A New Member of the Mouse Prolactin (PRL)-Like Protein-C Subfamily, PRL-Like Protein-Cα: Structure and Expression*

GUOLI DAI, BELINDA M. CHAPMAN, BING LIU, KYLE E. ORWIG‡, DANHUA WANG‡, ROBERT A. WHITE, BARRY PREUETT, AND MICHAEL J. SOARES

Department of Molecular & Integrative Physiology (G.D., B.M.C., B.L., K.E.O., D.W., M.J.S.), University of Kansas Medical Center, Kansas City, Kansas 66160; Section of Medical Genetics and Molecular Medicine (R.A.W., B.P.), Children’s Mercy Hospital, Kansas City, Missouri 64108

ABSTRACT

In this study, we establish the presence of a unique member of the PRL-like protein-C (PLP-C) subfamily in the mouse, PLP-Cα, characterize its complementary DNA and gene, and map its chromosomal location and pattern of expression during pregnancy. Mouse PLP-Cα encodes for a 239 amino acid protein and possesses from 69–71% identity with rat PLP-C, PLP-Cv, PLP-D, and PLP-H. Another feature characteristic of PLP-C subfamily members that is also present in mouse PLP-Cα is a 6-exon/5-intron gene structure including an aromatic domain encoded by exon 3. Southern analysis with mouse and rat PLP-C subfamily probes suggested the existence of a single mouse

PLP-Cα gene. Mouse PLP-Cα maps to chromosome 13 along with other members of the mouse PRL family. Expression of mouse PLP-Cα increases dramatically as gestation advances and is restricted to spongiotrophoblast and trophoblast giant cells of the junc-

tional zone. In summary, we have established the presence of a new PLP-C subfamily member in the mouse and demonstrated its simil-

arity in structure and expression to rat PLP-C subfamily members. This level of conservation between species expands the biological significance of the PLP-C subfamily and provides additional oppor-

unities for genetically evaluating its function. (Endocrinology 139: 5157–5163, 1998)

THE RODENT PRL gene family presently consists of at

least 15 different members that are expressed in the pituitary, uterus, and/or placenta (1). A subfamily has been identified that consists of members possessing a 6 exon/5 intron gene structure and is referred to as the PRL-like protein-C (PLP-C) subfamily. In the rat, five members of the PLP-C subfamily have been identified (four close relatives: PLP-C, PLP-Cv, PLP-D, and PLP-H; one distant relative: decidual/trophoblast PRL-related protein, d/1PRP), whereas in the mouse only d/1PRP has been isolated (2–9). These orphan ligands represent major secretory products of the rat placenta during the second half of gestation (10).

Members of the PRL family can be divided into classical and nonclassical categories based on their biological activity (1). Classical PRL family members, including PRL, placental lactogen-I (PL-I), PL-I variant (PL-Iv), and PL-II, bind the PRL receptor and stimulate PRL-like bioactivities. The PLP-C subfamily have been identified (four close relatives: PLP-C, PLP-Cv, PLP-D, and PLP-H). Another feature characteristic of PLP-C subfamily members that is also present in mouse PLP-Cα is a 6-exon/5-intron gene structure including an aromatic domain encoded by exon 3. Southern analysis with mouse and rat PLP-C subfamily probes suggested the existence of a single mouse

members do not use the PRL receptor signaling pathway

(11–13). Only limited information is available on possible cellular targets for some members of the rat PLP-C subfamily

(13, 14).

Understanding the physiology of the PLP-C subfamily and other nonclassical PRL family members would be signifi-

antly advanced by the availability of a mouse model and the application of gene manipulation strategies. In this study, we establish the presence of a new member of the PLP-C family in the mouse, PLP-Cα, characterize its complementary DNA (cDNA) and gene, and map its chromosomal location and pattern of expression during pregnancy.

Materials and Methods

Reagents

All restriction enzymes, polymerases, and DNA ligase were pur-

chased from New England Biolabs, Inc. (Beverly, MA). DNA extraction kits were purchased from Quiagen (Chatsworth, CA). Nitrocellulose and nylon membranes were obtained from Schleicher & Schuell, Inc. (Keene, NH). Radiolabeled nucleotides were purchased from DuPont NEN (Boston, MA). Prime-it random primer labeling kits and Pfu polymerase were obtained from Stratagene (La Jolla, CA). TR7671 Reagent for RNA extraction, oligonucleotide primers, and SuperScript cDNA syn-

thesis kits were obtained from Life Technologies (Gaithersburg, MD). TOPO TA Cloning kits were purchased from Invitrogen (San Diego, CA). Unless otherwise noted, all chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals and tissue preparation

CD-1 mice were obtained from Charles River Laboratories, Inc. (Wilm-

ington, MA). The animals were housed in an environmentally con-

trolled facility, with lights on from 0600–2000 h and allowed free access

Received July 8, 1998.

Address all correspondence and requests for reprints to: Michael J. Soares, Ph.D., Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas 66160-7401. E-mail: msoares@kumc.edu.

* This work was supported by grants from the National Institute of Child Health and Human Development (HD-20676, HD-29797, HD-33994; to M.J.S.) and the Paul Patton Memorial Trust (to R.A.W.).

‡ Supported in part by fellowships from the Kansas Health Foundation and the Lalor Foundation. Present address: Laboratory of Reproductive Physiology, School of Veterinary Medicine, University of Penn-

sylvania, Philadelphia 19104-6009.

§ Supported in part by fellowships from the Kansas Health Foundation.
to food and water. Timed pregnancies were generated and tissue dis-
sections were performed as previously described (15). The presence of
a copulatory plug was designated day 1 of pregnancy. Protocols for the
care and use of animals were approved by the University of Kansas
Animal Care and Use Committee.

Cloning and characterization of the PLP-C

Total RNA was extracted with TRIzol reagent from mouse placental
tissues isolated from day 19 of pregnancy. Five micrograms of total RNA
and 0.5 μg of oligo (deoxythymidine) were used for the RT reaction. PCR
reactions were performed using Pfu polymerase with a set of primers
based on the rat PLP-Cv cDNA sequence (5'9 primer: 5'9 AGAACTCATC-
CTGCATTGG 3'9; 3'9 primer: 5'9 GCATAGCCCAAGCAGACATAA 3'9) for 30 cycles (denature, 94 C for 1 min; anneal, 60 C for 2 min;
extension, 72 C for 2 min). The amplified product was subcloned into
pCR2.1-TOPO vector flanked by M13 reverse primer and T7 promoter
with the TOPO TA Cloning kit. DNA sequencing was performed using
an PE Applied Biosystems Model 310 sequencer and PE Applied Bio-
systems Dye Terminator Cycle Sequencing kits (Foster City, CA). Both
strands of the cDNAs were completely sequenced. Comparisons of
PLP-Ca with other members of the PRL family were performed with
CLUSTAL W (version 1.6, European Molecular Biology Laboratory,
Heidelberg, Germany, 16).

Isolation and characterization of the PLP-Ca gene

A genomic DNA library generated from a 129/SvEv mouse strain
liver and packaged in the Lambda FIX II vector was a generous gift of
Lexicon Genetics, Inc. (Houston, TX). Approximately, 1×10⁶ pfu were
screened with a mouse PLP-Ca cDNA as previously described (5). Three
positive clones obtained were amplified. Integrity of the clones was
verified by PCR with primers originally used to clone the PLP-Ca cDNA.
One of the two full length clones was used to inoculate LE392
Escherichia coli. A series of forward and reverse oligonucleotide primer
sets based on the mouse PLP-Ca cDNA were designed and used to sequence exons
and exon-intron boundaries. These primers were also used to estimate
5'9 and 3'9 flanking DNA and intron sizes by PCR analysis and agarose
electrophoresis. DNA sequencing was performed using an PE Applied
Biosystems Model 310 sequencer and PE Applied Biosystems Dye Ter-
minal Cycle Sequencing kits (Foster City, CA).

Southern blot analysis

Genomic DNA was isolated from mouse liver and individually di-
gested with BamHI, EcoRI, or HindIII. Digested samples were electro-
phoretically separated in 0.8% agarose gels, transferred to nylon mem-
branes, and probed with [32P]-labeled mouse PLP-Ca, rat PLP-C, or rat
PLP-Cv cDNAs. Hybridizations were performed in hybridization buffer
(50% formamide, 6×SSPE, 5×Denhardt's reagent, 1% SDS, and 100
μg/ml salmon sperm DNA) at 42 C overnight. Membranes were washed
under low stringency conditions consisting of two 15 min washes in 7×
SSPE with 0.1% SDS at room temperature followed by two 15-min
washes in 1×SSPE with 0.5% SDS solution at 37 C.

Chromosomal assignment of the mouse PLP-Ca gene

Chromosomal mapping of the mouse PLP-Ca gene was determined using The Jackson Laboratory Interspecific Backcross Panel (17).
Genomic DNAs from C57BL/6, Mus spretus and a (M. spretus × C57BL/
6)F1 × M. spretus (BSS type) backcross were analyzed by Southern blotting as previously described (18). Approximately 5 μg of genomic DNAs from the C57BL6/J and M. spretus progenitors were digested with 28 different restriction enzymes to find a suitable restriction fragment length variation (RFLV) for mapping. Southern blots were probed with the mouse PLP-Cα cDNA. Approximately 2 μg of DNA from the BSS type backcross panel were digested for each sample with BclI overnight.

Segregation of alleles was compared with other loci from a database at The Jackson Laboratory Backcross DNA map Panel Service (17).

**Analysis of PLP-Cα expression**

**Northern blot analysis.** Northern blots were performed as previously described in our laboratory (19, 20). RNA was extracted from tissues essentially as described by Chomczynski and Sacchi (21), using TRIzol. Total RNA (15 μg) was separated on a 1% agarose gel and transferred to a nylon membrane. Blots were probed with [32P]-labeled mouse PLP-Cα cDNA. The ribosomal protein L7 (rpL7) control probe was generated by PCR (22). Specific oligonucleotide primers amplified a 246 bp rpL7 fragment that was random primer labeled using Klenow and [32P]-dATP.

**In situ hybridization**

PLP-Cα messenger RNA (mRNA) was detected in frozen tissue sections as previously described (19, 23). The full-length mouse PLP-Cα cDNA was subcloned into pGEM-T vector flanked by T7 and SP6 promoters, linearized, and used as a template for the synthesis of [35S]-labeled sense and antisense RNA probes.

**Results**

**PLP-Cα cDNA and gene characterization**

In our attempt to identify members of the PLP-C subfamily in the mouse, we used two approaches: 1) survey of the National Center for Biotechnology Information dbEST database and 2) a PCR strategy using primers from putative rat homologs. Other than mouse d/tPRP, which has been previously characterized (8, 9), we were unable to find any mouse homologs of the rat PLP-C subfamily in the dbEST database. However, our efforts with the PCR strategy yielded a cDNA that was amplified from day 19 placental RNA using primers based on the nucleotide sequence of rat PLP-Cv. The cDNA was subcloned, sequenced, and shown to encode for a 239 amino acid protein that possesses from 69–71% identity with rat PLP-C, PLP-Cv, PLP-D, and PLP-H (Figs. 1 and 2; Table 1; GenBank Accession number, AF090140). We named this mouse PLP-C subfamily member, PLP-Cα, because of its significant homology with all members of the PLP-C subfamily but lack of additional overall homology with any one member. Based on homology with the rat PLP-C protein, it was determined that the mouse PLP-Cα contains a 29 amino acid signal peptide (2). The mature mouse PLP-Cα encodes for a protein with a number of features similar to other...
members of the PLP-C subfamily including a homologously positioned putative N-linked glycosylation site and six homologously situated cysteine residues, and the characteristic 14 amino acid aromatic domain (Fig. 2).

The mouse PLP-Cα cDNA was then used to screen a mouse genomic library resulting in the identification of three positive phage clones. One of the two full length clones was further characterized by restriction enzyme mapping, PCR, and sequencing. The PLP-Cα gene has a 6-exon/5-intron gene organization (Figs. 2 and 3) similar to other members of the PLP-C subfamily (5, 20). Southern analysis with a mouse PLP-Cα (Fig. 4) and rat PLP-C subfamily probes (data not shown) suggested the existence of a single mouse PLP-Cα gene.

Chromosomal mapping

The mouse PLP-Cα cDNA was used to determine the chromosomal location of the gene encoding PLP-Cα by analyzing the segregation of a BclI RFLV in genomic DNAs derived from the offspring of a (M. spretus × C57BL/6J)F1 × M. spretus backcross. The gene symbol, Prlpc, has been assigned to the mouse PLP-Cα locus and has been approved by the International Mouse Nomenclature Committee. A BclI RFLV for Prlpc was identified by the presence of a 2.6 kb genomic DNA fragment in C57BL/6J or the presence of a 3.3 kb fragment in M. spretus (Fig. 5A). Mapping data have been

TABLE 1. Sequence comparison between mouse PLP-Cα and other members of the rodent PRL familya

<table>
<thead>
<tr>
<th>PRL family memberb</th>
<th>Percent identity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleotide</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>d/tPRP</td>
<td>68</td>
</tr>
<tr>
<td>PLF-RP</td>
<td>47</td>
</tr>
<tr>
<td>PRL</td>
<td>13</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
</tr>
<tr>
<td>PLP-C</td>
<td>77</td>
</tr>
<tr>
<td>PLP-Cv</td>
<td>81</td>
</tr>
<tr>
<td>PLP-D</td>
<td>81</td>
</tr>
<tr>
<td>PLP-H</td>
<td>81</td>
</tr>
<tr>
<td>d/tPRP</td>
<td>68</td>
</tr>
<tr>
<td>PRL</td>
<td>10</td>
</tr>
</tbody>
</table>

a See references (3–9, 24, 27, 31) for sequences.
b PLP-Cα, PRL-like protein-Cα; d/tPRP, decidual/trophoblast prolactin-related protein; PLF-RP, proliferin-related protein; PLP-Cv, PLP-C variant.

Fig. 4. Southern blot analysis of mouse PLP-Cα from mouse genomic DNA. Genomic DNA was isolated from mouse liver and individually digested with BamHI (B), EcoRI (E), or HindIII (H). Digested samples were then electrophoretically separated in 0.8% agarose gels, transferred to nylon membranes, and probed with [32P]-labeled mouse PLP-Cα cDNA. Please note the relatively simple hybridization patterns observed with the three restriction enzymes.

mPLP-Cα Genomic Clone (12.9 Kb)

Fig. 3. Schematic representation of the 12.9 kb mouse PLP-Cα genomic clone. Sequence analysis of exon (E)/intron (I) boundaries revealed that the mouse PLP-Cα gene is comprised of six exons and five introns. The beginning of exon 1 is defined as the putative translation start site (ATG), and the end of exon 6 is defined as the polyadenylation site (AATAAA). Shaded boxes correspond with actual exon sizes. The sizes of the 5′ and 3′ flanking regions were determined by PCR and agarose gel electrophoresis.
deposited with the Mouse Genome Database under Accession Number J:46755. Haplotype analysis of these mapping data (Fig. 5B) indicated that the Prlpc locus is closely linked to Dtprp (mouse decidual/trophoblast PRL-related protein gene) and Pl1 (mouse placental lactogen-I gene) on chromosome 13 in the mouse. Allelic segregation patterns for Prlpc, Dtprp, and Pl1 are identical, indicating a distance of less than 1 centimorgan among these three genes. The calculated map distances between Prlpc and two adjacent loci, Gpld1 (glycosylphophatidylinositol-specific phospholipase D) and D13Bwg0938e (DNA segment, Chr 13, Brigham and Women’s Genetics 0938 expressed), including 95% confidence limits were determined:

\[\text{Gpld1-1.1 } \pm 1.1 \text{ cM-Prlpc-2.1 } \pm 1.5 \text{ cM-D13Bwg0938e}\]

**PLP-Cα expression patterns**

The tissue distribution of mouse PLP-Cα mRNA was determined by Northern blot analysis (Fig. 6). PLP-Cα expression was detected in chorioallantoic placental tissues from days 13, 16, and 19 of pregnancy. Expression of PLP-Cα increased progressively from midgestation to the end of pregnancy. PLP-Cα transcripts were not detected in mouse deciduoma, brain, thymus, heart, lung, diaphragm, liver, spleen, kidney, or ovary. This pattern of expression is identical to the expression patterns of rat PLP-C and PLP-Cv (5). Integrity of the RNA was verified by hybridization with the rpL7 probe.

*In situ* hybridization was used to determine the cellular localization of PLP-Cα in mouse uteroplacental tissues from day 8 to day 19 of gestation. PLP-Cα mRNA was localized to spongiotrophoblast and trophoblast giant cells from the junctional zone of the chorioallantoic placenta (Fig. 7, day 19 placental tissues). Antisense PLP-Cα hybridization signals increased in intensity as gestation progressed (data not shown). Sense probes did not exhibit significant hybridization with the placental tissues (Fig. 7). The cellular localization of PLP-Cα mRNA is identical to the intraplacental site of expression for rat PLP-C subfamily members: PLP-C, PLP-D, and PLP-H (2, 3, 6, 7).

**Discussion**

In this report, we have identified and characterized a new member of the mouse PRL gene family. This new member is part of the PLP-C subfamily. We have given this new ligand the name PLP-Cα because of its significant homology with all members of the rat PLP-C subfamily but lack of additional overall homology with any one member. Mouse PLP-Cα is similar to its rat counterparts in sequence, gene structure, tissue distribution, and temporal expression patterns (2, 3, 5–7).

Structural features of the mouse PLP-Cα gene are consistent with its inclusion in the PLP-C subdivision of the PRL gene family. Mouse PLP-Cα possesses a 6-exon/5-intron organization that is typical of other PLP-C subfamily members (5, 20) but differs from the 5-exon/4-intron pattern shared by

---

**Fig. 6. Placental-specific expression of mouse PLP-Cα.** Total RNA was collected from placental tissues (day 13, day 16, and day 19), day 8 deciduoma tissue, brain, thymus, heart, lung, diaphragm, liver, spleen, kidney, and ovary. The distribution of PLP-Cα mRNA in mouse tissues was determined by Northern blot analysis. PLP-Cα expression was restricted to placental tissues and increased as gestation progressed. The control probe for rpL7 was used to demonstrate loading accuracy and the integrity of the RNA.
other PRL family members (PRL, PL-II, and proliferin genes; 24–30). The additional exon is situated between exons 2 and 3 of the prototypical PRL gene structure and codes for a well-conserved aromatic domain that is a feature of all PLP-C subfamily members (2, 3, 5–7) and the more distantly related proliferin-related protein (31). The aromatic domain and other commonalities among PLP-C subfamily members are apparent; however, a true appreciation of their structural and functional relevance awaits discovery of the biological actions of these orphan ligands.

The mouse PLP-\(C_\alpha\) gene, Prlpc, maps to mouse chromosome 13 along with other members of the mouse PRL family (8, 9, 32, 33). The accumulation of data regarding the clustering of PRL family members on the same chromosome supports the hypothesis that individual members arose from duplication and divergent evolution from a common ancestral gene (34–36). The mouse Prlpc locus is closely linked to the \(Prl\) locus which is within a conserved region of homology with rat chromosome 17 and human chromosome 6. PLP-C subfamily members have been localized to rat chromosome 17 (3–5). A human homolog for the PLP-C subfamily has not been reported. The specific alignments of PRL family members on chromosome 13 have not been presented; however, such information may provide some insights regarding the coordinated patterns of expression for each of the family members.

The tissue-specific and temporal expression pattern of PLP-\(C_\alpha\) in the mouse closely parallels expression patterns for its counterparts in the rat. In both species, spongiotrophoblast cells and trophoblast giant cells are responsible for the expression of this cohort of ligands (2, 3, 6, 7; present study). Placental expression is initiated at midgestation and continues until parturition (2, 3, 5–7; present study). The high levels of expression and conservation across species are consistent with the potential physiological importance of PLP-\(C_\alpha\) during pregnancy.

Relationships between rat and mouse PLP-C subfamilies imply the existence of differing selective pressures on their origins. The rat contains four closely related PLP-C subfamily members (PLP-C, PLP-Cv, PLP-D, and PLP-H), whereas the mouse, at least for now, contains only a single PLP-C subfamily member (PLP-\(C_\alpha\)) bearing similarity to these rat members. As noted above, this mouse PLP-C subfamily member has approximately similar extents of homology with each of the four rat PLP-C subfamily members. Furthermore, we have not been able to isolate specific mouse homologs for rat PLP-C subfamily members other than for d/tPRP. These observations are somewhat surprising but may suggest different patterns of evolution for the PLP-C subfamily in the mouse vs. the rat. It will be of considerable interest to determine the spectrum of biological roles for each of the PLP-C subfamily members and to evaluate whether mouse PLP-\(C_\alpha\) possesses a broader range of actions that includes the range of actions represented by the closely related rat PLP-C subfamily (PLP-C, PLP-Cv, PLP-D, and PLP-H).

In summary, we have identified a new member of the PLP-C family in the mouse, PLP-\(C_\alpha\), characterized its cDNA and gene, and determined its chromosomal position and pattern of expression during gestation. Collectively, these findings are significant for two important reasons: 1) the high degree of PLP-\(C_\alpha\) conservation with members of the rat PLP-C subfamily implies physiological relevance during pregnancy; and 2) the availability of a mouse model creates new genetic-based opportunities for studying the physiology of the PLP-C subfamily.
Acknowledgments

The authors wish to thank Michael Rusnak of Lexicon Genetics Inc. for the gift of the mouse genomic library and Lucy Rowe and Mary Barter of The Jackson Laboratory for performing the linkage analyses.

References

8. Lin J, Poole J, Linzer DIH 1997 Three new members of the mouse PRL/growth hormone family are homologous to proteins expressed in the rat. Endocrinology 138:5541–5549
33. Lin J, Poole J, Linzer DIH 1997 Two novel members of the PRL/growth hormone family are expressed in the mouse placenta. Endocrinology 138:5535–5540