparent 1:2 complex, was also observed in the case of cPL. A comparison of the parameters of binding and of interaction between cPL and rbPRLR-ECD or rbMG microsomal fraction to that of bPRLR-ECD (see Figs 5 and 6), shows the apparent affinity for rabbit-derived proteins to be 200 to 400 times higher and this probably explains the compromised ability to form an apparent 1:2 complex with bPRLR-ECD. Despite this, the concentration of cPL required for half-maximal simulation of β-casein synthesis in rabbit MG acini was only 15-fold lower (EC50 = 2 × 10^{-9} M) than in the corresponding ovine culture (EC50 = 30 × 10^{-9} M). These results raise the
question as to the correlation between the binding of cPL to rabbit and ovine lactogenic receptors and the biological activity transduced as a result of this interaction. A possible explanation for this apparent discrepancy may be our recent suggestion that transient dimerization of PRLRs, lasting a few seconds or less, is sufficient to elicit a full biological response (Gertler et al. 1996). This suggestion is supported by the finding that receptor-associated JAK2 or other kinases are instantly activated by mutual trans-phosphorylation, subsequent to homodimerization of the receptor, forming docking sites for other downstream proteins (Goffin & Kelly 1997). Once this happens, the existence of the receptor homodimer is no longer required for signal transduction. Therefore, the shorter persistence of the receptor homodimer in ovine MG compared with rabbit MG does not cause any major biological disadvantage, and despite the 200- to 400-fold differences in the affinity constants, the difference in dose–response of the biological signal (β-casein synthesis) in both tissues is much less (see Fig. 8). Thus, the classical pharmacological theory that the biological activity is directly related to receptor occupancy does not possibly apply to cytokine receptors such as PRLR in which transient homodimerization is sufficient to initiate the transduction of the biological signal.

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