



HAL
open science

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

E. Sakal, Christophe Bignon, N. Chapnik-Cohen, Nathalie Daniel-Carlier, Jacqueline Paly, L. Belair, Jean Djiane, A. Gertler

► To cite this version:

E. Sakal, Christophe Bignon, N. Chapnik-Cohen, Nathalie Daniel-Carlier, Jacqueline Paly, et al.. Cloning, preparation and characterization of biologically active recombinant caprine placental lactogen. *Journal of Endocrinology*, 1998, 159 (3), pp.509-518. 10.1677/joe.0.1590509 . hal-02698216

HAL Id: hal-02698216

<https://hal.inrae.fr/hal-02698216>

Submitted on 1 Jun 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

E Sakal¹, C Bignon², N Chapnik-Cohen¹, N Daniel², J Paly²,
L Belair², J Djiane² and A Gertler¹

¹Institute of Biochemistry, Food Science and Nutrition, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovot 76100, Israel and ²Unité d'Endocrinologie Moléculaire, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France

(Requests for offprints should be addressed to A Gertler)

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 re-naturation 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.

Journal of Endocrinology (1998) **159**, 509–518

Introduction

Placental lactogens (PLs) are secreted from placentae 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 (PLRs) 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

pregnancy is different from that of the ovine; while in the latter the placenta becomes the primary source of progesterone in the second and the third trimesters of pregnancy, in the caprine the corpus luteum alone is responsible for progesterone production throughout the whole pregnancy (Hulet & Shelton 1980). The role of cPL in controlling this process has not yet been clarified. Caprine milk is gaining growing attention as a substitute for bovine milk, mainly in the cases of allergy syndromes or for adults lacking lactase. As cPL has not been cloned before, the present work was aimed at cloning and preparing substantial quantities of recombinant cPL that will facilitate studies in the homologous species. 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).

Materials and Methods

Materials

Recombinant hGH was obtained from Biotechnology General Inc. (Ness-Zioma, 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. Ovine prolactin (NIDKK AFP-8277E) was obtained from the National Hormone and Pituitary Program (Bethesda, MD, USA). Carrier-free Na^[125I] was purchased from the New England Nuclear Corp. (Boston, MA, USA). Isopropyl-1-thio-β-D-galactopyranoside (IPTG), RPMI-1640 medium, lysozyme, Triton X-100 and bovine serum albumin (BSA, RIA grade) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Superdex 75 HR 10/30 column, Q-Sepharose and SP-Sepharose (fast flow) were purchased from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). SDS-PAGE reagents were purchased from BioRad Laboratories (Richmond, CA, USA).

Cloning of cPL cDNA

The cPL cDNA was cloned by reverse transcription (RT)-PCR. One microgram total RNA from goat placenta was reverse transcribed, and a fraction of the reaction was PCR-amplified with Hi-Taq polymerase (Bioprobe Systems SA, Montreuil, France), between primers cPL5' (GGA TTT CTC TCC AAT CCT CAT G) and cPL3' (ATA AAA CTC AAA GAA GCT TTT GAC), deduced from the ovine PL sequence (Colosi *et al.* 1989) in the following program: (94 °C for 6 min) × 1, (94 °C for 1 min, 50 °C for 30 s, 72 °C for 1 min) × 20, (72 °C

for 10 min) × 1. The PCR product was gel purified and, after extraction, ligated to pGEM-T vector (Promega, Madison, WI, USA). Six white colonies were screened by PCR with universal M13 sequencing primers for the presence of insert, and the first four were sequenced.

Subcloning of cPL cDNA into pET8c expression vector

To facilitate the expression of the unfused protein in a prokaryotic expression vector, the signal sequence was removed from the cPL cDNA and restriction sites and a new translation start was added. The cPL cDNA (clone 4) was used as template in a PCR experiment with primers cPL3 (GAT ATC GAA TTC CCA TGG AGA ACT ATC CAC CAT AC) and cPL4 (AAG CTT CTG CAG GGA TCC TTA GCA TGA GGT CAA TCG GCA), and *Pfu* DNA polymerase (Stratagene Ltd, Cambridge, UK) in the following program: (94 °C for 5 min) × 1, (94 °C for 1 min, 55 °C for 30 s, 75 °C for 2 min) × 15, (75 °C for 15 min) × 1. The PCR product was separated from the template by gel electrophoresis and, after digestion by EcoRI and BamHI, subcloned into EcoRI/BamHI-digested pBluescript. After sequencing, the insert from one colony was subcloned from pBluescript into pET8c pre-digested with NcoI/BamHI. Automatic DNA sequencing was performed to confirm the proper sequence.

Expression, refolding and purification of cPL

Transformed *E. coli* BL21 cells were grown in Terrific Broth medium to OD₆₀₀=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 mM, 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 l 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

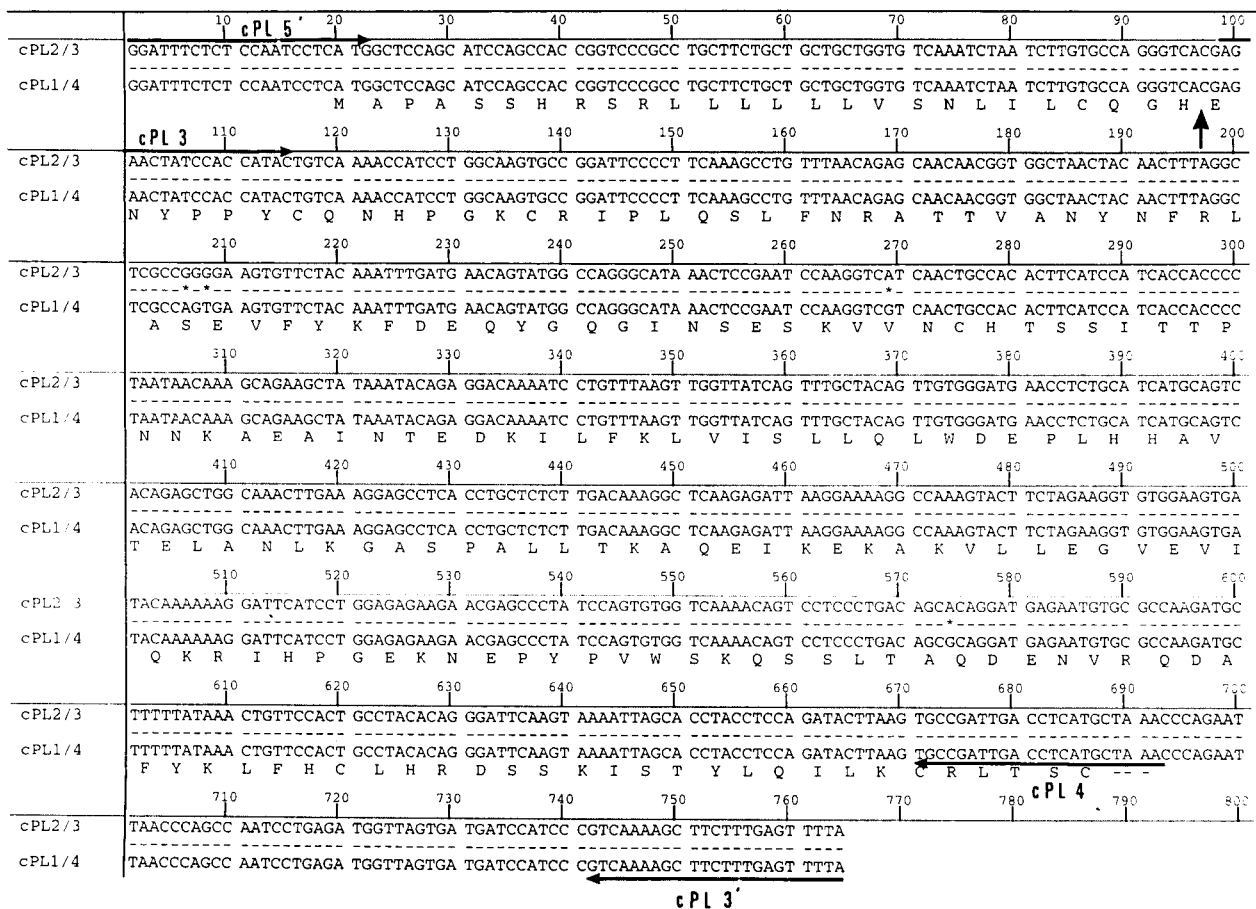


Figure 1 DNA sequence analysis revealed two bands of sequences illustrated by clones 1 and 4 and by clones 2 and 3. The vertical arrow indicates the predicted first amino acid of the mature protein, and the horizontal arrows the respective primers (see text).

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 radiolabeled ¹²⁵I-oPL as a ligand. Iodination of oPL was performed according to the protocol described by Gertler *et al.* (1984).

	10	20	30	40	50	60	70	80
oPL	MAPASSHREH	QWTCNLVRS	RLLLLLVVSN	LILCQQAQH	PPYCRNQPGK	CQIPLQNLFD	RATTVANYNS	KLAGEVMNRF
	-----**	#####	-----*---	-----***	-----*---	-*-----*	-----*	*--*-----*
cPL	MAPASSHR--	-----S	RLLLLLVVSN	LILCQGHENY	PPYCNHPGK	CRIPLQSLFN	RATTVANYNF	RLASEVFYKF
	-----##	#####	#-----#---	-#-----#-	#-----#-	-----#	---#-##-#	---#-##-#
bPL	MAPASSHRGH	QWICDLVRS	CLLLLLVVSN	LLLCQGAEDY	APYCKNQPGN	CRIPLQSLFE	RATLVASNNY	RLAREMFNEF
	90	100	110	120	130	140	150	160
oPL	DEYQGQGIN	ESKVIN-CHT	SSITPNNKA	EAINTEDEAL	FKLVISLLHS	WDEPLHHAVT	ELANSKGTSP	ALLTKAQEIK
	-----	---*--&---	-----	-----	-----**	-----	---*---*--	-----
cPL	DEYQGQGIN	ESKVVN-CHT	SSITPNNKA	EAINTEDEAL	FKLVISLLQL	WDEPLHHAVT	ELANLKGASP	ALLTKAQEIK
	##-#-#-#	#-##-#-#	###-----#	#-#-----#-	##-----##	-----#---	---###---	##-##-#-#
bPL	NKQFGEGKNF	TSKFINSCHT	EFMTTPNNKE	AAANTEDEAL	LRLVISLLHS	WDEPLHQAAT	ELLHRNGASP	DILARAKEIE
	170	180	190	200	210	220	230	237
oPL	EKAKVLVEGV	EVIQKRIHPG	EK-NEPYPVW	SEQSSLTSD	ENVRQVAFYR	LFHCLHRDSS	KIYTYLRILK	CRLTSCET
	-----*---	-----	--&-----	-*-----*--	-----*---	-----	---*-----*	-----
cPL	EKAKVLVEGV	EVIQKRIHPG	EK-NEPYPVW	SKQSSLTQAD	ENVRQDAFYK	LFHCLHRDSS	KISTYLQILK	CRLTSC
	#-#-----	-#-----#-	--&-----	-##-----#-	-#-----#	#-----	-----###---	--#-#-
bPL	DKTKVLVEGV	EMIQKRVHPG	EKKNEPYPVW	SEKSSLTADD	EDVRQTAFYR	MFHCLHRDSS	KISTYINLLK	CRFTPC

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).

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% Ultrosor SF (Biosepra, S.A., Villeneuve-la-Garenne, France), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 50 μ g/ml gentamycin. After 4 days culture, the acini were incubated with different concentrations of oPRL or cPL 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.

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) previously equilibrated with 25 mM NaAc buffer, pH 5.0. Elution was carried out using a discontinuous NaCl gradient in the same buffer at a rate of 90 ml/h, and 10 ml fractions were collected (Fig. 3B). The pure monomeric cPL (as evidenced by gel filtration on a Superdex column) was eluted as a wide peak (second peak, fractions 26–31) with 0.35 M NaCl. Caprine PL monomeric fractions from both columns (see Fig. 3 underlined) were dialyzed against 0.1% NaHCO₃ and lyophilized. Inclusion bodies prepared from 2.5 l fermentation culture yielded ~35 mg protein

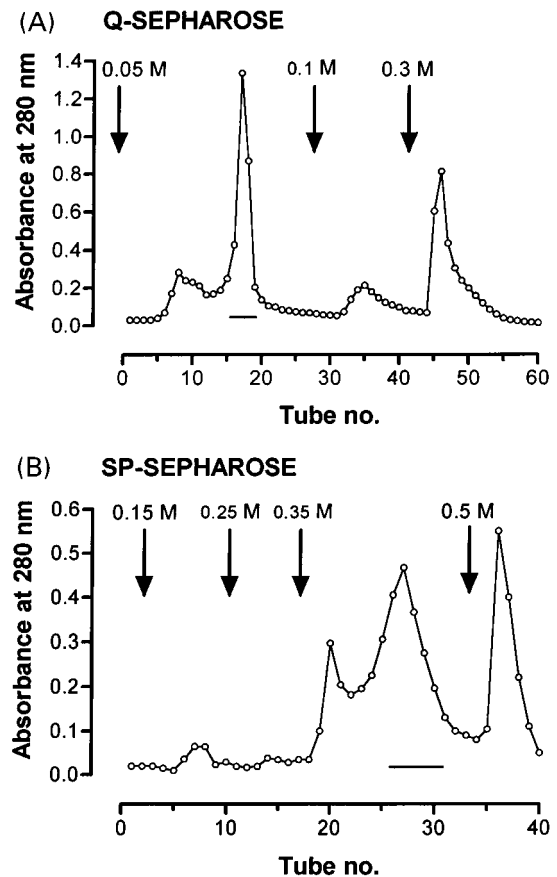


Figure 3 Purification of proteins extracted from inclusion bodies on Q-Sepharose and SP-Sepharose columns. (A) The Q-Sepharose column (2.5 × 7 cm) was equilibrated with 10 mM Tris-HCl, pH 9.0, at 4 °C. The refolded proteins were applied to the column at a rate of 120 ml/h and eluted with a discontinuous 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

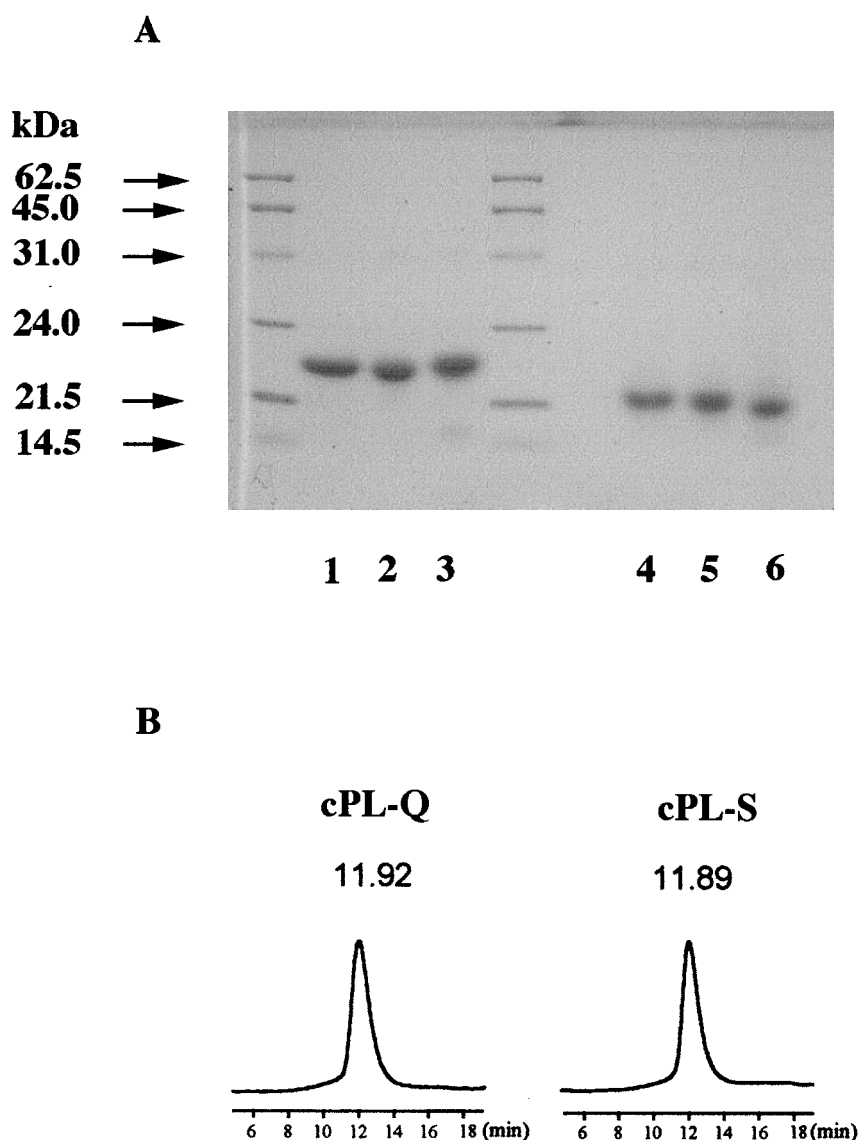


Figure 4 (A) SDS-PAGE of oPL, cPL-Q and cPL-S on 12.5% SDS-polyacrylamide gel in the presence (lanes 1, 2, and 3) and in the absence (lanes 4, 5 and 6) of β -mercaptoethanol. The gel was stained with Coomassie Brilliant Blue R. Molecular mass markers are shown on the left. (B) Determination of the cPL-Q 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.

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

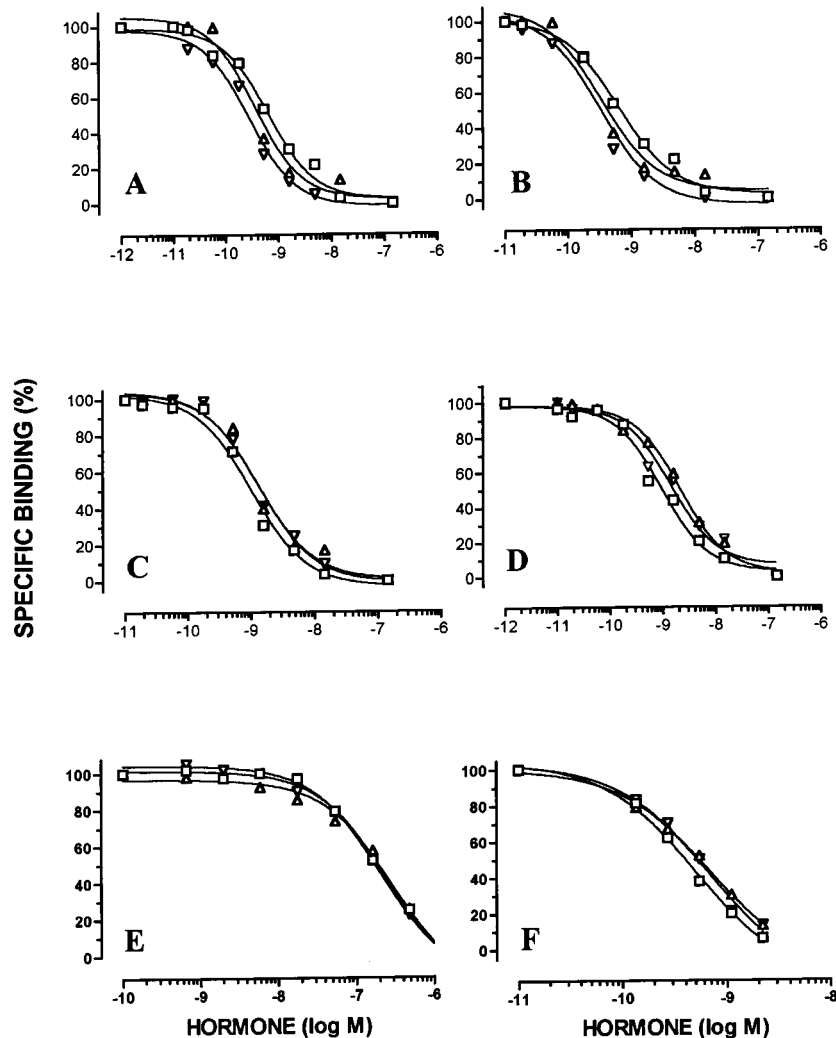


Figure 5 Competition of unlabeled oPL (\square), cPL-Q (\triangle) and cPL-S (∇) with ^{125}I -oPL for binding to (A) hGHR-ECD, (B) rbGHR-ECD, (C) rbPRLR-ECD, (D) rPRLR-ECD, (E) bPRLR-ECD and (F) rbMG. The specific binding was 27% in (A), 22% in (B), 19% in (C), 21% in (D), 15% in (E) and 35% in (F). Full lines were calculated using the PRIZMA curve-fitting program.

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 ($R^2 > 0.97$) and higher than for the two-site competition curves. Though IC_{50} values for oPL, cPL-Q and cPL-S were similar in general (Fig. 5 and Table 1), both 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 IC_{50} values for binding to bPRLR-ECD were higher than for other receptors and the specific binding of the ^{125}I -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

Table 1 The IC₅₀ values of oPL, cPL-Q and cPL-S calculated from the binding experiments presented in Fig. 5.

Receptor	IC ₅₀ values (nM)		
	oPL	cPL-Q	cPL-S
rbPRLR-ECD	1.03	1.32	1.39
rbGHR-ECD	0.63	0.33	0.37
rPRLR-ECD	0.95	2.13	1.40
hGHR-ECD	0.63	0.37	0.28
bPRLR-ECD	238	273	201
rbMG microsomes	0.51	0.68	0.68

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

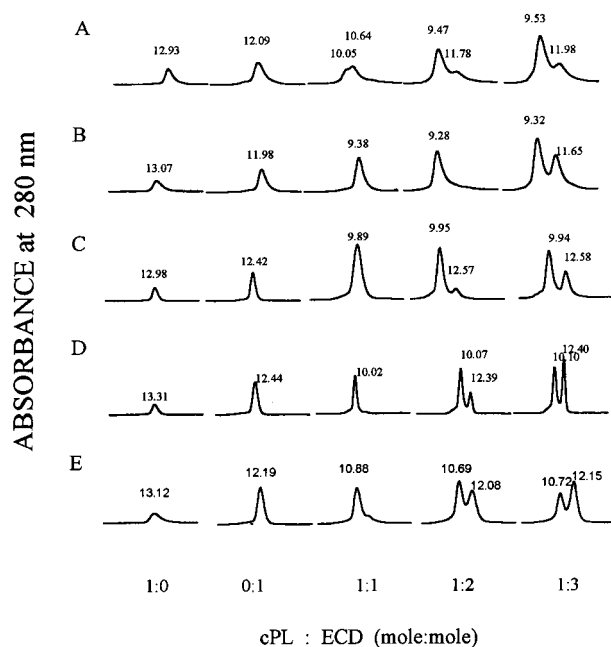


Figure 6 Gel filtration of cPL-Q complexes with (A) hGHR-ECD, (B) rbGHR-ECD, (C) rbPRLR-ECD, (D) rPRLR-ECD and (E) bPRLR-ECD on a Superdex 75 HR 10/30 column. Complex formation was carried out during 20 to 30 min incubation at room temperature in TN buffer using various ECD:cPL ratios. The initial hormone concentration was constant (1.6 μ M). Aliquots (200 μ l) 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.

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,

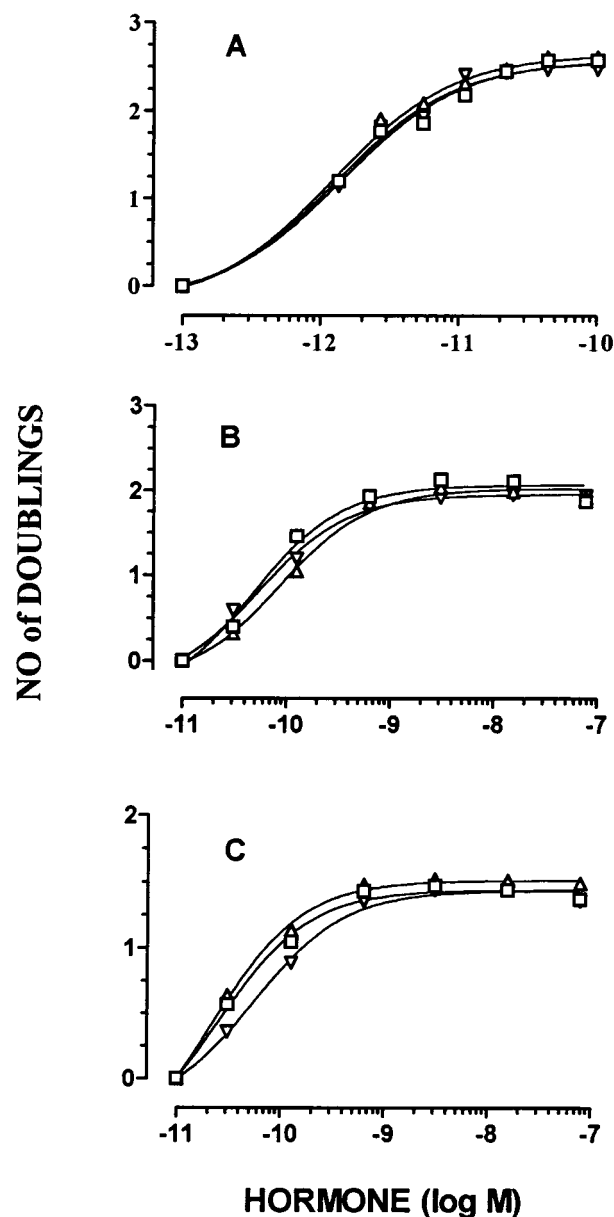


Figure 7 Effect of oPL (\square), cPL-Q (Δ) and cPL-S (∇) on the proliferation of (A) Nb₂-T1C lymphoma cells, (B) FDC-P1-hGHR9D11 cells, and (C) FDC-P1-rbGHR3B9 cells. Number of doublings was calculated as described previously (Gertler *et al.* 1985).

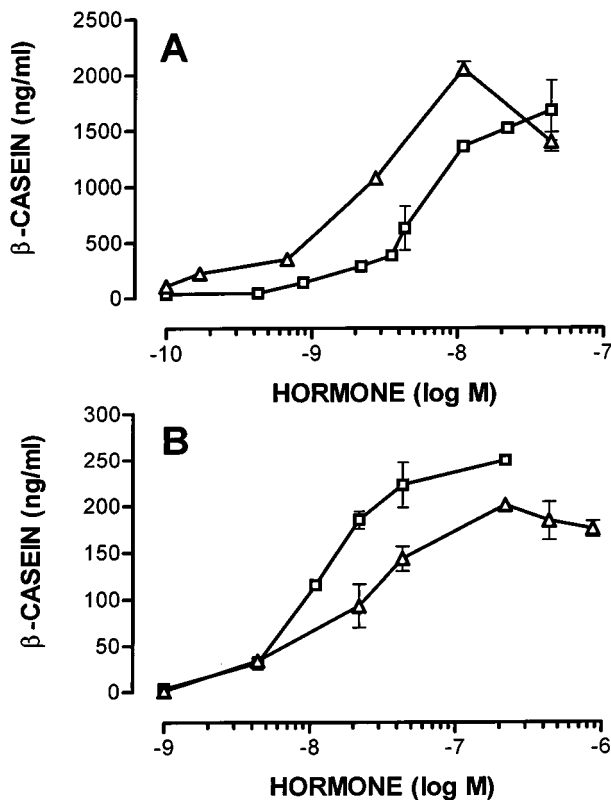


Figure 8 Effect of cPL-Q (Δ) and oPRL (□) on β-casein production in (A) rabbit and (B) ovine mammary gland acini culture. The results are means ± S.D. of two replicates.

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, ($EC_{50}=2 \times 10^{-9}$ vs 6×10^{-9} M), whereas in sheep culture it was slightly lower ($EC_{50} \sim 30 \times 10^{-9}$ 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 bioassay. Somatogenic activity of cPL-Q and cPL-S was almost identical to that of oPRL in two heterologous bioassays in FDC-P1 cells transfected with either hGHRs (Fig. 7B) or rbGHRs (Fig. 7C). The respective EC_{50} values for cPL-Q, cPL-S and oPRL 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 stimulation of β-casein synthesis in rabbit MG acini was only 15-fold lower ($EC_{50}=2 \times 10^{-9}$ M) than in the corresponding ovine culture ($EC_{50}=30 \times 10^{-9}$ 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.

Acknowledgements

The authors thank the National Hormone and Pituitary Program (Bethesda, MD, USA) for ovine prolactin.

References

- Anthony RV, Liang R, Kayl EP & Pratt SL 1995 The growth hormone/prolactin gene family in ruminant placenta. *Journal of Reproduction and Fertility* (Suppl) **49** 83–95.
- Bignon Ch, Sakal E, Belair L, Chapnik-Cohen N, Djiane J & Gertler A 1994 Preparation of recombinant extracellular domain of rabbit prolactin receptor expressed in *Escherichia coli* and its interaction with lactogenic hormones. *Journal of Biological Chemistry* **269** 3318–3324.
- Byatt JC, Wallace CR, Bremel RD, Collier RJ & Bolt DJ 1987 The concentration of bovine placental lactogen and the incidence of different forms in fetal cotyledons and in fetal serum. *Domestic Animal Endocrinology* **4** 231–241.
- Byatt JC, Welply JK, Leimgruber RM & Collier RJ 1990 Characterization of glycosylated bovine placental lactogen and the effect of enzymatic deglycosylation on receptor binding and biological activity. *Endocrinology* **127** 1041–1049.
- Byatt JC, Warren WC, Eppard PJ, Staten NR, Krivi GG & Collier RJ 1992 Ruminant placental lactogens: structure and function. *Journal of Animal Sciences* **70** 2911–2923.
- Colosi P, Thordarson G, Hellmiss R, Singh K, Forsyth IA, Gluckman PD & Wood WI 1989 Cloning and expression of ovine placental lactogen. *Molecular Endocrinology* **3** 1462–1469.
- Christinger HW, Elkins PA, Sandowski Y, Sakal E, Gertler A, Kossiakoff AA & de Vos AM 1998 Crystallization of ovine placental lactogen in a 1:2 complex with the extracellular domain of the rat prolactin receptor. *Acta Crystallographica* (In Press).
- Forsyth IA 1986 Variations among species in the endocrine control of mammary growth and function: the roles of prolactin, growth hormone and placental lactogen. *Journal of Dairy Science* **69** 866–878.
- Freemark M & Comer M 1989 Purification of a distinct placental lactogen receptor, a new member of growth hormone/prolactin receptor family. *Journal of Clinical Investigations* **83** 883–889.
- Galosi SS, Gertler A, Elberg G & Laird DM 1991 Distinct placental and prolactin (lactogen) receptors in bovine endometrium. *Molecular and Cellular Endocrinology* **78** 229–236.
- Gertler A, Ashkenazi A & Madar Z 1984 Binding sites of human growth hormone and ovine and bovine prolactins in the mammary gland and the liver of lactating dairy cow. *Molecular and Cellular Endocrinology* **34** 51–57.
- Gertler A, Walker A & Friesen HG 1985 Enhancement of human growth hormone stimulated mitogenesis of Nb2 node lymphoma cells by tetradecanoyl-phorbol-13-acetate (TPA). *Endocrinology* **116** 1636–1644.
- Gertler A, Hauser SD, Sakal E, Vashdi D, Staten NR, Freeman JJ & Krivi GG 1992 Preparation, purification and determination of the biological activities of twelve N-terminus truncated recombinant analogues of bovine placental lactogen. *Journal of Biological Chemistry* **271** 12655–12659.
- Gertler A, Grosclaude J & Djiane J 1996 Affinity and stoichiometry of interaction between extracellular domains (ECDs) of prolactin receptor (PRLR) from three species and various lactogenic hormones. *Journal of Biological Chemistry* **271** 24482–24491.
- Goffin V & Kelly PA 1997 The prolactin/growth hormone family: structure/function relationship. *Journal of Mammary Gland Biology and Neoplasia* **2** 7–17.
- Hulet CV & Shelton M 1980 Sheep and goat. In *Reproduction of Farm Animals*, pp 346–354. Ed ESE Hafez. Philadelphia: Lea & Febinger.
- Jahn G, Dusanter-Fourt I, Kelly PA, Houdebine LM & Djiane J 1987 Measurement by radioimmunoassay of casein content in rabbit mammary gland during pregnancy and after prolactin stimulation in organ culture. *Proceedings of the Society for Experimental Biology and Medicine* **184** 19–23.
- Laemmli UK 1970 Cleavage of structure proteins during assembly of the head of bacteriophage T4. *Nature* **227** 680–685.
- Rowlinson SW, Barnard R, Bastiras S, Robins AJ, Brinkworth R & Waters MJ 1995 A growth hormone agonist produced by targeted mutagenesis at binding site 1. *Journal of Biological Chemistry* **270** 16833–16839.
- Rowlinson SW, Waters MJ, Lewis, UJ & Barnard R 1996 Human growth hormone fragments 1–43 and 44–193: *in vitro* somatogenic activity and receptor binding characteristics in human and non-primate systems. *Endocrinology* **137** 90–95.
- Sakal E, Bignon Ch, Kantor A, Leibovitch H, Shamay A, Djiane J & Gertler A 1997 Large-scale preparation of recombinant ovine placental lactogen. *Journal of Endocrinology* **152** 317–327.
- Sandowski Y, Nagano M, Bignon C, Djiane J, Kelly PA & Gertler A 1995 Recombinant extracellular domain of rat prolactin receptor interacts with different affinity and stoichiometry with various lactogenic hormones. *Molecular and Cellular Endocrinology* **115** 1–11.
- Shamay A, Pines M, Waksman M & Gertler A 1990 Proliferation of bovine mammary epithelial cells *in vitro* is modulated by G-proteins. *Molecular and Cellular Endocrinology* **69** 217–226.
- Tchelet A, Staten NR, Creely DP, Krivi GG & Gertler A 1995 Extracellular domain of prolactin receptor from bovine mammary gland: expression in *Escherichia coli*, purification and characterization of its interaction with lactogenic hormones. *Journal of Endocrinology* **144** 393–403.
- Wallis M 1992 The expanding growth hormone/prolactin family. *Journal of Molecular Endocrinology* **9** 185–188.

Received 3 April 1998

Accepted 27 July 1998