

Identification of two new nonclassical members of the rat prolactin family

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ABSTRACT

The prolactin (PRL) family is comprised of a group of hormones/cytokines that are expressed in the anterior pituitary, uterus, and placenta. These proteins participate in the control of maternal and fetal adaptations to pregnancy. In this report, we have identified two new nonclassical members of the rat PRL family through a search of the National Center for Biotechnology Information dbEST database. The cDNAs were sequenced and their corresponding mRNAs characterized. Overall, the rat cDNAs showed considerable structural similarities with mouse proliferin-related protein (PLF-RP) and prolactin-like protein-F (PLP-F), consistent with their classification as rat homologs for PLF-RP and PLP-F. The expression of both cytokines/hormones was restricted to the placenta. The intraplacental sites of PLF-RP and PLP-F synthesis differed in the rat and the mouse. In the

mouse, PLF-RP was expressed in the trophoblast giant cell layer of the midgestation chorioallantoic and choriovitelline placentas and, during later gestation, in the trophoblast giant cell and spongio-trophoblast layers within the junctional zone of the mouse chorioallantoic placenta. In contrast, in the rat, PLF-RP was first expressed in the primordium of the chorioallantoic placenta (ectoplacental cone region) and, later, exclusively within the labyrinth zone of the chorioallantoic placenta. In the mouse, PLP-F is an exclusive product of the spongio-trophoblast layer, whereas in the rat, trophoblast giant cells were found to be the major source of PLP-F, with a lesser contribution from spongio-trophoblast cells late in gestation. In summary, we have established the presence of PLF-RP and PLP-F in the rat.

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INTRODUCTION

Pregnancy requires the regulated availability and delivery of resources throughout fetal development. Control of these gestation-associated processes are very much linked to the uterus and placenta and, in species with hemochorial placentation, to decidual and trophoblast cells. These cells orchestrate maternal changes required for the establishment and maintenance of pregnancy, at least in part, through the secretion of cytokines and hormones. The uteroplacental prolactin (PRL) family of cytokines/hormones participates in the regulation of physiological mechanisms ensuring

viviparity (Soares *et al.* 1998). Individual members of the PRL family have been shown to possess classical PRL-like actions, implicating their participation in the control of maternal ovarian and mammary gland development and function (Thordarson *et al.* 1986, Colosi *et al.* 1988a, Galosy & Talamantes 1995), while other members possess nonclassical actions and may contribute to the establishment of vascular connectivity between maternal and fetal compartments (Jackson *et al.* 1994, Linzer 1995), immune regulation (Robertson *et al.* 1982, 1994, Cohick *et al.* 1996, Dai *et al.* 1996a, Müller *et al.* 1999), and the control of hematopoiesis (Lin & Linzer 1999).

The rat and mouse possess at least 15 different members of the PRL family (Soares *et al.* 1998). Some members of the PRL family have been characterized in both the mouse and the rat, whereas other members have only been characterized in either the rat or the mouse. The existence of cross-species homologs for members of the PRL family potentially expands the significance of each individual member and the availability of experimental models for studying their biology. The deposition of expressed sequence tags (ESTs) from rat placental cDNA libraries by the University of Iowa Rat Gene Discovery Program into the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) dbEST database, prompted a search for new members of the rat PRL family.

Inspection of the dbEST database resulted in the identification of new members of the rat PRL family, including putative rat homologs for two members of the mouse placental PRL family, proliferin-related protein (PLF-RP) and prolactin-like protein-F (PLP-F). PLF-RP and PLP-F are nonclassical PRL family members that have only been characterized in the mouse (Linzer & Nathans 1985, Lin *et al.* 1997a, Müller *et al.* 1998c). The existence of a possible hamster PLF-RP homolog has been proposed (Barnes & Renegar 1996). Both PLF-RP and PLP-F are exclusively synthesized by trophoblast cells of the mouse placenta (Linzer & Nathans 1985, Lin *et al.* 1997a, Müller *et al.* 1998c). PLF-RP has been shown to be a prominent negative placental regulator of angiogenesis (Jackson *et al.* 1994). The biological actions of PLP-F have yet to be reported.

In this report, we present data on rat PLF-RP and PLP-F cDNAs, and describe their expression patterns, as well as their intraplacental cellular sites of synthesis. Both genes are exclusively synthesized by the placenta. Most interestingly, the intraplacental sites of PLF-RP and PLP-F synthesis differ in the rat and the mouse placentas.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS) and donor horse serum (HS) were purchased from JRH Bioscience (Lenexa, KS, USA). All restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). Rat PLF-RP and PLP-F cDNAs were obtained from the University of Iowa Rat Gene Discovery Program through either the American Type Culture Collection (ATCC, Manassas, VA, USA) or Research Genetics (Huntsville, AL, USA).

A rat cDNA for heart fatty acid binding protein (hFABP) was obtained from Dr Jeffrey I Gordon, Washington University, St Louis, MO, USA (Heuckeroth *et al.* 1987). DNA extraction kits were purchased from Qiagen (Chatsworth, CA, USA). Nitrocellulose and nylon membranes were obtained from Schleicher and Schuell (Keene, NH, USA). Radiolabeled nucleotides were purchased from DuPont-NEN (Boston, MA, USA). TRIzol reagent for RNA was obtained from Life Technologies (Gaithersburg, MD, USA). Unless otherwise noted, all other chemicals and reagents were purchased from Sigma (St Louis, MO, USA).

Animals and tissue preparation

Holtzman rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA). CD-1 mice were obtained from Charles River Inc. (Wilmington, MA, USA). The animals were housed in an environmentally controlled facility, with lights on from 0600 to 2000 h, and allowed free access to food and water. Timed pregnancies and tissue dissections were performed as previously described (Soares 1987, Orwig *et al.* 1997b). The day on which a copulatory plug was found was designated day 0 of rat pregnancy. Protocols for the care and use of animals were approved by the University of Kansas Animal Care and Use Committee.

Cell culture

Rcho-1 trophoblast cells were cultured as previously indicated (Faria & Soares 1991). Briefly, Rcho-1 trophoblast cells were maintained in a proliferative subconfluent state in NCTC-135 culture media supplemented with 10 mM HEPES, 50 μ M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 20% FBS in a 37 °C incubator under 95% air/5% CO₂. After 3 days of culture in NCTC-135 culture medium supplemented with 20% FBS, cultures of Rcho-1 trophoblast cells were switched to NCTC-135 culture medium containing 10% HS serum to facilitate differentiation (Hamlin *et al.* 1994, Hamlin & Soares 1995, Peters *et al.* 1999). Rcho-1 trophoblast cells are capable of differentiating along the junctional zone trophoblast giant cell phenotype. Trophoblast giant cell differentiation was verified by the morphological detection of trophoblast giant cells and/or the expression of placental lactogen (PL)-I (Peters *et al.* 1999). The HRP-1 trophoblast stem cell line, which was derived from labyrinthine trophoblast and exhibits directional transport properties (Soares *et al.* 1987, Shi *et al.* 1997), was maintained in RPMI-1640 medium containing

50 μ M 2-mercaptoethanol, 1 mM sodium pyruvate, antibiotics, and 20% FBS, and was used as a control in the expression studies.

Characterization of PLF-RP and PLP-F cDNAs

Examination of the dbEST database from day 14 rat placenta revealed the presence of several cDNA clones exhibiting a high degree of homology with mouse PLF-RP and PLP-F. We obtained these clones from the University of Iowa Rat Gene Discovery Program. DNA sequencing was performed using an Applied Biosystems Model 310 sequencer and Applied Biosystems Dye Terminator Cycle Sequencing kits (Foster City, CA, USA). Both strands of the cDNAs were completely sequenced. Comparisons of PLF-RP and PLP-F sequences with other members of the PRL family were performed with CLUSTAL W (version 1.6) (Thompson *et al.* 1994).

Analysis of PLF-RP and PLP-F expression

The expression of PLF-RP and PLP-F mRNAs in the rat and mouse was assessed by biochemical and cytochemical procedures.

Northern blot analysis The expression of PLF-RP and PLP-F mRNA was determined by Northern blotting as previously described in our laboratory (Faria *et al.* 1990, Orwig *et al.* 1997b). Total RNA was extracted from various rat tissues, essentially as described by Chomczynski and Sacchi (1987), using TRIzol. Blots were probed with 32 P-labeled rat PLF-RP and PLP-F, and β -actin cDNAs (Orwig *et al.* 1997a,b).

In situ hybridization PLF-RP and PLP-F mRNAs were detected in frozen tissue sections as previously described (Faria *et al.* 1990, Rasmussen *et al.* 1997). Full-length rat PLF-RP and PLP-F cDNAs were linearized and used as templates for the synthesis of 35 S-labeled sense and antisense RNA probes. Hybridizations with sense and antisense RNA probes for PL-II (Dai *et al.* 1996a), hFABP (Heuckeroth *et al.* 1987, Knipp *et al.* 1999b) and PRL-like protein-C (PLP-C) (Deb *et al.* 1991b, Dai *et al.* 1996b) were used as additional controls for identifying placental cell subpopulations.

RESULTS

Rat PLF-RP cDNA characterization

A rat cDNA exhibiting sequence homology to mouse PLF-RP was obtained from the University

of Iowa Rat Gene Discovery Program (dbEST Id: 1656282). Both strands of the full-length putative rat PLF-RP cDNA were sequenced (Fig. 1). The cDNA exhibited 84% nucleotide identity with the previously described mouse PLF-RP cDNA (Linzer & Nathans 1985) and was found to encode for a 245 amino acid protein with 81% amino acid identity with mouse PLF-RP (Fig. 2). Based on homology with mouse PLF-RP, it was further predicted that rat PLF-RP contained a 30 amino acid signal peptide. Both rat and mouse PLF-RP also contain six homologously positioned cysteine residues. A notable difference between the rat and mouse structures was the presence of two putative *N*-linked glycosylation sites in the rat and three putative *N*-linked glycosylation sites in the mouse. The most carboxy-terminally located putative *N*-linked glycosylation site of mouse PLF-RP was absent in rat PLF-RP (Fig. 2).

Rat PLP-F cDNA characterization

Four rat cDNAs exhibiting sequence homology to mouse PLP-F were obtained from the University of Iowa Rat Gene Discovery Program (dbEST Id: 1539768, 1577422, 1752882, and 1753166). Only one of the clones (dbEST Id: 1753166) was found to be full length. Both strands of the full-length putative PLP-F cDNA were sequenced (Fig. 3). The cDNA exhibited approximately 80% nucleotide identity with the previously described mouse PLP-F cDNAs (Lin *et al.* 1997a, Müller *et al.* 1998c) and was found to encode for a 250 amino acid protein with 68% amino acid identity with the mouse PLP-F protein (Fig. 4). Based on homology with mouse PLP-F, it was further predicted that rat PLP-F contains a 29 amino acid signal peptide. This signal peptide is one amino acid shorter than the mouse PLP-F signal peptide. Both rat and mouse PLP-F contain six homologously positioned cysteine residues and three homologous putative *N*-linked glycosylation sites. Please note that the overall homology of rat and mouse PLP-F was somewhat less than that found for rat and mouse PLF-RP.

PLF-RP and PLP-F Northern analyses

Northern blot analyses from several rat tissues (brain, diaphragm, heart, kidney, liver, lung, ovary, spleen, and placenta) indicated that both PLF-RP and PLP-F mRNA expression was restricted to placental tissues (data not shown). Similar findings have been observed for mouse PLF-RP and PLP-F (Linzer & Nathans 1985, Lin *et al.* 1997a, Müller *et al.* 1998c). Rat PLF-RP and PLP-F mRNAs migrated at approximately 1 kb (Fig. 5).

CCTCGAGGCCAAGAATTTCGGCACGAGGGCATATTTCCAGAGTCAAGAGCTAAGCCTGGGTAA	62
-30	-20
GACTCTGCAGAG ATG CCC CCT TCT TTG ATT CAA TCT TGC TCC TCA GGG GCT	113
M P P S L I Q S C S S G A	
-10	
CTC CTG ATG CTG TTA ATG GCA AAT CTC TTC CTG TGG GAG AAG GTA TCC	161
L L M L L M A N L F L W E K V S	
-1 1	10
TCT GCA CCC ATA AAT GCC AGC GAG ACT ACC CTC AAT GAC TTG AAG GAC	209
S [↑] A P I N A S E T T L N D L K D	
20	30
TTG TTT GAT AAT GCA ACT GTA ATT TCT GGA AAG ATG GCT GAG CTT GGT	257
L F D N A T V I S G K M A E L G	
40	
TTT GCC ATG CGC AAA GAA TTT TTC AGC AAT TCA TTC TCT TCA GAC ATA	305
F A M R K E F F S N S F S S D I	
50	60
TTC ACT AAT ATT ATA TTA GAT CTG CAC AAG AGT AAG GAG AAT ATA ATC	353
F T N I I L D L H K S K E N I I	
70	
AAG GCT TTC AAC AGC TGC CAT ACT GTT CCT ATC AAT TTT CCT GAA ACT	401
K A F N S C H T V P I N F P E T	
80	90
ACT GAG GAT ATC CGA ATG ACC TCG TTT GAA GAG TTT TTG AAA ATG ATT	449
T E D I R M T S F E E F L K M I	
100	110
CTT CAG ACA CTG CTG GCC TGG AAA GAC CCT CTG CAA CAT CTA GTG ACA	497
L Q T L L A W K D P L Q H L V T	
120	
GAA CTC AGT GCT TTG CCA GGA TGC CCT TAT AGT ATC CTA TCA AAG GCC	545
E L S A L P G C P Y S I L S K A	
130	140
AAA GCC ATT GAG GCT AAA AAC AAA GAC CTT CTA GAG TAC ATC AAA AGA	593
K A I E A K N K D L L E Y I K R	
150	
ATA ATA TCC ATG GTT AAT CCT GCA ATC AAA GAA AAT GAA GAT TAC CCA	641
I I S M V N P A I K E N E D Y P	
160	170
ATC TGG TCA GAT TTG GAC TCT CTG CAG GCA GCT GAT AAA GAA ATT CAA	689
I W S D L D S L Q A A D K E I Q	
180	190
TTT TTT GCT CTT TAT ATG TTT TCC TTC TGC TTA CGT TCT GAC CTA GAA	737
F F A L Y M F S F C L R S D L E	
200	
TCA CTA GAT TTT CTA GTC AAT TTC CTA AAA TGT CTG CTT CTT TAT AAT	785
S L D F L V N F L K C L L L Y N	
210	
GAT AAT GTC TGC TAC TCA GGA TTT TGA AAAATTGCATGATCTATCTTGAAACT	839
D N V C Y S G F -	
TCTAATTTTATGGCCTTTGAATATATGATTAATCTTATGTGTCTATAAATAAAAGGAGACA	901
TCAGATATGTCAAGGATGG	920

FIGURE 1. Nucleotide and predicted amino acid sequences for rat PLF-RP. Encoded amino acids are indicated by single letter designations beneath their respective codons. Translation is assumed to begin at the first ATG (nucleotides 75–77) and continue until the termination codon, TGA (nucleotides 810–812). An arrow indicates the predicted signal peptide cleavage site between Ser⁻¹ and Ala⁺¹. The identity of this site as the cleavage site is based on homology with mouse PLF-RP (Linzer & Nathans 1985, Colosi *et al.* 1988b). Putative N-linked glycosylation sites are denoted by the amino acids enclosed in a black box. (GenBank Accession No. AF 139809.)

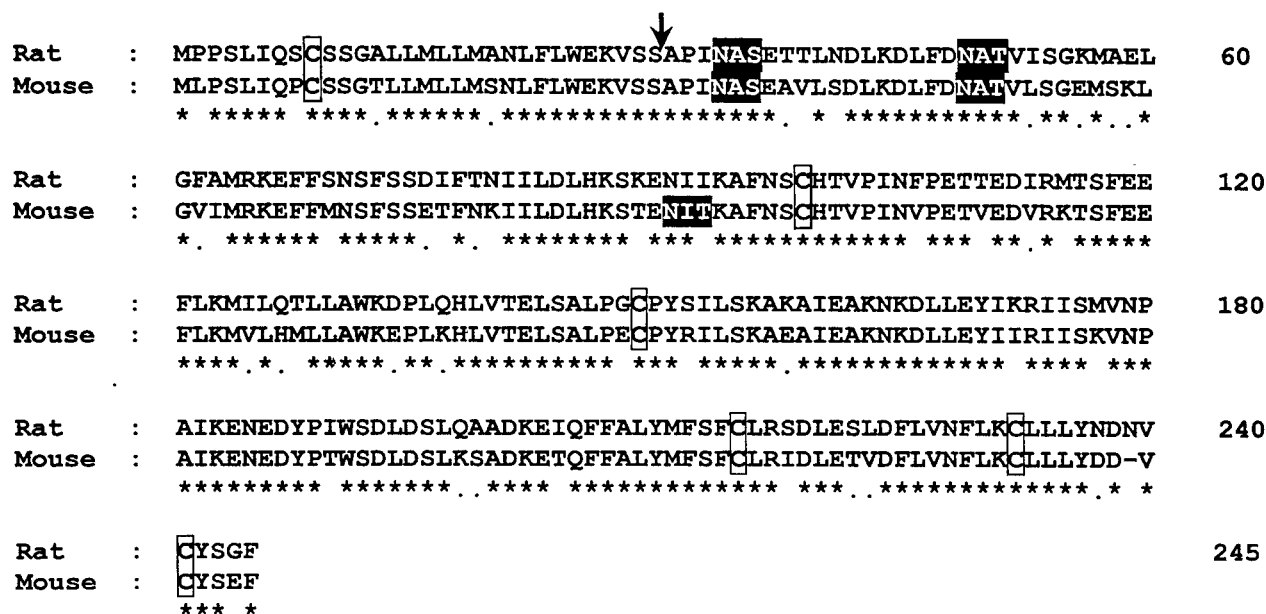


FIGURE 2. Amino acid sequence comparison of rat (present study) and mouse PLF-RP (Linzer & Nathans 1985). Please note that both sequences contain six cysteine residues positioned in homologous locations (shown as outlined boxes); however, they differ in the presence of putative *N*-linked glycosylation sites (black shaded boxes). Rat PLF-RP possesses two putative *N*-linked glycosylation sites, whereas mouse PLF-RP possesses three putative *N*-linked glycosylation sites. Asterisks below the sequences denote identity and dots below the sequences denote similarity.

Northern analyses of PLF-RP and PLP-F transcripts in placental tissues isolated from days 11 to 21 of gestation were performed. On day 11 of gestation, PLF-RP mRNA was preferentially expressed in chorioallantoic placenta primordia (Fig. 5). As gestation progressed, PLF-RP mRNA was predominantly expressed in the labyrinth zone of the chorioallantoic placenta (Fig. 5). PLP-F mRNA was detected in choriovitelline and chorioallantoic placentas from day 11 of gestation (Fig. 5). As gestation advanced, PLP-F was restricted to the junctional zone of the chorioallantoic placenta (Fig. 5). The integrity of the RNA was verified by hybridization of the samples with β -actin cDNA (Fig. 5).

Additional information on trophoblast lineages responsible for PLF-RP and PLP-F expression in the rat was obtained from Northern analyses of two rat trophoblast cell lines, Rcho-1 trophoblast cells and HRP-1 trophoblast stem cells. Rcho-1 trophoblast cells and HRP-1 trophoblast stem cells represent cell populations characteristic of the junctional zone trophoblast giant cell lineage (Faria & Soares 1991, Peters *et al.* 1999) and labyrinthine trophoblast cell precursors (Soares *et al.* 1987, Shi *et al.* 1997) respectively. PLF-RP transcripts were not detected in either trophoblast cell line, whereas

PLP-F mRNA expression was readily detected in differentiated Rcho-1 trophoblast cells but not in the HRP-1 trophoblast stem cell line (Fig. 6).

Collectively, the Northern blot experiments are consistent with PLF-RP expression associated with the labyrinth zone but not of a cell lineage represented by either the Rcho-1 trophoblast cell line or the HRP-1 trophoblast stem cell line. On the other hand, the source of PLP-F was attributed to both the choriovitelline and chorioallantoic placentas. Within the chorioallantoic placenta, PLP-F was restricted to the junctional zone and, based on the Rcho-1 trophoblast cell pattern of expression, trophoblast giant cells are likely contributors.

Cellular localization of PLF-RP and PLP-F expression in the developing placenta

Since nomenclature and descriptions of the morphology of the rodent placenta differ in the published literature, we provide an overview of the organization of the rodent chorioallantoic placenta.

Organization of the chorioallantoic placenta

The mature rodent chorioallantoic placenta (mouse or rat) arises from a population of trophoblast stem cells referred to as the ectoplacental cone (Rossant

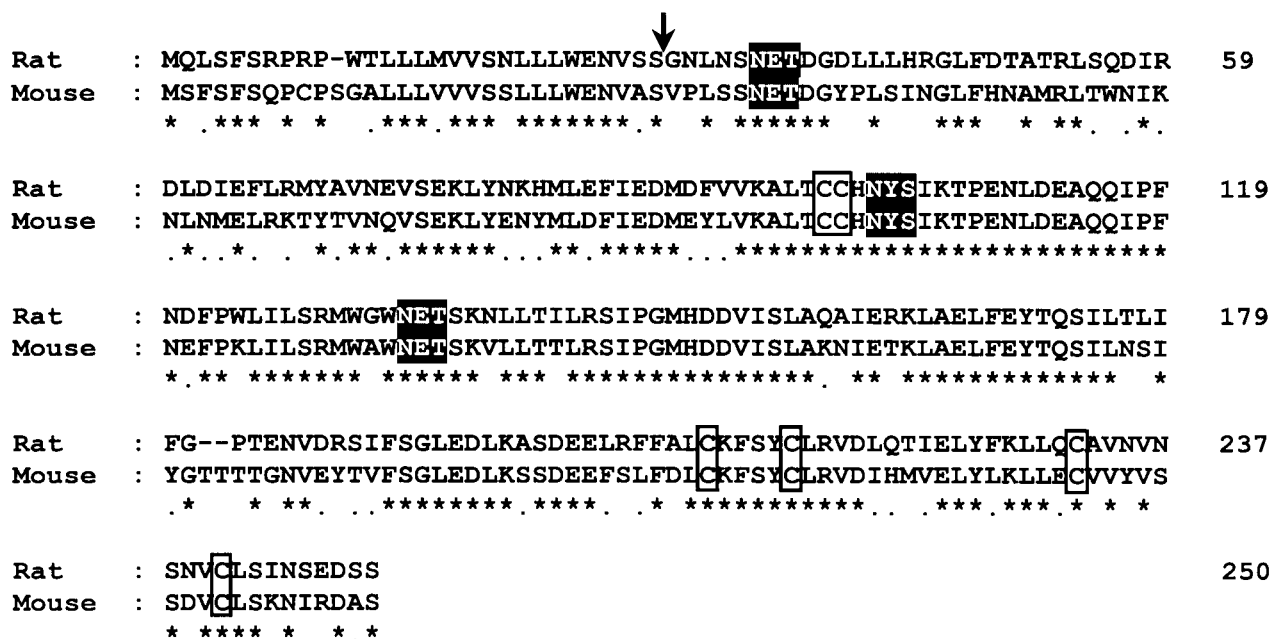


FIGURE 4. Amino acid sequence comparison of rat (present study) and mouse PLP-F (Lin *et al.* 1997a, Müller *et al.* 1998c). Please note that both sequences contain six cysteine residues and three putative N-linked glycosylation sites positioned in homologous locations. Asterisks below the sequences denote identity and dots below the sequences denote similarity.

1995, Soares *et al.* 1996). As a result of interactions with the maternal stromal compartment and fetal mesenchyme, the ectoplacental cone develops into two recognizable zones: the junctional zone and the labyrinth zone (Soares *et al.* 1996) (Fig. 7). The junctional zone is situated at the maternal interface and is comprised of three differentiated trophoblast lineages, trophoblast giant cells, spongiotrophoblast, and glycogen cells, whereas the labyrinth zone is located at the fetal interface and consists of trophoblast giant cells, syncytial trophoblast cells, and fetal mesenchyme and vasculature (Soares *et al.* 1996). Trophoblast giant cells and spongiotrophoblast cells are principally involved in the biosynthesis of members of the PRL family (Soares *et al.* 1998). Labyrinthine trophoblast cells contribute largely to the bidirectional transport of nutrient and wastes between maternal and fetal compartments (Knipp *et al.* 1999a).

In situ localization of PLF-RP and PLP-F The Northern blot analyses were not entirely consistent with previous reports on PLF-RP and PLP-F mRNA expression patterns in the mouse. In order to resolve the cellular sources of PLF-RP and PLP-F within the developing rat placenta we performed *in situ* hybridization. PLF-RP and PLP-F mRNA distributions were also examined

in mouse placental tissues, for comparison. Similar to previous reports, PLF-RP mRNA was localized to trophoblast giant cells and spongiotrophoblast cells of the junctional zone of the mouse placenta, and PLP-F was localized to the spongiotrophoblast layer (Colosi *et al.* 1988b, Carney *et al.* 1993, Lin *et al.* 1997a) (Fig. 8). Cellular expression patterns for PLF-RP and PLP-F in the rat placenta were compared with expression patterns of known markers for specific trophoblast cell populations (PLP-C, junctional zone trophoblast giant cells and spongiotrophoblast cells, Deb *et al.* (1991b); PL-II, junctional zone and labyrinthine trophoblast giant cells, Deb *et al.* (1991a); hFABP, syncytial trophoblast layer of the labyrinth zone, Watanabe *et al.* (1991), Knipp *et al.* (1999b)) (Fig. 9).

Rat PLF-RP PLF-RP was first detected on day 10 of gestation in the ectoplacental cone region of the rat chorioallantoic placental primordium (Fig. 9E and F). At later days of pregnancy, PLF-RP was predominantly expressed in the labyrinth zone of the rat chorioallantoic placenta (Fig. 9G). These results were consistent with the Northern analyses but contrasted with the cellular localization of mouse PLF-RP (Fig. 8C and D). The labyrinthine distribution of PLF-RP mRNA (Fig. 9G) in the

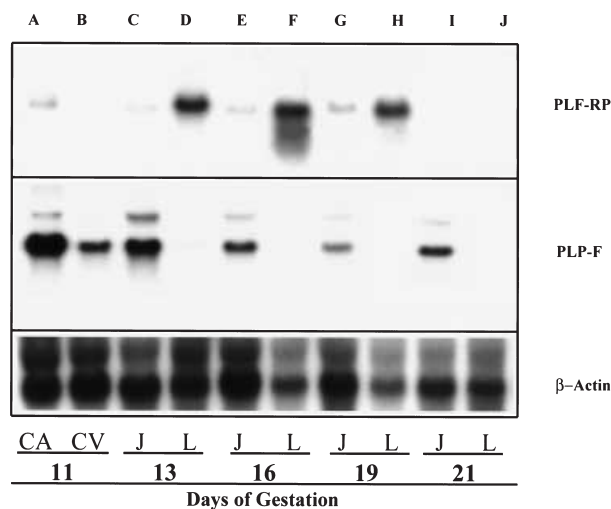


FIGURE 5. Expression of PLF-RP and PLP-F mRNA in the rat placenta during the second half of gestation: Northern blots showing the temporal and spatial patterns of expression of PLF-RP and PLP-F mRNAs in the placenta. Placental tissues were isolated, dissected into junctional and labyrinth zones, and extracted for total RNA. Total RNA (20 µg/lane) was fractionated on 1.4% agarose gels, transferred to nylon membranes, and probed with [³²P]dATP-labeled cDNAs. Hybridizations were carried out with ³²P-labeled PLF-RP, PLP-F, and β-actin. Please note the predominance of PLF-RP in the labyrinth zone and the predominance of PLP-F in the junctional zone of the chorioallantoic placenta. CA, chorioallantoic placenta; CV, choriovitelline placenta; J, junctional zone; L, labyrinth zone.

rat chorioallantoic placenta was similar to the labyrinthine expression pattern of PL-II, which is punctate and confined to trophoblast giant cells (Fig. 9D), but different from the hFABP expression pattern which forms columns and is confined to the syncytial trophoblast layers (Fig. 9C).

Rat PLP-F In the rat, PLP-F was primarily a product of the trophoblast giant cells and spongiotrophoblast cells (Fig. 9H–K). At day 10 of gestation, trophoblast giant cells (Fig. 9H and I) were the exclusive sites of PLP-F expression, whereas by day 16 of pregnancy spongiotrophoblast cells were also contributing to the biosynthesis of PLP-F (Fig. 9J). Spongiotrophoblast cell PLP-F expression showed an additional increase by day 19 of pregnancy (Fig. 9K). Again, these results in the rat contrast with the mouse, in which PLP-F was shown to be expressed exclusively in the spongiotrophoblast compartment (Lin *et al.* 1997a, present study).

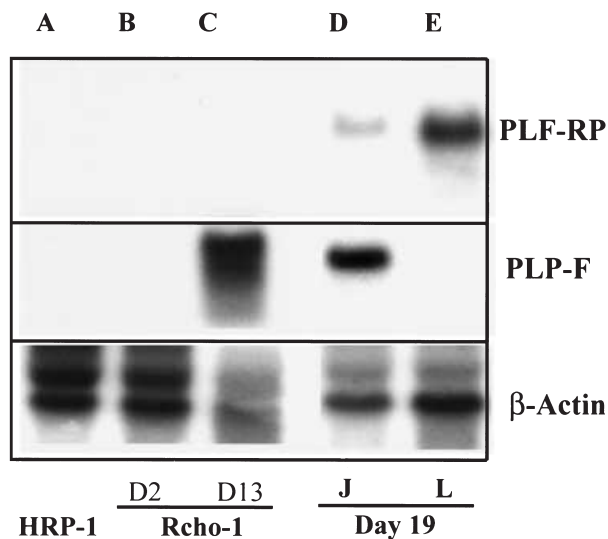


FIGURE 6. Expression of PLF-RP and PLP-F in rat trophoblast cells: Northern blots showing expression of PLF-RP, PLP-F, and β-actin in HRP-1 and Rcho-1 trophoblast cells. Cells were harvested and extracted for total RNA. Total RNAs (20 µg/lane) were fractionated on 1.4% agarose gels, transferred to nylon membranes, and probed with [³²P]dATP-labeled cDNAs. Hybridizations were carried out with ³²P-labeled PLF-RP, PLP-F, and β-actin cDNAs. Lane A, HRP-1 trophoblast cells; lane B, undifferentiated Rcho-1 trophoblast cells, day 2 (D2); lane C, differentiated Rcho-1 trophoblast cells, day 13 (D13); lane D, day 19 junctional zone (J); lane E, day 19 labyrinth zone (L). Please note that PLF-RP was not expressed in either trophoblast cell model, while PLP-F was prominently expressed in differentiated Rcho-1 trophoblast cells.

DISCUSSION

The PRL family of cytokines/hormones has expanded over the past few years. This most recent expansion is chiefly related to the establishment of EST databases derived from rat and mouse embryonic and extraembryonic cDNA libraries. Perusal of ESTs from the University of Iowa Rat Gene Discovery Program within the NCBI dbEST database led to the identification of two new members of the rat PRL family.

The two newly identified rat PRL family members exhibit marked structural similarities with mouse PLF-RP and PLP-F. These similarities include the conservation of six cysteine residues and two putative *N*-linked glycosylation sites in rat and mouse PLF-RP, and six cysteine residues and three putative *N*-linked glycosylation sites in rat and mouse PLP-F (Linzer & Nathans 1985, Lin *et al.*

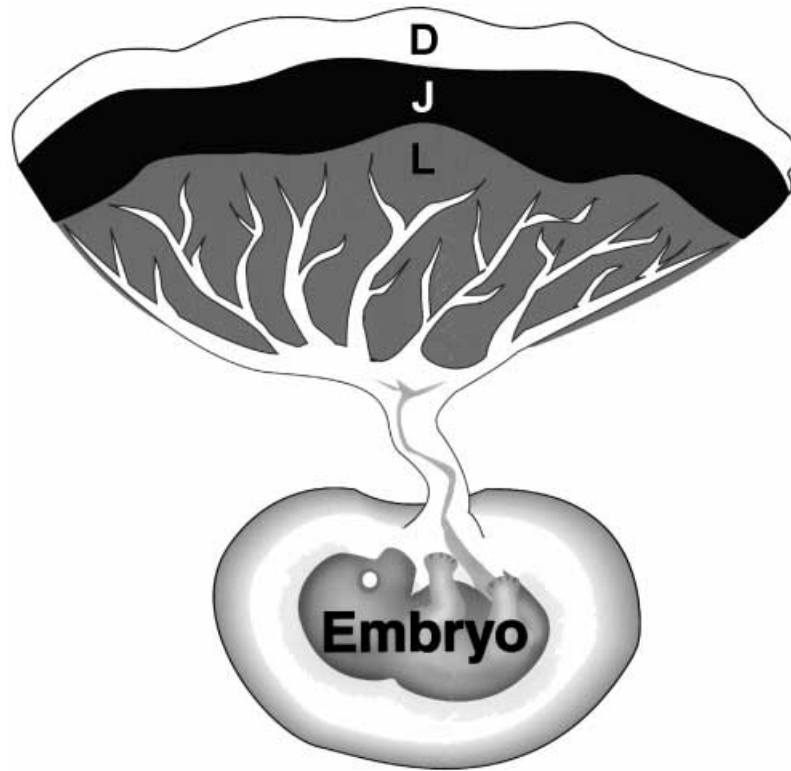


FIGURE 7. Schematic diagram of mature rodent chorioallantoic placenta. The placenta is divided into two major regions: junctional zone (J) and labyrinth zone (L). The junctional zone is comprised of three differentiated trophoblast cell lineages: trophoblast giant cells, spongiotrophoblast cells, and glycogen cells. The labyrinth zone contains two differentiated trophoblast cell lineages: trophoblast giant cells and syncytial trophoblast cells, together with associated fetal mesenchyme and vasculature. D, decidua.

1997a, Müller *et al.* 1998c, present study). The degree of similarity between the rat and mouse cDNAs is comparable to that observed for other PRL family members possessing rat and mouse homologs (PRL, Maurer *et al.* (1981), Cooke & Baxter (1982), Linzer & Talamantes (1985); PL-I, Colosi *et al.* (1987), Robertson *et al.* (1990), Hirose *et al.* (1994), Dai *et al.* (1996a); PL-II, Duckworth *et al.* (1986a), Jackson *et al.* (1986), Dai *et al.* (1996a); PLP-A, Duckworth *et al.* (1986b), Lin *et al.* (1997b), Müller *et al.* (1998b); PLP-B, Duckworth *et al.* (1988), Lin *et al.* (1997b), Müller *et al.* (1988b); decidual prolactin-related protein, Roby *et al.* (1993), Lin *et al.* (1997b), Orwig *et al.* (1997b)). Based on the significant structural homologies, the two newly identified cDNAs were classified as rat homologs of PLF-RP and PLP-F. Despite the notable similarities in structure,

differences in the cellular sites of PLF-RP and PLP-F expression in the rat and mouse are apparent.

PLF-RP

In the mouse, PLF-RP is expressed initially in the giant cell layer of the chorioallantoic and choriovitelline placental primordia and subsequently in the giant and spongiotrophoblast cell layers of the mature chorioallantoic placental junctional zone (Colosi *et al.* 1988b, Carney *et al.* 1993, present study). In contrast, PLF-RP expression begins in the chorioallantoic placental primordium (ectoplacental cone region) and continues predominantly in labyrinthine trophoblast of the chorioallantoic placenta (present study). The spatial pattern of

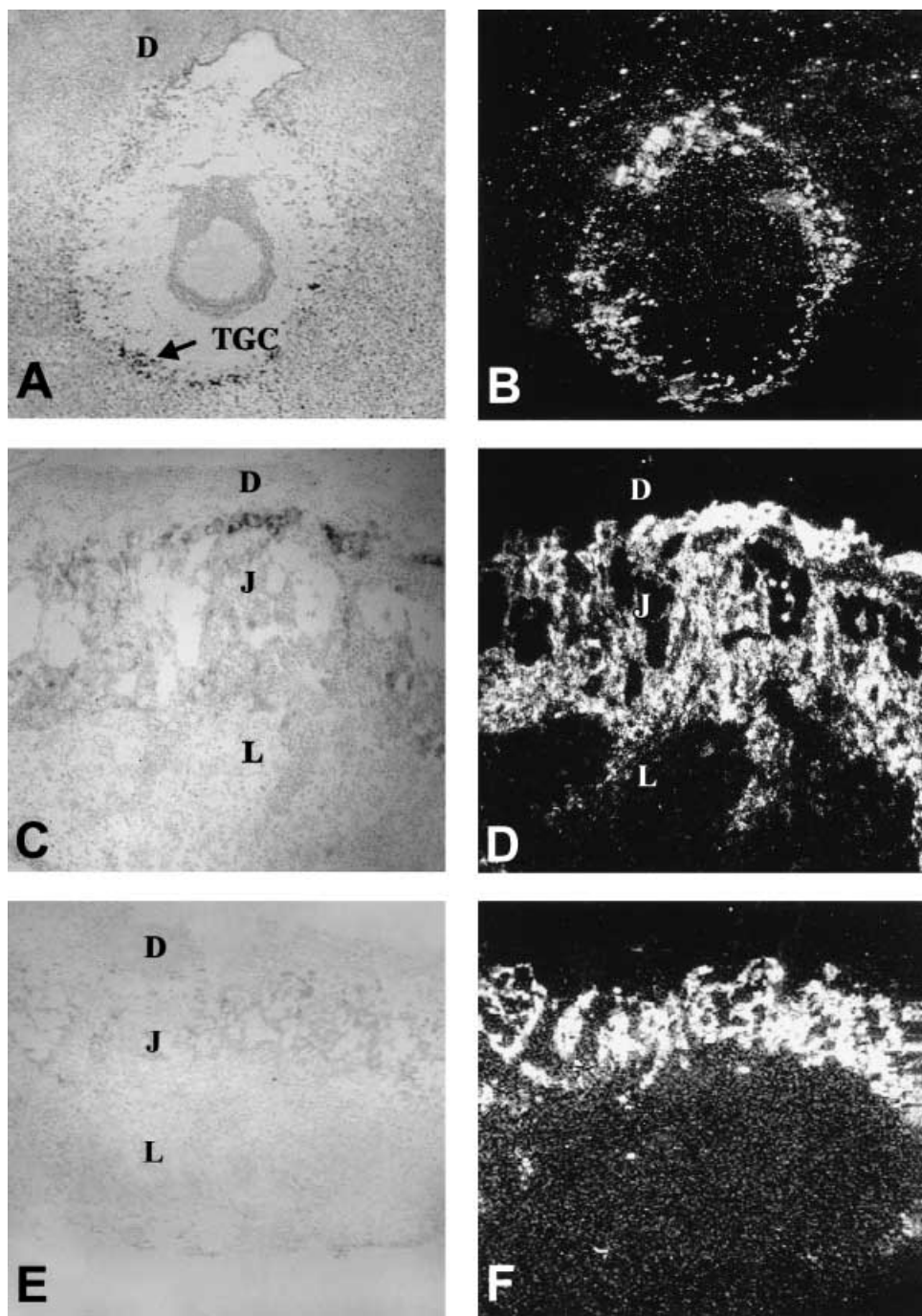


FIGURE 8. Cell and tissue specific localization of PLF-RP and PLP-F in mouse conceptus and placental tissues. The *in situ* detection of mRNA expression was performed on frozen tissue sections. Full-length rat PLF-RP and PLP-F cDNAs were used as templates for the synthesis of ^{35}S -labeled sense and antisense RNA probes. (A) Bright field representation using a PLF-RP antisense probe on a day 8 conceptus tissue section. (B) Dark field representation using a PLF-RP antisense probe on a day 8 conceptus tissue section. (C) Bright field representation using a PLF-RP antisense probe on a day 16 placental tissue section. (D) Dark field representation using a PLF-RP antisense probe on a day 16 placental tissue section. (E) Bright field representation using a PLP-F antisense probe on a day 16 placental tissue section. (F) Dark field representation using a PLP-F antisense probe on a day 16 placental tissue section. Original magnifications, $\times 40$. D, decidua; J, junctional zone; L, labyrinth zone; TGC, trophoblast giant cell.

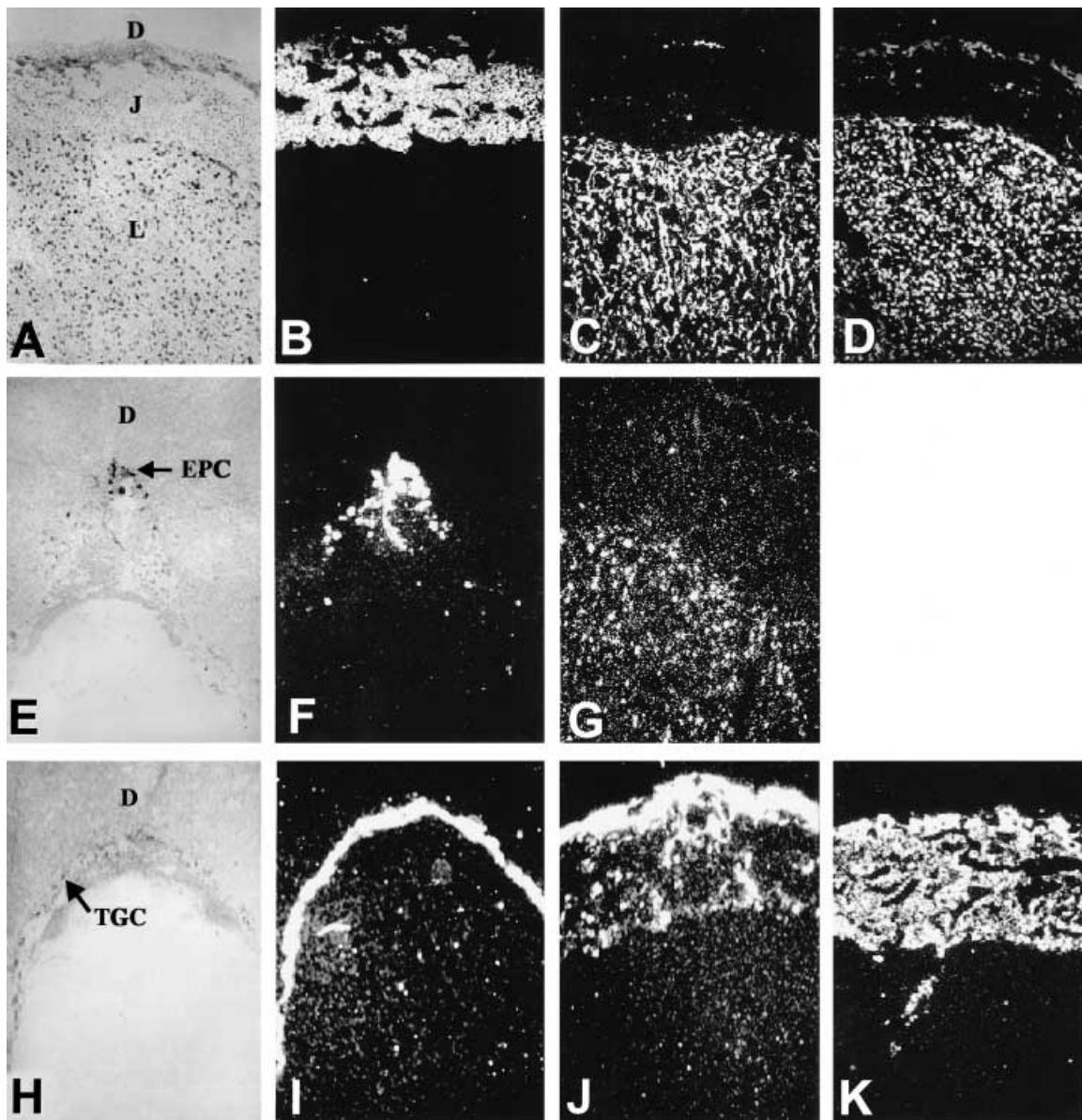


FIGURE 9. Cell and tissue specific localization of PLF-RP and PLP-F in rat conceptus and placental tissues. The *in situ* detection of mRNA expression was performed on frozen tissue sections. Full-length rat PLF-RP and PLP-F cDNAs were used as templates for the synthesis of ^{35}S -labeled sense and antisense RNA probes. Hybridizations with sense and antisense RNA probes for PL-II (Dai *et al.* 1996a), hFABP (Heuckeroth *et al.* 1987), and PLP-C (Deb *et al.* 1991b, Dai *et al.* 1996b) were used as additional controls for identifying placental cell subpopulations. (A) Bright field representation using a PL-II antisense probe on a day 19 placental tissue section. (B) Dark field representation using a PLP-C antisense probe on a day 19 placental tissue section. (C) Dark field representation using a hFABP antisense probe on a day 19 placental tissue section. (D) Dark field representation using a PL-II antisense probe on a day 19 placental tissue section. (E) Bright field representation using a PLF-RP antisense probe on a day 10 conceptus tissue section. (F) Dark field representation using a PLF-RP antisense probe on a day 10 conceptus tissue section. (G) Dark field representation using a PLF-RP antisense probe on a day 16 placental tissue section. (H) Bright field representation using a PLP-F antisense probe on a day 10 conceptus tissue section. (I) Dark field representation using a PLP-F antisense probe on a day 10 conceptus tissue section. (J) Dark field representation using a PLP-F antisense probe on a day 16 placental tissue section. (K) Dark field representation using a PLP-F antisense probe on a day 19 placental tissue section. Original magnifications, $\times 40$. D, decidua; J, junctional zone; L, labyrinth zone; EPC, ectoplacental cone; TGC, trophoblast giant cell.

PLF-RP expression in the rat labyrinth zone was punctate and most closely resembled the cellular distribution of PL-II, which is restricted to labyrinthine trophoblast giant cells (Campbell *et al.* 1989, Deb *et al.* 1991a, present study). However, temporally, PL-II and PLF-RP expression patterns in the labyrinth zone were not entirely coincident (Campbell *et al.* 1989, present study). PL-II expression continues to increase as gestation progresses, whereas PLF-RP expression decreases prior to the end of pregnancy. Further insight into the cellular source of PLF-RP was revealed by examination of its expression patterns in two rat trophoblast cell lines. Rcho-1 trophoblast cells are a stem cell population restricted to differentiation along the junctional zone trophoblast giant cell lineage (Faria & Soares 1991, Hamlin *et al.* 1994, Peters *et al.* 1999), while HRP-1 trophoblast stem cells are proposed to be progenitors for the labyrinthine syncytial cell lineage (Soares *et al.* 1987, Shi *et al.* 1997). PLF-RP mRNA was not detectable in either trophoblast cell line. These observations further reinforce the apparent phenotypic differences between trophoblast giant cells located in the junctional zone and those in the labyrinth zone. Junctional zone trophoblast giant cells express most members of the PRL family, while labyrinthine trophoblast giant cells exhibit a more restricted expression profile, limited to PL-II and, possibly, PLF-RP.

The significance of this apparent species difference in PLF-RP expression is difficult to resolve fully. Based on structural similarities, we would predict that the biological actions of mouse and rat PLF-RP are similar. Since mouse PLF-RP is a potent anti-angiogenic factor (Jackson *et al.* 1994), it would seem likely that rat PLF-RP would share a similar biological activity. The different locations of PLF-RP biosynthesis in the rat and the mouse placentas may influence access to their potential target vasculature. In the mouse, PLF-RP has been proposed to act primarily on maternal blood vessels (Jackson *et al.* 1994, Linzer 1995), while in the rat, PLF-RP may have a more significant role in modulating the invasion of fetal blood vessels during the formation of the chorioallantoic placenta, thus demarcating the boundaries for genesis of the junctional and labyrinth zones. A site of production in the labyrinth zone may also provide PLF-RP with greater access to the fetal compartment, in a manner similar to that reported for PL-II (Ogren & Talamantes 1988). The presence of rat PLF-RP in the fetal circulation and the nature of its physiological actions remain to be determined.

PLP-F

In the mouse, PLP-F is an exclusive product of the spongiotrophoblast layer, whereas in the rat, trophoblast giant cells were found to be the major source of PLP-F, with a lesser contribution from spongiotrophoblast cells late in gestation (Lin *et al.* 1997a, present study). The trophoblast giant cell source of PLP-F in the rat was further verified by the demonstration of PLP-F expression in differentiated Rcho-1 trophoblast cells. Trophoblast giant cells and spongiotrophoblast cells arise through the activation of distinct regulatory programs at temporally disparate developmental phases (Guillemot *et al.* 1994, Cross *et al.* 1995, Rossant *et al.* 1998). Trophoblast giant cells represent the first differentiated trophoblast lineage originating shortly after implantation, and are situated at the maternal-placental interface (Rossant 1995, Soares *et al.* 1996). Spongiotrophoblast cells develop concurrently with the establishment of the chorioallantoic placenta at midgestation and are located beneath the trophoblast giant cell layer (Rossant 1995, Soares *et al.* 1996). As a result, it is apparent that PLP-F has access to potentially different physiological compartments, depending on its sites of synthesis in the rat and in the mouse. The significance of these observations awaits insight into the biology of PLP-F. Although the biological actions of PLP-F have yet to be determined, its expression pattern at the maternal interface from early to late gestation indicates functions associated with maternal adaptations to pregnancy.

Overview

The identification of PLF-RP and PLP-F in the rat is significant. Their presence in the rat indicates that they are not a novelty unique to a single species. Cross-species conservation suggests a greater overall importance of these two cytokines/hormones. A more extensive survey of PLF-RP and PLP-F homologs would provide additional insight. The existence of PLF-RP and PLP-F in the rat also expands our experimental repertoire for studying their function. The key to furthering our understanding of the biology of PLF-RP and PLP-F will be through the implementation of strategies to identify targets of their actions. An approach utilizing alkaline phosphatase-cytokine fusion proteins has proved very useful for identifying targets for both classical and nonclassical members of the PRL family (Müller *et al.* 1998a, 1999). The combination of the identification of cellular targets via the alkaline

phosphatase–ligand fusion protein strategy with the generation of null mutant mice should significantly improve our appreciation of the biology of PLF-RP and PLP-F.

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