

Mutations of ovine and bovine placental lactogens change, in different ways, the biological activity mediated through homologous and heterologous lactogenic receptors

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Abstract

The biological activities of ovine (o) and bovine (b) placental lactogens (PLs) and their mutated analogues were compared using several binding and *in vitro* bioassays. In almost all cases, the biological activities of these analogues mediated through rat (r) prolactin receptor (PRLR) showed little or no change, despite a remarkable decrease in their capacity to bind to the extracellular domain of rPRLR and despite compromised stability of the 2:1 complexes. These results indicate that mutations impairing the ability of oPL or bPL to form stable complexes with lactogenic receptors do not necessarily lead to a decrease in the biological activity, because the transient existence of the homodimeric complex is still sufficient to initiate the signal transduction. In contrast, oPL and bPL analogues completely, or almost completely, lost their ability to activate homologous PRLRs, and some of them even acted as site-2 antagonists. To explain the difference between the activity transduced through homologous and that transduced through heterologous PRLRs, we propose the novel term 'minimal time of homodimer persistence'.

This concept assumes that in order to initiate the signal transduction, the associated kinase JAK2 has to be transphosphorylated and this requires a 'minimal time' of homodimer existence. In the case of homologous interaction between ruminant PLs and homologous PRLRs, this 'minimal time' is met, though the interaction with homologous PRLRs has a shorter half-life than that with heterologous PRLRs. Therefore oPL or bPL are active in cells possessing both homologous and heterologous PRLRs. Mutations of oPL or bPL lead to reduced affinity and, consequently, the 'time of homodimer persistence' is shortened. Although in the case of heterologous interaction the 'minimal time' is still sufficient to initiate the biological activity, in homologous interactions, which are already weaker than heterologous interactions, further destabilization of the complex shortens its persistence to below the 'minimal time', leading to full or partial loss of biological activity.

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Introduction

The placenta of ruminants and other species synthesize and secrete unique proteins, belonging to the growth hormone/prolactin (GH/PRL) family, termed placental lactogens (PLs). Ovine (o) (Martal & Djiane 1975, Warren *et al.* 1990), bovine (b) (Murthy *et al.* 1982) and caprine (c) (Currie *et al.* 1990) PLs have been isolated from placenta and found to be proteins, composed of 198–200 amino acids, which are structurally closer to their respective

PRLs than to GHs (Byatt *et al.* 1992). Bovine PL is glycosylated, though the glycosylation is not important for its biological activity (Byatt *et al.* 1992). We and others have prepared recombinant oPL (Colosi *et al.* 1989, Sakal *et al.* 1997), bPL (Krivi *et al.* 1989) and, recently, also cPL (Sakal *et al.* 1998), as well as several bPL and oPL, mutants (Vashdi-Elberg *et al.* 1995, 1996, Helman *et al.* 1997, 1998, Herman *et al.* 1999). We and others have also documented the fact that ruminant PLs are capable of activating either human (h) or rabbit

(rb) growth hormone receptor (GHR)-mediated biological activities in several *in vitro* models (Byatt *et al.* 1991, Vashdi *et al.* 1992). Recently, however, we have shown that oPL, bPL and cPL are inactive in homologous bioassays, namely in cells transfected with oGHRs, and even act as antagonists, in contrast to oGH, whose agonistic activity was equal to that of hGH (Herman *et al.* 1999). Despite the differences in their biological activity, oGH and PLs bound with similar affinity to the oGHR extracellular domain (oGHR-ECD), indicating that the binding occurs through site 1 of the hormone. Gel filtration of complexes between oPL and oGHR-ECD showed 1:1 stoichiometry, confirming this conclusion. We therefore concluded that ruminant PLs antagonize the activity of oGH in homologous systems because they cannot homodimerize oGHRs, whereas in heterologous systems they act as agonists. In contrast, ruminant PLs exhibit oPRLR-mediated activity not only in heterologous systems (Byatt *et al.* 1990, Gertler *et al.* 1992, Vashdi-Elberg *et al.* 1995, 1996, Sakal *et al.* 1997) but also in an acini culture of ruminant mammary gland (Sakal *et al.* 1997, 1998).

In the present work, we have therefore posed the question of why, despite the different affinities for the homologous and heterologous lactogenic receptors, ruminant PLs can initiate biological activity in both systems. To answer this question, we prepared several mutated analogues of both oPL and bPL and showed that the mutation systems may change differently the activity in homologous versus heterologous. A plausible explanation for this phenomenon is also proposed.

Materials and Methods

Materials

Recombinant bPL, bPL G133R, bPL K73D, bPL T188D, oPL, oPL T185D, oPL G130R, hGH and non-glycosylated recombinant rat (r) and bovine PRLR-ECDs were prepared as described previously (Sakal *et al.* 1997, Krivi *et al.* 1989, Gertler *et al.* 1992, Vashdi-Elberg *et al.* 1996, Helman *et al.* 1997, 1998). Carrier-free ¹²⁵I-labelled Na was purchased from New England Nuclear Corp. (Boston, MA, USA). Molecular-weight markers for SDS-PAGE, RPMI-1640 medium, lysozyme, nalidixic acid, Triton X-100 and BSA (radioimmunoassay grade) were obtained from Sigma Chemical Co. (St Louis, MO, USA). SDS-PAGE reagents and the protein assay kit were purchased from BioRad Laboratories (Richmond, CA, USA). Fetal calf serum (FCS) and horse serum were purchased from Labotal Co. (Jerusalem, Israel) and a Superdex 75 HR 10/30 column and Q-Sepharose (fast flow) were obtained from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). All other chemicals were of analytical grade.

Construction of oPL K71E, oPL T185F, bPL A26W and A26W/G133K expression vectors

Ovine PL, bPL or bPL G133R expression vectors were modified with the Quickchange mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions, using, respectively, two sets of two complementary primers: 5'-CTGCCACACGTCATC CATCACCACCCCTAATAACGAAGCAGAAGC-3' and 5'-GCTTCTGCTTCGTTATTAGGGGTGGTGA TGGATGACGTGTGGCAG-3' for oPL K71E; 5'-CCCCTTCAAAGCTTGTGGAGAGATGGACATT GGTGGCTAGCAAC-3' and 5'-GTTGCTAGCCAC CAATGTCCATCTCTCAAACAAGCTTTGAAGGG G-3' for bPL A26W and bPL A26W/G133K. These primers were designed to contain specific restriction sites (AflIII, HindIII respectively), while conserving the same amino acid sequence for colony screening. The procedure included 12 PCR cycles and the use of *Pfu* polymerase enzyme for the reaction (Stratagene). The template used for mutant construction was wild-type oPL or bPL in pMON3922 (Obukowicz *et al.* 1992). The mutated construct was then digested with the restriction enzyme DpnI, which is specific to methylated and hemimethylated DNA (target sequence: 5'-G^{m6}ATC-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 screened for mutation, using the specific restriction site, and showed 80% efficiency. Two colonies were sequenced and confirmed to contain the mutation and no undesired misincorporation of nucleotides. Ovine PL-analogue T185F expression vector was constructed as described previously for oPL T185D (Herman *et al.* 1999), using PCR technology. The sequences of the sense and antisense primers were 5'-GGAGATATACCATGGCACA GCATCCACC-3' and 5'-GCACTTAAGTATCCGG AGGTAGAAGTAAATTTTAC-3' respectively. Automatic DNA sequencing was performed to confirm the proper sequence.

Construction of oPRLR expression vector

The full-length cDNA of oPRLR was contributed by Dr J Djiane (INRA, Jouy-en-Josas, France) in a PLK-neo plasmid. The insert was completely digested with KpnI and partially cut with EcoRI. The partial cut was formed in order to avoid digestion at the EcoRI site located in the middle of the oPRLR cDNA. The purified cDNA was then inserted into a pcDNA3 expression vector (Invitrogen Co., Leek, The Netherlands) previously digested with KpnI and EcoRI. The insert-containing vector was transfected into XL1 competent cells. Five colonies were analysed and all were found to be insert-positive. A large amount of oPRLR cDNA was then prepared from one of the colonies for subsequent use in the

293 cell transfection bioassay. Bovine PRLR cDNA in a pcDNA3.1 expression vector (Scott *et al.* 1992) was kindly provided by Dr L Schuler (University of Wisconsin, Madison, WI, USA).

Expression, refolding and purification of oPL and bPL analogues

Escherichia coli MON105 cells transformed with the expression plasmids containing oPL K71E, oPL T185F, bPL A26W or bPL A26W/G133K were incubated in 500 ml Terrific Broth (TB) medium (Tartof & Hobbs 1987) with shaking at 200 r.p.m. at 37 °C in 2 litre flasks to an A_{600} of 0.9, after which nalidixic acid (25 mg/flask) was added. The cells were incubated for an additional 4 h and harvested by 5-min centrifugation at 10 000 g; the supernatant was then decanted and the pellet was stored at -20 °C. Over 95% of the expressed protein was found in the inclusion bodies, which were prepared as described previously for bPL (Gertler *et al.* 1992). The inclusion-body pellet obtained from 2.5 l bacterial culture was solubilized in 200 ml 4.5 M urea buffered with 10 mM Tris base. The pH was increased to 11.3 with NaOH, then cysteine was added, to 0.1 mM for oPL K71E and oPL T185F, or to 2 mM for bPL A26W and bPL A26W/G133K. The clear solution was stirred at 4 °C for 1 h, diluted with 2 vols cold water for oPL K71E. Bovine PL A26W and A26W/G133K were treated differently. After stirring for 1.5 h, 3 vols 0.67 M arginine were added and the solution was stirred for another 24 h. Subsequently, the proteins were further dialysed for an additional 48 h against 5 × 10 l 10 mM Tris-HCl (pH 9) and then 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 5 ml fractions were collected. The protein concentration was determined from the absorbance at 280 nm, and the monomer content was determined by gel-filtration chromatography on a Superdex 75 column.

Binding experiments

Binding to soluble bPRLR-ECD and rPRLR-ECD and to the microsomal fraction prepared from ovine mammary gland was carried out as described previously (Sandowski *et al.* 1995, Sakal *et al.* 1997). The ligand was ¹²⁵I-labelled hGH and the competitors were oPL, bPL or their analogues. Iodination of hGH was performed according to a previously described protocol (Gertler *et al.* 1984).

Determination of monomer content and complex formation

HPLC 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 rPRLR-ECD and bPRLR-ECD and oPL or oPL analogues, using methods described previously (Bignon *et al.* 1994, Sakal *et al.* 1997).

In vitro bioassays in transiently transfected 293 cells

Additional bioassays were carried out in a HEK 293 cell line transiently transfected with bPRLR, oPRLR or rPRLR 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 (TK) promoter. The transfection and bioassay were carried out as described previously and the effects of the hormones and their analogues were determined by measuring the luciferase activity in cell lysates (Tchelet *et al.* 1997, Herman *et al.* 1999).

In vitro bioassays in Nb₂ rat lymphoma cells

An *in vitro* bioassay, in which the signal is transduced through lactogenic receptors, was performed by using a rat Nb₂-11C lymphoma-cell proliferation protocol, with slight modifications to the original assay (Gertler *et al.* 1985).

Sheep mammary gland acini culture

Ewe mammary gland was quickly recovered, kept on ice, then cut and kept in Hank's balanced salt solution (HBSS) medium. Connective tissue and muscle were removed from each slice, and the remaining epithelial mammary tissue was then cut into small fragments with a scalpel and incubated for 2 h in Earle's balanced salt solution (EBSS) medium with collagenase III (200 IU/ml) (Gibco BRL, Paisley, Scotland) and hyaluronidase (200 IU/ml) (Sigma Chemical Co.) and 2% (w/v) BSA at 37 °C. After digestion, the cellular suspension was filtered to remove the remaining connective and undigested tissue. The acini suspension was decanted rapidly to eliminate the less heavy fibroblastic cells. Two washings were performed for 20 min at 37 °C in the presence of 0.01% DNase I, grade II (Boehringer, Mannheim, Germany) followed by 5 washings without the enzyme. The acini suspension was then plated in 24-well plates in DMEM/HAM-F12 medium (Gibco BRL) containing 2% Ultrosor SF (Biosepra, S.A., Villeneuve-la-Garenne, France), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 50 µg/ml gentamicin. After 4 days of culture, the acini were incubated with different concentrations of oPRL, oPL or oPL analogues for 48 h. The β-casein secreted into the medium was determined by radioimmunoassay as described previously (Jahn *et al.* 1987). Statistical analysis was performed using a two-tailed *t*-test.

Results

Binding assays

^{125}I -hGH was used as a ligand in binding assays for both the microsomal fraction prepared from ovine mammary gland and that from bPRLR-ECD because radiolabelled oPL, bPL and oPRL showed very poor binding (Gertler *et al.* 1984, Sakal *et al.* 1997). However, because of the low affinity of bPL and oPL toward the homologous receptor, much higher concentrations of the competitors were used in the binding assays in which an ovine microsomal fraction or a bPRLR-ECD was used. In competitive experiments using the microsomal fraction of ovine mammary gland (Fig. 1A), oPL exhibited the lowest inhibitory concentration (IC_{50}) value (2.9×10^{-8} M), whereas the corresponding IC_{50} values for oPL G130R, oPL T185D and K71E were higher (4.9×10^{-8} , 27.6×10^{-8} and 36.6×10^{-8} M respectively). On the other hand, the affinities of oPL, oPL T185D, oPL T185F and oPL K71E toward bPRLR-ECD were almost identical and the respective IC_{50} values were 1.28×10^{-7} , 2.35×10^{-7} , 1.38×10^{-7} and 1.84×10^{-7} M (Fig. 1B), whereas the binding capacity of oPL G130R was totally abolished. In a heterologous binding assay, based on interaction with rPRLR-ECD, the affinities of oPL T185D, oPL T185F, and oPL K71E were more drastically reduced as compared with oPL, and the corresponding IC_{50} values were 8.9×10^{-8} , 17.3×10^{-8} and 5.7×10^{-8} M respectively, as compared with the IC_{50} of oPL (0.63×10^{-8} M) (Fig. 1C). The ability of oPL G130R to bind rPRLR-ECD was very poor ($\text{IC}_{50} > 10^{-6}$ M).

The ability of bPL analogues K73D, T188D, G133R, A26W and A26W/G133K to compete for binding to lactogenic receptors was compared with that of bPL, using both heterologous and homologous binding assays. Compared with bPL, the bPL analogues G133R and A26W/G133K lost over 99% of their ability to bind to rPRLR-ECD, and the corresponding IC_{50} values were, respectively, 0.17×10^{-8} , 165×10^{-8} and 48.2×10^{-8} M (Fig. 1D). Other analogues such as K73D, T188D and A26W had also lost 90–95% of their binding ability, and their corresponding IC_{50} values were, respectively, 1.67×10^{-8} , 3.24×10^{-8} and 2.08×10^{-8} M. In contrast, only small differences in the binding capacities of bPL, bPL K73D, bPL T188D and bPL A26W toward bPRLR-ECD were observed, and the respective IC_{50} values were 4.53×10^{-7} , 3.67×10^{-7} , 3.10×10^{-7} and 4.78×10^{-7} M. However, bPL A26W/G133K and bPL G133R bound less strongly, even at the highest concentrations, and the extrapolated IC_{50} values were, respectively, 20.3×10^{-7} and 94.2×10^{-7} M (Fig. 1E).

Gel-filtration experiments

The stoichiometry of the complexes formed by the interaction of rPRLR-ECD with oPL, bPL or their analogues

was determined by gel filtration at several rPRLR-ECD:analogue ratios, at a constant concentration ($2 \mu\text{M}$) of the latter. Measurement of the sizes of the eluted peaks and their retention times (RTs) enabled us to analyse and calculate the stoichiometry and the molecular mass of the hormone-receptor complex. Gel filtration (Fig. 2A) confirmed former results in which formation of 1:2 oPL:rPRLR-ECD was shown (Sakal *et al.* 1997). The same gel-filtration profile was also obtained after 1:5 dilution of the complexes (not shown), but, at a 25-fold dilution, the gel-filtration profile indicated partial dissociation to a 1:1 complex (not shown). Complexes of oPL analogues T185D, T185F and K71E showed a similar gel-filtration profile. However, higher RT values of the complex were observed, and an excess of R-ECD was already apparent at the 2:1 ratio. Although these results are only semi-quantitative, they hint that the mutation destabilizes the 1:2 complex. In contrast to these oPL analogues, oPL G130R did not form any detectable complex with rPRLR-ECD. It should be noted that the free hormone cannot be seen (Fig. 2A, the last line) because the RT values of oPL G133R and rPRLR-ECD were so close that the two peaks merged.

Bovine PL and its T188D, A26W and A26W/G133K analogues formed rather stable 1:2 complexes with rPRLR-ECD (Fig. 2B). At an approximate hormone:ECD ratio of 1:1, only one peak representing the complex was observed (RT=9.13–9.44), with some excess of the hormone. However, at a 1:2 ratio of hormone:ECD, the size of this peak increased, but the RT hardly changed. Some excess of the ECDs could be seen with A26W and A26W/G133K analogues, indicating lesser stability of the complex. These results indicate that even at an initial 1:1 bPL:ECD ratio, formation of a 1:2 complex was dominant and thus a mixture of the latter along with free hormone was observed. At a 1:3 bPL:ECD ratio, the peak of the complex remained essentially unchanged and the ECD excesses were more visible. In contrast, bPL analogue G133R formed only a 1:1 complex, even at a 3:1 molar excess of rPRLR-ECD (Helman *et al.* 1998). As 2:1 complexes of bPRLR-ECD with unmodified oPL or bPL are very unstable and rapidly dissociate to 1:1, they have never been identified by gel filtration (Tchelet *et al.* 1995, Sakal *et al.* 1997). Therefore, no studies of complex formation between oPL or bPL analogues and bPRLR-ECD were conducted.

Biological activity of oPL and oPL analogues G130R, K71E, T185D and T185F

The biological activity of oPL and its analogues was tested in several bioassays based on signal transduction conducted by either heterologous or homologous PRLRs. In rat lymphoma Nb₂ cells (Fig. 3A), the activities of oPL and oPL analogues K71E, T185F and T185D were almost identical and the corresponding effective concentration

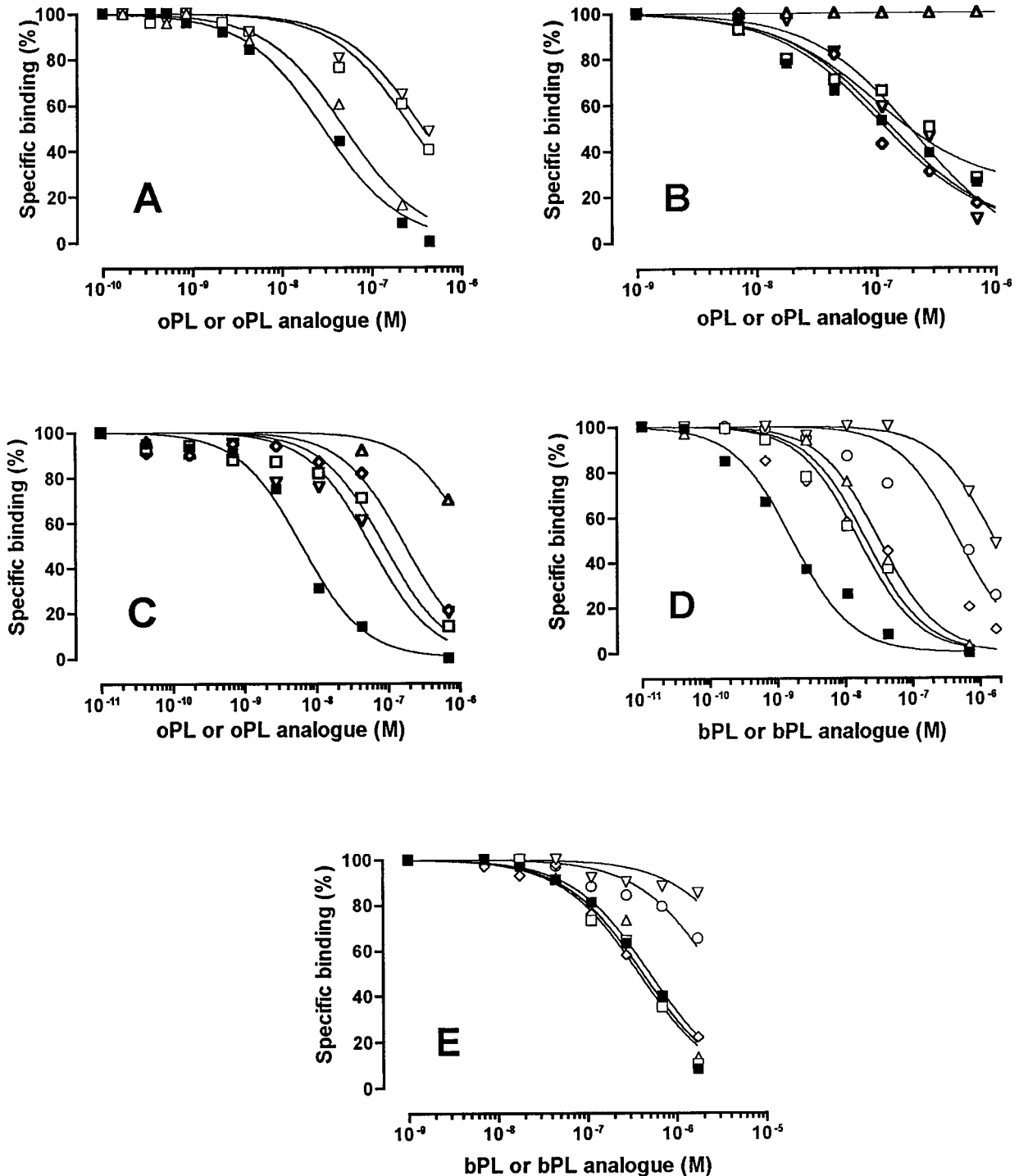


Figure 1 Competition of unlabelled oPL (■), oPL G130R (△), oPL K71E (▽), oPL T185D (□) and oPL T185F (◇) with the binding of ¹²⁵I-hGH to ovine mammary gland microsomal fraction (A), bPRLR-ECD (B) and rPRLR-ECD (C), and competition of unlabelled bPL (■), bPL G133R (▽), bPL K73D (□), bPL T188D (△), bPL A26W (◇) and bPL A26W/G133K (○) with the binding of ¹²⁵I-hGH to rPRLR-ECD (D) and bPRLR-ECD (E). Results of all specific bindings in the absence of a competitor were normalized. The specific binding was 27% in (A), 16% in (B), 8.5% in (C), 20% in (D), and 14% in (E). Full lines were calculated using the PRIZMA curve-fitting program (GraphPad Software, San Diego, CA, USA). Each experiment was performed 2–3 times and a representative experiment is shown.

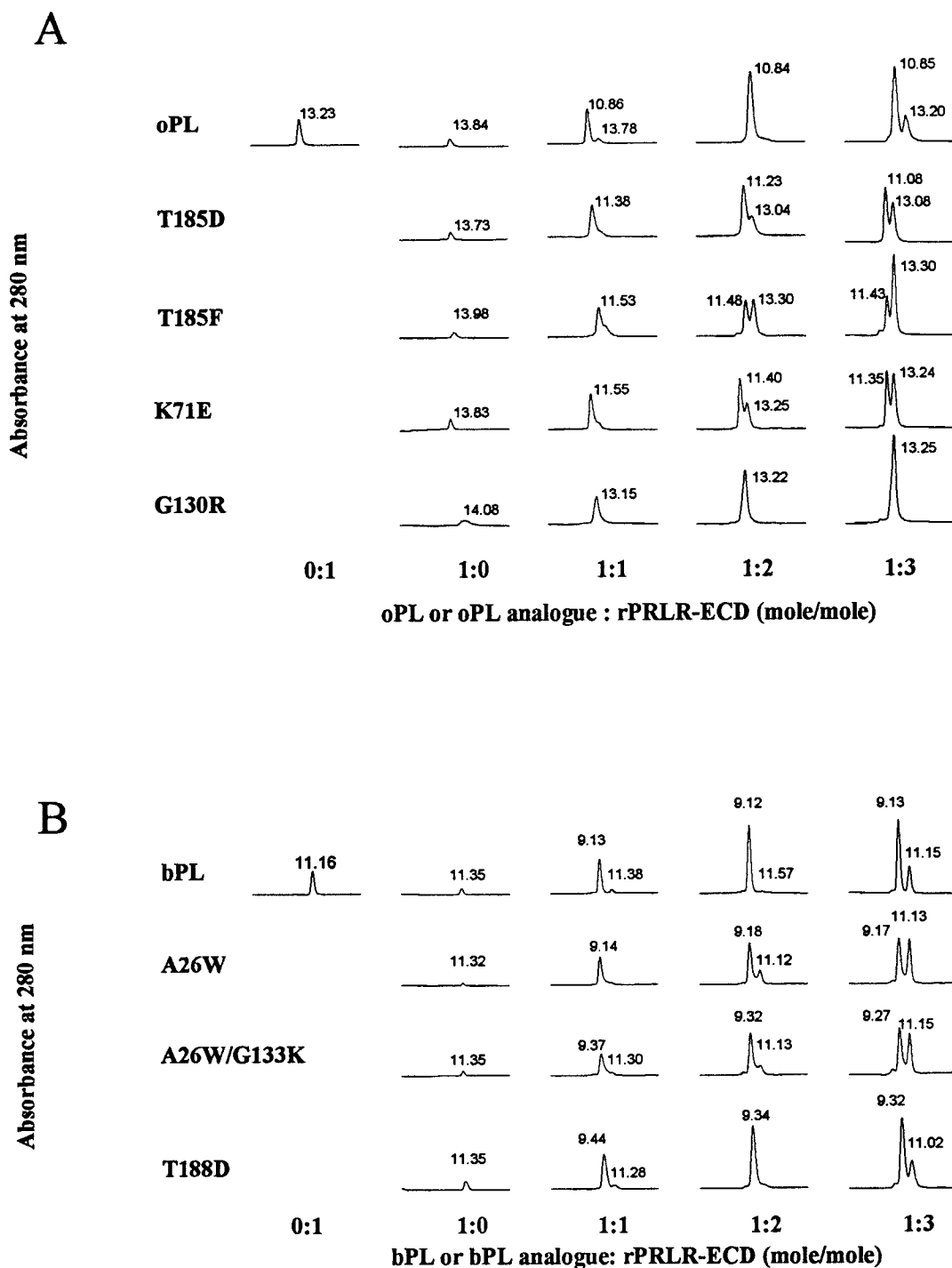


Figure 2 Gel filtration of rPRLR-ECD complexes with oPL and oPL analogues (A) or bPL and bPL analogues (B) on a Superdex 75 HR 10/30 column. Complex formation was carried out during a 20–30 min incubation at room temperature in TN buffer (25 mM Tris-HCl and 150 mM NaCl, pH 8), using various ECD:hormone molar ratios, then aliquots (200 μ l) of the incubation mixture were applied to the column, pre-equilibrated with the same buffer. The initial hormone concentration (2 μ M) was constant in all cases. The column was developed at 0.8 ml/min (A) and 1.0 ml/min (B) and calibrated (in A) with BSA (66 kDa, RT=11.13 min), egg albumin (45 kDa, RT=12.18 min), the extracellular domain of the hGH receptor (28 kDa, RT=13.02 min) and ovine placental lactogen (23 kDa, RT=13.84 min). The protein concentration in the eluate was monitored by measuring the absorbance at 280 nm. Each experiment was conducted at least three times.

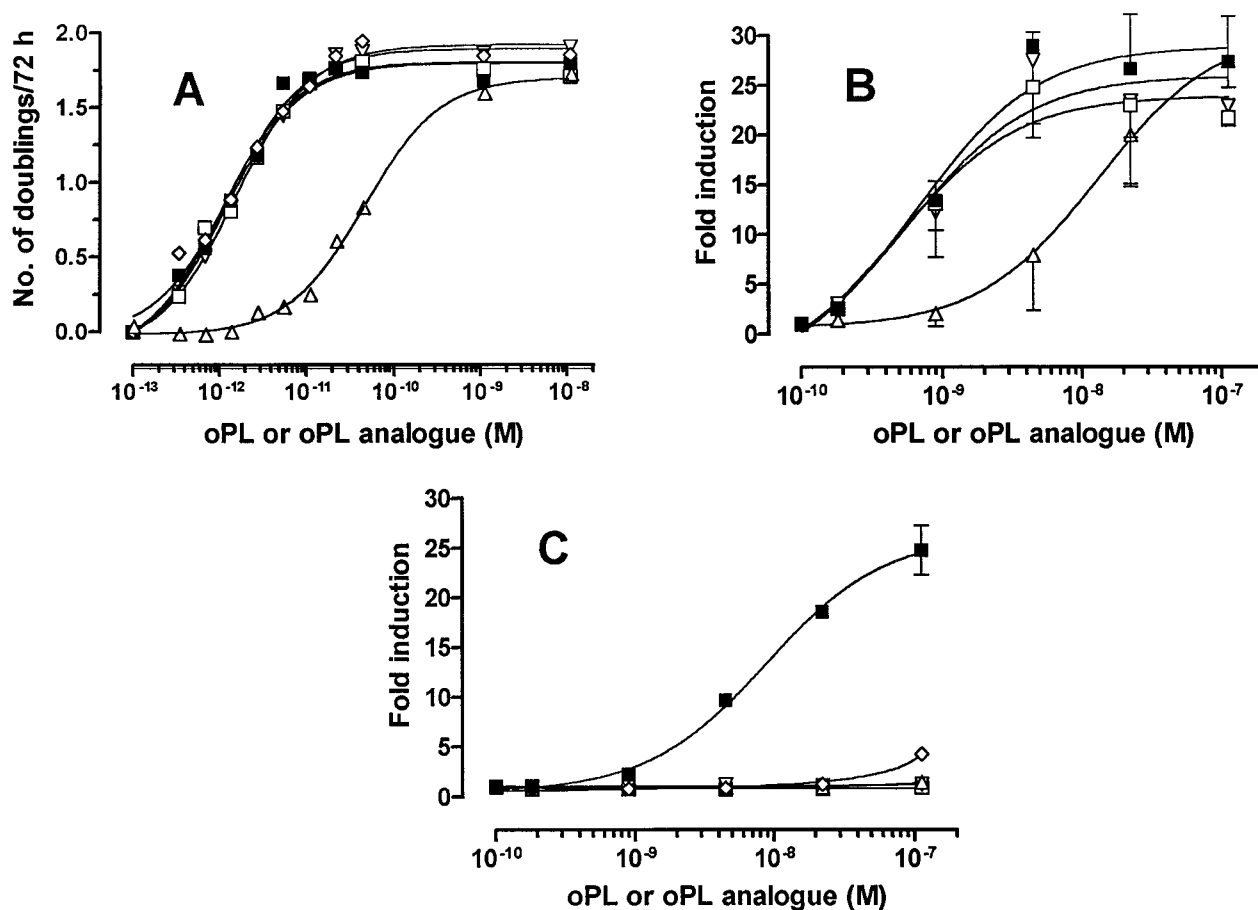


Figure 3 Activities of oPL (■), oPL G130R (△), oPL K71E (▽), oPL T185D (□) and oPL T185F (◇) in Nb₂-11C rat lymphoma cells (A) and in 293 cells transiently transfected with rPRLRs (B) or oPRLRs (C). Each experiment was performed in triplicate (Nb₂ cells) or duplicate (HEK 293 cells). The results are shown as means \pm S.D., and full lines were calculated using the PRIZMA curve-fitting program.

(EC₅₀) values were 1.06×10^{-12} , 1.57×10^{-12} , 1.34×10^{-12} , 1.15×10^{-12} M, whereas the activity of oPL G130R was \sim 40-fold lower (EC₅₀ = 4.4×10^{-11} M). Similar results were also obtained in 293 cells transiently transfected with the long form of rPRLR, and the EC₅₀ values for oPL and its K71E, T185D and G130R analogues were, respectively, 6.35×10^{-10} , 5.76×10^{-10} , 4.56×10^{-10} and 1.38×10^{-8} M (Fig. 3B). In contrast, in 293 cells transiently transfected with the long form of oPRLR and the luciferase as a reporting gene, only oPL was active (EC₅₀ = 0.89×10^{-8} M), whereas K71E, G130R and T185D were devoid of biological activity and only weak activity of T185F (2.6-fold induction) was observed at the highest concentration (Fig. 3C). No antagonistic activity, however, was observed when the analogues were given at a 100:1 analogue:oPL ratio (not shown). Similar results were also obtained using ovine mammary gland acini culture. As shown in Fig. 4A, β -casein secretion was stimulated by both oPRL and oPL, though the latter was less active. The values obtained at

the three highest concentrations of oPRL were significantly ($P > 0.05$) higher than the respective concentration of oPL (Fig. 4A). However, three oPL analogues tested in this assay (G130R, K71E and T185D) were devoid of any activity (not shown), but at a fivefold excess they were capable of significantly ($P > 0.05$ to $P > 0.001$) inhibiting oPRL and oPL activity (Fig. 4B).

Biological activity of bPL and bPL analogues K73D, G133R, T188D, A26W and A26W/G133K

The biological activity mediated through heterologous receptors was tested in the rat lymphoma Nb₂ cell line. In this assay, bPL K73D was as active as bPL (Helman *et al.* 1997), bPL T188D was twice as active, whereas bPL G133R, bPL A26W and bPL A26W/G133K were, respectively, 1.55-, 1.76- and 1.65-fold less active than bPL (Fig. 5A). Similar results were obtained in 293 cells transfected with rPRLR (Fig. 5B). The activities of bPL

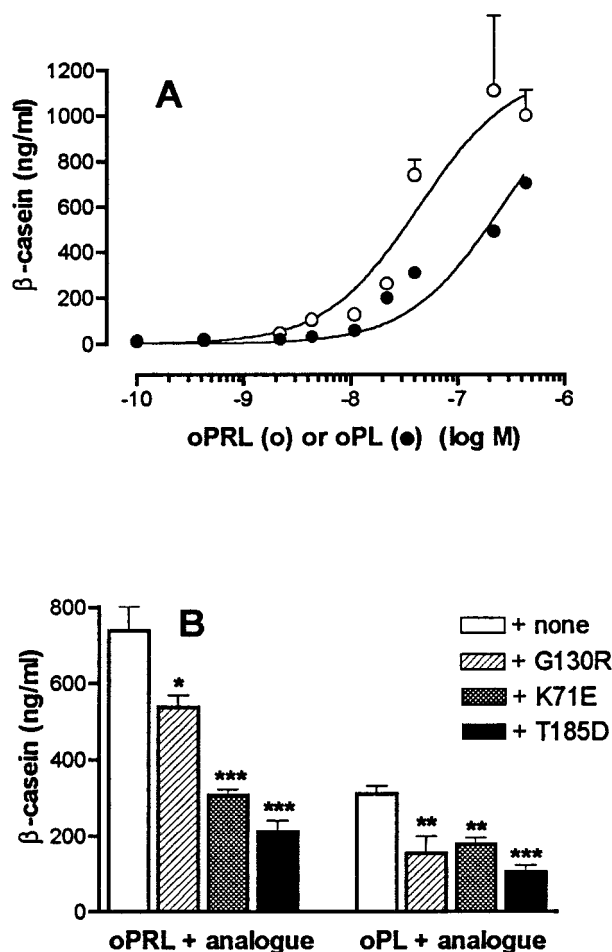


Figure 4 Effect of oPL (●) and oPRL (○) on β -casein production in ovine mammary gland acini culture (A). The results are means \pm S.D. values of two replicates, and the differences between oPL and oPRL were statistically significant ($P > 0.05$) at the three highest concentrations. In most cases, the S.D. values were too small to be seen on the figure. The antagonistic activities of oPL G130R, oPL K71E and oPL T185D (25×10^{-8} M) in acini cultures stimulated with 5×10^{-8} M oPRL or oPL (B) are shown. Bars marked with asterisks are significantly different from the control: * $P > 0.05$; ** $P > 0.01$; *** $P > 0.001$. The results are means \pm S.E.M. of three replicates.

and bPL analogues K73D and T188D were almost identical. The corresponding EC_{50} values were 6.21×10^{-10} , 9.95×10^{-10} , and 9.13×10^{-10} M, whereas the activities of bPL G133R, bPL A26W and bPL A26W/G133K were lower. The respective EC_{50} values were 2.67×10^{-9} , 3.50×10^{-9} and 2.53×10^{-9} M. Bovine PL stimulated the expression of LHRE-promoter-linked luciferase activity in 293 cells transiently transfected with homologous bPRLR (Fig. 5C). In these assays, bPL was active with an EC_{50} of 1.0×10^{-8} M, bPL K73D and bPL T188D were far less active, with respective extrapolated EC_{50} values of less than 10^{-6} M, while other

analogues were not active at all (Fig. 5C). Furthermore, the three analogues that completely lost their activity (bPL G133R, bPL A26W and bPL A26W/G133K) acted as antagonists and inhibited bPL activity in a dose-dependent manner, giving respective IC_{50} values of 5.1×10^{-7} , 2.6×10^{-7} and 5.0×10^{-7} M (Fig. 5D). The lack of the antagonistic of bPL T188D and bPL K73D activity could be the result of their low agonistic activity.

Discussion

To study the differences in the transduction of biological signals through homologous (ruminant) vs heterologous (rat) lactogenic receptors, several analogues of oPL and bPL were prepared. The mutations were aimed at modifying the interaction between PLs and the receptor by modifying their site-1 (oPL analogues T185D, T185F, K71E and bPL analogues T188D, K73D) or their site-2 (oPL analogues G130R and bPL analogues G133R, A26W and A26W/G133K).

The binding capacities and biological activities of oPL and bPL analogues in homologous (ruminant) and heterologous (rat) systems are summarized in Table 1. The biological activity mediated through rPRLRs was tested in two bioassays. Although the rat lymphoma Nb2 cell bioassay, which is based on measuring proliferation, may be affected by many intrinsic limiting factors, the bioassay in 293 cells transfected with PRLRs is less sensitive to limiting factors, since the response implies synthesis of a single protein; nonetheless, the effect of mutations was similar in both assays. It is clear that in almost all cases the biological activities of oPL and bPL analogues, as determined by heterologous interaction with rPRLR, are changed very little, if at all. The only exception was oPL G130R, the activity of which, in contrast to that of the analogous bPL G133R, in the rat lymphoma Nb₂ and 293 cell bioassay was drastically reduced. This probably results from the fact that the mutation affected not only site 2, as shown for its interaction with oGHR-ECD (see Herman *et al.* 1999), but also site 1. This is evidenced by its very low capacity to compete for binding to rPRLR-ECD and its inability to form a stable complex detectable by gel filtration (Fig. 2A). The low agonistic activity cannot be attributed to improper refolding, as this analogue is a potent competitor for binding to rbGHR-ECD, hGHR-ECD and oGHR-ECD (Herman *et al.* 1999). However, the agonistic activity of other oPL and bPL analogues in rat lymphoma Nb₂ and 293 cells was almost fully retained. It occurred despite (1) the 5- to 100-fold decrease in their capacity to bind to rPRLR-ECD, which, as discussed before, probably represents binding to site 1 only (Helman *et al.* 1997, 1998, Tchelet *et al.* 1997, Herman *et al.* 1999) and (2) the compromised stability of the 2:1 complexes, as demonstrated by gel filtration of diluted complexes. These findings substantiate our former suggestion that mutations

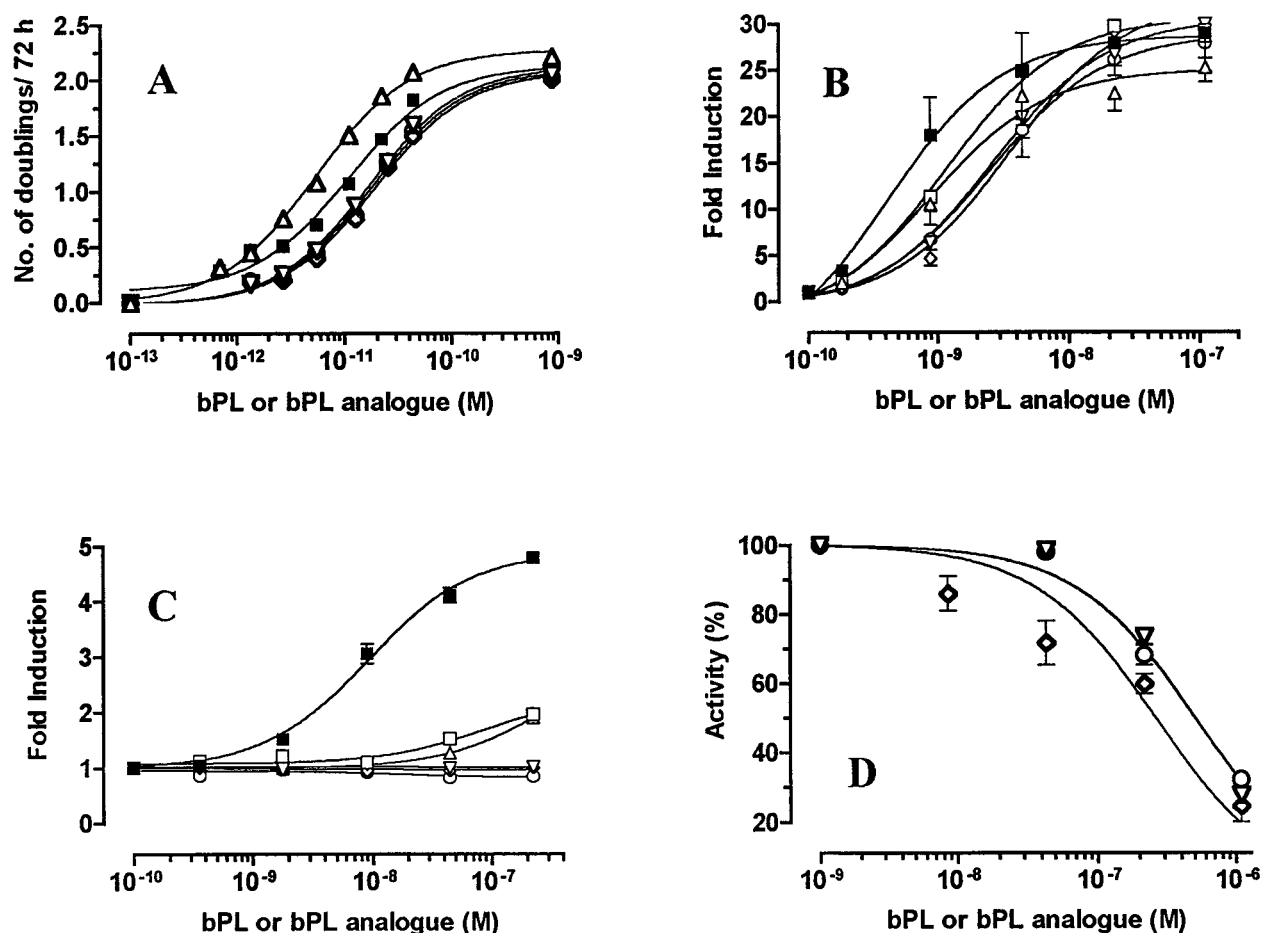


Figure 5 Activities of bPL (■), bPL G133R (▽), bPL K73D (□), bPL T188D (△), bPL A26W (◇) and bPL A26W/G133K (○) in Nb₂-11C rat lymphoma cells (A) in 293 cells transiently transfected with rPRLR (B) or bPRLR (C), and antagonistic activities of bPL G133R (▽), bPL A26W (◇) and bPL A26W/G133K (○) transfected with bPRLR (D). Luciferase activity that was induced by bPL (9×10^{-9} M) was normalized to 100%. The actual activity was higher than the control by fourfold. Each experiment was performed in triplicate (Nb₂ cells) or duplicate (HEK 293 cells). The results are shown as means \pm s.d., and full lines were calculated using the PRIZMA curve-fitting program.

impairing the ability of oPL or bPL to form stable complexes with lactogenic receptors do not necessarily lead to a decrease in the biological activity, because the transient existence of the homodimeric complex is still sufficient to initiate signal transduction, whereas the activity mediated through GHRs (which might require longer interaction) was lost (Helman *et al.* 1998).

In contrast to biological activity transduced through heterologous (rat) receptor, which was unchanged or only partially reduced (except in the case of oPL G130R), both oPL and bPL analogues completely or almost completely lost their ability to activate homologous PRL receptors. This was clearly demonstrated in HEK 293 cells transfected with bovine or ovine PRLRs and, in the case of oPL, also in primary cultures of mammary gland acini (Figs 3–5). Furthermore, some of these analogues even acted as site-2 antagonists in ovine mammary cells and in the HEK

293 cells transfected with bPRLs but not with oPRLRs. Although we have no valid explanation for the difference between the two experimental models, two possible reasons could be suggested: (1) the difference could result from the fact that in 293 cells oPRLRs are overexpressed; (2) the interaction of oPL and its analogues with oPRLR in intact ovine mammary is stabilized by additional factors existing in mammary cells but not in the HEK 293 cells. Further experiments are required to validate these suggestions.

To explain one possible reason for the difference between the signals transduced through homologous (ruminant) vs heterologous (rat) PRL receptors, we would like to propose a novel hypothesis based on an assumption that there is a 'minimal time of homodimer persistence' required to transduce the hormonal signal. This concept assumes that for the signal transduction to be initiated the

Table 1 Binding and biological activity of oPL, bPL and their analogues *in vitro* and stoichiometry of their complexes with rGHR-ECD

	Binding activity			Bioassays in cell lines or primary culture				Complex with rPRLR-ECD
	rPRLR-ECD	bPRLR-ECD	oMG microsomes	293 ¹ o/bPRLR	oMG acini	293 rPRLR	Nb ₂ rPRLR	
Hormone								
oPL	100	100	100	100	100	100	100	1:2
oPL T185D	7*	54	11*	0*	AN	140	92	1:2 (w)
oPL T185F	4*	93	NT	~2*	NT	NT	79	1:2 (w)
oPL G130R	<1*	0*	57	0*	AN	5*	3*	None
oPL K71E	11*	70	8*	0*	AN	110	68	1:2 (w)
bPL	100	100	NT	100	NT	100	100	1:2
bPL T188D	5*	146	NT	~1*	NT	68	200*	1:2
bPL K73D	10*	123	NT	~1*	NT	63	98 ³	1:1 (w) ²
bPL G133R	<1*	5*	NT	AN	NT	23*	65*	1:1 ³
bPL A26W	8*	95	NT	AN	NT	18*	57*	1:2
bPL A26W/G133K	<1*	22*	NT	AN	NT	25*	61*	1:2

¹The experiments with oPL or oPL analogues were performed in cells transfected with oPRLRs, and the experiments with bPL of bPL analogues were performed in cells transfected with bPRLRs. ²From Helman *et al.* (1997). ³From Helman *et al.* (1998). oMG=ovine mammary gland; NT=not tested; AN=antagonist; w=weak. The relative activity was determined by comparing the respective IC₅₀ (binding experiments) or EC₅₀ (bioassays) values. The differences between the different curves were determined from the respective s.d. values obtained by the PRIZMA curve-fitting program and were analysed with the two-tailed t-test. *Values that are significantly different ($P>0.05$) from the unmodified oPL or bPL.

associated kinase JAK2 has to be transphosphorylated and that this requires a 'minimal time' of homodimer existence. Once this goal has been achieved, the existence of the homodimer is no longer obligatory. Our present and former results (Vashdi-Elberg *et al.* 1995, Helman *et al.* 1997, 1998, Tchelet *et al.* 1997, Herman *et al.* 1999) showing that bPL analogues such as G133R and G133K, as well as others, lose their biological activity mediated by heterologous GHRs but fully retain the activity mediated through heterologous PRLRs, lead us to suggest that this 'minimal time' is shorter for PRLRs than for GHRs. In the case of the interaction between ruminant PLs and ruminant PRLRs, this 'minimal time' is still met, even though the interaction with homologous receptors has a shorter half-life than that with heterologous PRLRs (Gertler *et al.* 1996). Therefore oPL and bPL are active in both homologous and heterologous lactogenic assays. In the present work on several oPL and bPL analogues, residues at sites 1 or 2 which participate in the interaction with heterologous PRLRs were changed. As a result, the 'time of homodimer persistence' was shortened, though not below the minimum threshold required for activation of rPRLRs and therefore the activity was preserved. In contrast, homologous (ruminant) interactions with PRLRs are already weaker than heterologous interactions and, subsequently, the half-life of the receptor dimer is shorter (Tchelet *et al.* 1995, Gertler *et al.* 1996, Sakal *et al.* 1997). Therefore further destabilization of the complex shortens the complex's existence below the 'minimal time', leading to complete (oPL analogues T185D, G130R, K71E and bPL analogues G133R, A26W, A26W/G133K) or almost complete (oPL T185F, bPL T188D and bPL K73D) loss of biological activity. If the loss of activity results mainly from

a weakened interaction between the receptor and site 2 of the hormone, the analogue becomes a site-2 antagonist, as suggested for hGH by Wells & de Vos (1996). The 'minimal time' hypothesis also explains our previously published unexplained observation that point mutations of hGH abolish its lactogenic activity mediated through bPRLR but not that mediated through rPRLR (Binder *et al.* 1990).

Our current hypothesis of a 'minimal time' concept is also supported by the results of Pearce *et al.* (1999), who tested the biological activities of several hGH analogues in FDC-P1 cells expressing hGHR. It was found that despite the increased affinity for sites 1, 2 or both, none of these variants changed the EC₅₀ for cell proliferation or the levels of JAK2 phosphorylation. The same group also studied the effect of a reduction in site-1 affinity on cell proliferation by using hGH mutants in which affinity for site 1 is reduced from 5- to 500-fold. Cell proliferation was clearly shown to be unaffected until affinity was reduced about 30-fold relative to wild-type hGH. It was concluded that the GH binding affinity for its receptor surpasses the requirements for cellular activity, or that a difference in affinity of up to 1000-fold (resulting from either raising or reducing the affinity toward hGH or rbGH receptors) had no effect on biological activity (Pearce *et al.* 1999).

As we have recently reported the crystallization of the 1:2 oPL:rPRLR-ECD complex (Christinger *et al.* 1998) and elucidated its three-dimensional structure (Elkins *et al.* 2000), comparison of rPRLR-ECD and oPRLR-ECD structures by modelling (O Livnah & A Gertler, unpublished observations) has allowed us to look for structural differences responsible for the lower affinity of the latter. When comparing the differences in primary structure

between rPRLR-ECD and oPRLR-ECD based on the already determined three-dimensional structure of oPL: rPRLR, several differences may account for the lower affinity of oPL to oPRLR. We examined the differences at three sites of the complexed receptors: (1) ligand binding site 1, (2) ligand binding site 2 and (3) the receptor-receptor contact surface. In ligand binding site 1, there are three differences in amino acid residues that interact with oPL: I74 M [L3], T141I [L5] and H163K [L5-6] (Ekins *et al.* 2000). At site 2, there is only one mutation, I74 M [L3], and in the receptor-receptor contact area there are two mutations in R2:G162P and H163K. Taken together, these changes in the size and chemical properties of the amino acid residues, resulting in several bumps in the intermolecular contact, lead one to assume that oPL binding is likely to be somewhat different for oPRLR and rPRLR. These differences in interactions would result in alterations in the interdomain orientation of oPRLR, which in turn has a direct influence on the receptor-receptor interactions and thus causes lower affinity towards the ligand oPL.

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