

# Rcho-1 Trophoblast Cell Placental Lactogens: Complementary Deoxyribonucleic Acids, Heterologous Expression, and Biological Activities\*

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## ABSTRACT

In this report, we have investigated placental lactogens (placental lactogen-I, PL-I; PL-I variant, PL-Iv; PL-II) expressed by differentiated Rcho-1 trophoblast cells. A complementary DNA (cDNA) library to differentiated Rcho-1 trophoblast cells was constructed and screened with probes to detect PL-I and PL-II. Sequence analysis of three independent Rcho-1 PL-I cDNAs indicated that they significantly differed from the previously reported PL-I sequence but more closely resembled a related cDNA referred to as PL-I mosaic (PL-Im). Upon further analysis, Rcho-1 PL-I/PL-Im transcripts could be detected in Rcho-1 trophoblast cells and normal developing placental tissue; however, the previously reported PL-I transcript could not be identified from the same sources. Given these results, we reexamined the original PL-I cDNA by PCR and nucleotide sequence analyses. The sequence differed from the original report and was found to be identical to the Rcho-1 PL-I and PL-Im cDNA clones. Thus, PL-I,

Rcho-1 PL-I, and PL-Im are equivalent and should be referred to as PL-I. The PL-I gene was localized to chromosome 17 of the rat genome, similar to other PRL family members. Rcho-1 PL-II cDNAs were identical to the published PL-II sequence. PL-Iv cDNAs were isolated from differentiated Rcho-1 cells via an RT-PCR strategy and found to be identical to previously isolated PL-Iv cDNAs. Rcho-1 PL-I and PL-II cDNAs were subcloned into the pcDNA3 expression vector and recombinant protein produced in HRP-1 cells. Both recombinant Rcho-1 PL-I and PL-II proteins significantly stimulated the proliferation of lactogen-dependent rat Nb2 lymphoma cells and mouse mammary epithelial cells. In summary, we show that the Rcho-1 PL-I corresponds to PL-Im and Rcho-1 PL-Iv and PL-II are identical to their previously described placental counterparts. Additionally, both recombinant Rcho-1 PL-I and PL-II proteins are biologically active. (*Endocrinology* 137: 5020–5027, 1996)

THE RODENT placenta produces a family of proteins that include structural and functional homologs of pituitary PRL referred to as placental lactogens (PLs; 1, 2). PL-I, PL-I variant (PL-Iv), and PL-II possess PRL-like actions and are produced by trophoblast giant cells in a highly coordinated pattern during the development of the rat placenta (3–13). PL-I is expressed from implantation until midgestation, whereas PL-II is expressed from midgestation until parturition (6, 7, 10, 14). PL-Iv is expressed during the latter half of gestation by trophoblast giant cells and also by another population of trophoblast cells referred to as spongiotrophoblast cells (11, 12).

The actions of PL-I have been primarily studied via the generation of recombinant PL-I in Chinese hamster ovary (CHO) cells (5, 9). PL-I binds to PRL receptors (15, 16) and possesses a number of actions previously attributed to pi-

tuinary PRL. These activities include stimulation of rat Nb2 lymphoma cell proliferation (4, 9), mouse mammary gland epithelial cell  $\alpha$ -lactalbumin and insulin-like growth factor binding protein (29 kDa IGFBP) biosynthesis (5, 17), islet B-cell proliferation, and insulin secretion (18), and luteal cell progesterone production (19) and the inhibition of pituitary PRL secretion (20). PL-I is equipotent with pituitary PRL and the predominant lactogen of midgestation.

The biological activities of PL-Iv have been investigated with SDS-gel electrophoresis purified PL-Iv and heterologously expressed CHO cell recombinant PL-Iv (12, 13). Both preparations are capable of stimulating rat Nb2 lymphoma cell proliferation; however, neither is as potent as pituitary PRL (12, 13). Recombinant PL-Iv is also capable of competing with pituitary PRL for PRL receptors, again at a reduced potency (13). Our current information suggests that PL-Iv is a functional homolog of pituitary PRL with somewhat reduced biological potency.

The actions of PL-II have been studied with native PL-II purified from either the mouse, rat, or hamster placenta (21–24). PL-II was originally characterized based on its ability to bind PRL receptors and mimic PRL biological activities (15, 16, 21–25). Among the spectrum of PL-II actions resembling pituitary PRL are stimulation of a variety of cellular processes, including: rat Nb2 lymphoma cell proliferation

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(26), mouse mammary gland, epithelial cell  $\alpha$ -lactalbumin, and IGFBP (29 kDa) biosynthesis (17, 24, 27), islet B-cell proliferation and insulin secretion (18), and luteal cell progesterone production (19). PL-II is equipotent with PRL and is the most prominent circulating lactogenic hormone of the latter half of pregnancy.

Recently, another rat PL, related to PL-I and PL-Iv, was identified and termed PL-I mosaic (PL-Im; 28). The expression pattern of PL-Im corresponds to the expression pattern of PL-I (28). Additional relationships of PL-Im to PL-I and the role of PL-Im in the physiology of pregnancy have not been determined.

The Rcho-1 trophoblast cell line has proven to be a useful model for studying the trophoblast giant cell lineage and the PLS they synthesize. Several years ago, Teshima and colleagues developed a procedure for experimentally generating rat choriocarcinomas (29). These trophoblast tumors were found to be transplantable, possess significant endocrine effects on their hosts, and subsequently were shown to express PL-I (29–31). Cell lines with trophoblast characteristics were established from the choriocarcinomas (32, 33). One of these cell lines, referred to as Rcho-1 trophoblast cells, consists of a homogenous population of trophoblast stem cells that proliferate or can be induced to differentiate depending upon the conditions of their culture (33, 34). Rcho-1 trophoblast cell differentiation is incremental, recapitulating the normal ontogeny of trophoblast giant cell differentiation-specific gene expression, including the expression of members of the PRL gene family and steroid hydroxylases (33–37). PL-I and PL-II have been reported to be expressed by differentiating Rcho-1 trophoblast cells along with two other members of the family, PRL-like protein-A (PLP-A) and PLP-C (33, 34). Differentiation appears restricted to the trophoblast giant cell lineage in that Rcho-1 trophoblast cells are not capable of expressing spongiotrophoblast-restricted genes (33, 38).

The present study was undertaken to resolve significant uncertainties regarding PLS in Rcho-1 trophoblast cells. An accurate assessment of which members of the PL-I subfamily (PL-I, PL-Im, and PL-Iv) are present in Rcho-1 trophoblast cells has not been accomplished. Available antibodies and nucleotide probes cannot discriminate among PL-I, PL-Im, and PL-Iv. The identity of the PL-I subfamily members in Rcho-1 trophoblast cells have been based on the sizes of PL-I immunoreactive proteins and their temporal appearance during *in vitro* differentiation. In this report, we show that the Rcho-1 PL-I corresponds to PL-Im. PL-Iv and PL-II were also identified in Rcho-1 cells and possess characteristics identical to their previously described placental counterparts. We further demonstrate that recombinant Rcho-1 PL-I and PL-II are functional PRL homologs.

## Materials and Methods

### Reagents

FBS and donor horse serum (HS) were purchased from JRH Bioscience (Lenexa, KS). Reagents for PAGE were purchased from Bio-Rad (Hercules, CA). All restriction enzymes, polymerases, and DNA ligase were purchased from New England Biolabs (Beverly, MA). Transformation competent *Sure* bacterial cells, random primer labeling kits, messenger RNA (mRNA) isolation kits, and a ZAP Express comple-

mentary DNA (cDNA) synthesis kit were acquired from Stratagene (La Jolla, CA). DNA extraction kits were purchased from Qiagen (Chatsworth, CA). Nitrocellulose and nylon membranes were obtained from Schleicher and Schuell (Keene, NH). Ovine PRL was purchased from Nobl Laboratories Inc. (Sioux City, IA). T7 DNA sequencing kits were acquired from United States Biochemical (Cleveland, OH). PCR kits were obtained from Invitrogen (San Diego, CA), Promega (Madison, WI), and CLONTECH (Palo Alto, CA). The pcDNA3 expression vector was acquired from Invitrogen. Radiolabeled nucleotides were purchased from DuPont-NEN (Boston, MA). TRIzol Reagent for RNA extraction, reverse transcriptase PCR kits, and LipofectAmine reagent for transfection were obtained from Life Technologies (Gaithersburg, MD). Reagents for the detection of immune complexes by enhanced chemiluminescence were acquired from Amersham Corp. (Arlington Heights, IL). Unless otherwise noted, all other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

### Animals and tissue preparation

Holtzman rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The animals were housed in an environmentally controlled facility, with lights on from 0600–2000 h, and allowed free access to food and water. Timed pregnancies and tissue dissections were performed as previously described (39, 40). The presence of a copulatory plug or sperm in the vaginal smear was designated day 0 of pregnancy.

Virgin Balb/c mice were obtained from Charles River Laboratories (Wilmington, MA) and used in the assessment of PL-I and PL-II biological activities.

Protocols for the care and use of animals were approved by the University of Kansas Animal Care and Use Committee.

### Rcho-1 trophoblast cell culture

The Rcho-1 trophoblast cell line was derived from a rat choriocarcinoma and is capable of differentiating along the trophoblast giant cell lineage (33). Rcho-1 trophoblast cells were routinely maintained in subconfluent conditions with NCTC-135 culture medium supplemented with 20% FBS, 50  $\mu$ M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml of penicillin, and 100  $\mu$ g/ml of streptomycin in an atmosphere of 5% CO<sub>2</sub>/95% air at 37 C in a humidified incubator (33). Differentiation was induced by growing the cells to confluence in FBS supplemented culture medium and then replacing the serum supplementation with 10% HS (34, 41).

### Construction of the differentiated Rcho-1 trophoblast cell cDNA library

Poly A<sup>+</sup> enriched RNA (2.8  $\mu$ g) was extracted from a differentiated Rcho-1 trophoblast cell population and used for the first strand cDNA synthesis. The integrity of the RNA preparation was verified by electrophoresis. A cDNA library was constructed with the ZAP Express cDNA Synthesis Kit (Stratagene) according to the manufacturer's instruction. The quality of first and second strand synthesis was determined by electrophoresis. cDNAs were fractionated by size, quantitated, ligated into the ZAP Express vector, and then into Gigapack II Gold packaging extract (Stratagene). One round of amplification of the primary library was performed in XL-1 blue MRF' host cells. The library was titered, tested, and stored in 7% dimethylsulfoxide at –80 C.

### Isolation and characterization of the PL-I and PL-II cDNAs

The cDNA library to differentiated Rcho-1 trophoblast cells was screened with cDNAs to mouse PL-I (4) and to rat PL-II (3) as previously described (42, 43). Five positive plaques for Rcho-1 PL-I and six positive plaques of Rcho-1 PL-II were selected and excised. Rcho-1 PL-I and PL-II cDNAs directionally cloned into *Eco*RI and *Xho*I restriction sites within the pBK-CMV phagemid were used for restriction and nucleotide sequence analyses as previously described by our laboratory (42). DNA sequencing was performed by the dideoxy chain termination method (44) using oligonucleotide primers complementary to the T3 promoter, the T7 promoter, and to internal segments of the respective cDNAs. Sequencing reactions used [<sup>35</sup>S]dATP and were performed with T7 sequencing kits. Reaction products were resolved in 6% polyacrylamide

urea gels, dried, and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY).

### Chromosomal assignment

Cell hybrids used in the chromosomal localization of the Rcho-1 PL-I gene were derived from the fusion of mouse hepatoma cells (BWTG3) with adult rat hepatocytes and have been used in the chromosomal assignment of several rat genes (45–47). Chromosomal preparations were generated as previously reported (45, 48). DNA was extracted and analyzed by PCR using primers specific for Rcho-1 PL-I (see Fig. 1). The PCR reaction was performed for 35 cycles (denature: 94 C for 30 sec; annealing and extension: 68 C for 6 min). Reaction products were electrophoretically separated in 1% agarose gels, transferred to nylon membranes, and probed with a [<sup>32</sup>P]-labeled Rcho-1 PL-I cDNA (49).

### PCR analyses

Total RNA was extracted from differentiated Rcho-1 trophoblast cells, choriovitelline placental tissue (isolated from day 11 of gestation), and junctional zone placental tissue (isolated from day 19 of gestation) with the TRIzol reagent. RT-PCR was performed with primers specific for Rcho-1 PL-I, rat PL-Iv, for the originally described PL-I, or rat  $\beta$ -actin (see Fig. 1). RT-PCR was performed according to the manufacturer's instructions (SuperScript preamplification system for first strand cDNA synthesis kit, Life Technologies). Five micrograms of total RNA samples and 0.5  $\mu$ g of oligo(deoxythymidine) were used for the reverse transcriptase reaction. The PCR reaction was performed for 30 cycles (denature: 94 C for 1 min; annealing: 60 C for 2 min; extension: 72 C for 2 min). Amplified products were subcloned into the pGEM-T vector (Promega) and their identity verified by sequencing. Subsequent samples were electro-

#### Rcho-1 PL-I primers:

upstream primer (8-28)\*: 5'-TGACTTTGACTCTTTTCGGGCT-3'  
downstream primer (754-734): 5'-GCTCTGAATACACCGAGAGCG-3'

#### PL-Iv primers:

upstream primer (80-100): 5'-TGGCCTCCAACCAACTGTGC-3'  
downstream primer (481-461): 5'-TCTGAGTCTCTTGAATATTT-3'

#### Original PL-I primers:

Set No. 1:  
upstream primer (8-28): 5'-TGACTTTGACTCTTTTCGGCGC-3'  
downstream primer (584-564): 5'-CAAATGGCAAAAAGATGATGC-3'  
Set No. 2:  
upstream primer (110-130): 5'-CCACTGATGATCTATATCATT-3'  
downstream primer (466-446): 5'TGCTTTCATCAGATGAGTCA-3'

#### Rat $\beta$ -actin primers:

upstream primer (100-119): 5'-ATCGTGGCCGCCCTAGGCA-3'  
downstream primer (343-323): 5'-TGGCCTTAGGGTTACAGAGGGG-3'

\*Nucleotide sequence positions relative to the ATG translation site.

FIG. 1. Primer sequences used in the PCR analysis of Rcho-1 PL-I (present report), PL-Iv (13), original PL-I (10), and rat  $\beta$ -actin (63). All nucleotide positions are relative to the ATG translational start site.

phoretically separated in 1% agarose gels, stained with ethidium bromide, transferred to nylon membranes, and probed with [<sup>32</sup>P]-labeled Rcho-1 PL-I or PL-Iv cDNAs (49). PCR amplification of products from a rat genomic library with primers for Rcho-1 PL-I or for the originally identified PL-I was evaluated as described above for the chromosomal localization.

### Heterologous expression of PL-I and PL-II

BamHI-XhoI restriction fragments of Rcho-1 PL-I and PL-II cDNAs were isolated from their respective pBK-CMV phagemid vectors and directionally subcloned into the pcDNA3 eukaryotic expression vector (Invitrogen). The PL-I and PL-II expression vectors were then transfected into HRP-1 cells using a liposome-mediated delivery system. The HRP-1 cell line represents a transplantable cell population with both trophoblast and yolk sac attributes (50–51). HRP-1 cells were routinely maintained in RPMI-1640 culture medium containing 10% FBS and the above supplements in an atmosphere of 5% CO<sub>2</sub>/95% air at 37 C in a humidified incubator. Cells were seeded in 35-mm tissue culture dishes (3 × 10<sup>5</sup>), grown to approximately 70% confluence, and then transfected with 10  $\mu$ g of the respective DNA construct following linearization with BglIII. DNA-liposome complexes were prepared by the addition of 20  $\mu$ l of LipofectAmine. HRP-1 cells were then incubated with the complexes for 5 h at 37 C in a CO<sub>2</sub> incubator. Following the incubation, the cells were transferred to normal growth medium. Selection with G418 (1 mg/ml) was initiated 48 h after transfection and continued for 2 weeks. Cells containing the Rcho-1 PL-I expression vector construct are referred to as HI cells, whereas the cells containing the PL-II expression vector construct are referred to as HII cells. Conditioned medium from the HI, HII, and parent HRP-1 cell lines were generated in confluent cultures exposed to serum-free DMEM/Ham's F10 culture medium, concentrated 30–35 times using an Amicon ultrafiltration apparatus (mol wt cutoff: 10,000), and stored frozen until further analysis.

### Western blot analysis of PL-I and PL-II

Western blot analyses were performed as previously described (52, 53). Samples were separated by PAGE in 12.5% gels under reducing conditions. Proteins from the gels were electrophoretically transferred to nitrocellulose. Polyclonal antibodies generated against synthetic peptides corresponding to amino acids 1–19 of PL-I (34) and amino acids 56–70 of PL-II (53) were used as probes. Immune complexes were detected using the enhanced chemiluminescence system as previously described (13).

### Biological activities of recombinant PL-I and PL-II

**Nb2 lymphoma cell bioassay.** PRL-like biological activities were assessed through the use of the rat Nb2 lymphoma cell proliferation assay (54). Nb2 lymphoma cells were routinely grown in Fischer's medium supplemented with 50  $\mu$ M 2-mercaptoethanol, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 10% HS, and 10% FBS in an atmosphere of 5% CO<sub>2</sub>/95% air at 37 C in a humidified incubator. Twenty-four hours before initiating the assay, cells were diluted to a concentration of 100,000 cells/ml with Fischer's medium supplemented with 2-mercaptoethanol, antibiotics, and 10% HS (stationary medium). At the initiation of the assay, cells were washed and aliquoted into 16 mm wells (100,000 cells/ml-well) of a 24-well culture plate. HI, HII, and HRP-1 cell concentrated condition medium, or ovine PRL were added at various concentrations and incubated for an additional 72 h. Samples of treated cells were collected and counted in a Sysmex Microcell counter (model CC-110, TOA Medical Electronics, Kobe, Japan). Treatments were performed in triplicate.

**Mouse mammary epithelial cell proliferation.** PRL-like actions were also monitored through the assessment of mammary epithelial cell responses to the PRL-related proteins (55). Epithelial cells were obtained by collagenase dissociation of minced glands from virgin Balb/c mice and purification by Percoll gradient centrifugation (56). Isolated mammary epithelial cells were cultured in collagen gels for 10 days in serum-free medium (Ham's F12/DMEM, 1:1, vol:vol) containing insulin (10  $\mu$ g/ml) plus the test substances. Some cultures were also treated with progres-

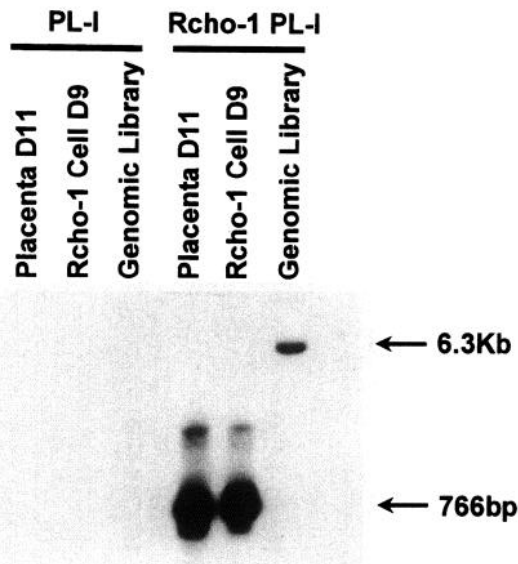


FIG. 2. PCR analysis of PL-I mRNA in Rcho-1 trophoblast cells and day 11 trophoblast tissue and the PL-I gene in a rat genomic library. Total RNA was extracted from differentiated Rcho-1 trophoblast cells and choriovitelline placental tissue (isolated from day 11 of gestation) with the TRIzol Reagent. RT-PCR was performed with primers specific for Rcho-1 PL-I (present study) and for the originally described PL-I (10; data from primer set No. 1 is shown), or rat  $\beta$ -actin (see Fig. 1). PCR amplification of products from a rat genomic library with primers for Rcho-1 PL-I or for the originally identified PL-I was also evaluated. Amplified products were electrophoretically separated in 1% agarose gels stained with ethidium bromide transferred to nylon membranes and probed with a [ $^{32}$ P]-labeled Rcho-1 PL-I cDNA. Please note that the Rcho-1 PL-I was identified in Rcho-1 trophoblast cell midgestation choriovitelline placental tissues and from the rat genomic library whereas the original PL-I (10) amplification product was not generated from any of the sources.

terone (50 ng/ml). At the termination of culture, proliferation was assessed by DNA fluorometric assay using cell standards (56).

#### Statistical analysis

The data were analyzed by ANOVA. The source of variation from significant F ratios was determined with the Newman-Keuls multiple comparison test (57).

### Results

#### Isolation and characterization of Rcho-1 trophoblast cell PL-I and PL-II cDNA clones

A cDNA library representing differentiated Rcho-1 trophoblast cells was generated. The sizes of cDNAs contained in the library ranged from 300bp to 10 kilobase pairs. The titer of the library was  $4.2 \times 10^8$  pfu/ml consisting of greater than 95% recombinant phages. Screening of the cDNA library ( $5 \times 10^5$  pfu) for PL-I and PL-II resulted in the identification of several hundred positive plaques. Five positive PL-I plaques and six positive PL-II plaques were selected for *in vivo* excision. pBK-CMV phagemid clones generated from *in vivo* excision were tested with several different restriction enzymes. Three of the PL-I clones (PL-I-31, PL-I-33, PL-I-35) and four of the PL-II clones (PL-II-31, PL-II-32, PL-II-33, PL-II-35) showed similar restriction maps, respectively. PL-I-33 and PL-II-33 were selected for further analysis.

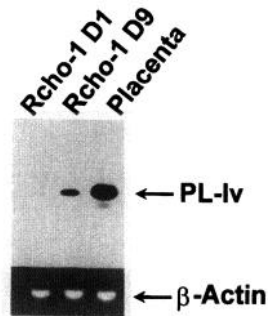


FIG. 3. RT-PCR analysis of PL-Iv expression in Rcho-1 trophoblast cells and normal placental tissue. Total RNA was extracted from differentiated Rcho-1 trophoblast cells and junctional zone placental tissue (isolated from day 19 of gestation) with the TRIzol Reagent. RT-PCR was performed with primers specific for rat PL-Iv (13) or rat  $\beta$ -actin (see Fig. 1). Amplified products were electrophoretically separated in 1% agarose gels stained with ethidium bromide transferred to nylon membranes and probed with a [ $^{32}$ P]-labeled PL-Iv cDNA. The rat  $\beta$ -actin amplified product was detected by ethidium bromide staining. Please note that PL-Iv products were specifically amplified from differentiated Rcho-1 trophoblast cells and from the junctional zone of the chorioallantoic placenta. The identity of the amplified products were verified by nucleotide sequencing.

Sequence analysis of three independent Rcho-1 PL-I cDNAs indicated that they significantly differed from the previously reported PL-I sequence (10) but more closely resembled a related cDNA referred to as PL-I mosaic (PL-Im; 28). Coding regions for Rcho-1 PL-I and PL-Im were identical. Forty-four nucleotides of Rcho-1 PL-I 5'-untranslated sequence were identified in the cDNAs (5'-agatttctgctgg-gagctactgtcttgatctgctcagaaATG-3') not previously reported in the PL-Im sequence. Two other single base differences were noted in 3' untranslated nucleotide sequence and have been submitted to GenBank. Rcho-1 PL-I transcripts could be detected in Rcho-1 trophoblast cells and normal developing placental tissue by RT-PCR; however, the previously reported PL-I transcript could not be identified from the same sources by RT-PCR (Fig. 2). Furthermore the Rcho-1 PL-I gene was amplified from a rat genomic library with the same set of primers (Fig. 2). We were not successful in amplifying any products from the rat genomic library with either set or combination of primers designed to detect the originally reported PL-I (Fig. 2). Detection of amplified products was determined by ethidium bromide staining and Southern analysis with exposure of x-ray film for extended time periods. Given these results, we reexamined the original PL-I cDNA (10) by PCR and nucleotide sequence analyses. The sequence differed from the original report (10) and was found to be identical to the Rcho-1 PL-I (present study) and PL-Im cDNA (28) clones. Thus, PL-I, Rcho-1 PL-I, and PL-Im are equivalent and should be referred to as PL-I.

Rcho-1 PL-II cDNA sequences were virtually identical to the published PL-II sequence (3; data not shown). An additional 43 nucleotides of PL-II 5' untranslated sequence plus the translation start site were identified (5'-aagcagtctagtgtgtctccactcgagaactctcaagATG-3'). The ATG start site replaces the initial GTG (valine) of the originally published PL-II sequence (3). One additional difference was noted in 3' flanking nucleotide sequence and has been submitted to GenBank.

PL-Iv was specifically amplified by RT-PCR from differentiated (day 9 of culture) but not undifferentiated (day 1 of culture) Rcho-1 trophoblast cells (Fig. 3). The nucleotide sequence of the amplified Rcho-1 PL-Iv product was identical to the previously published PL-Iv sequence (13; data not shown).

#### Chromosomal assignment

The Rcho-1 PL-I gene was localized using somatic cell hybrids that segregate rat chromosomes. PCR-based identification of the Rcho-1 PL-I gene in the cell hybrids cosegregated with rat chromosome 17. Discordant clones were not observed. Independent discordant clones for the other rat chromosomes are shown in Table 1. The data is consistent with the localization of the Rcho-1 PL-I gene to chromosome 17 of the rat genome.

#### Heterologous expression of PL-I and PL-II

Conditioned medium from HI cells (HRP-1 cells transfected with the Rcho-1 PL-I cDNA expression vector) contained immunoreactive PL-I as determined by Western blot analysis. Rcho-1 PL-I expressed by HI cells exhibited a faster mobility than native PL-I expressed by Rcho-1 trophoblast cells (32–34 kDa vs. 36–40 kDa, see Fig. 4). Faster migrating recombinant PL-I immunoreactive species have similarly been reported for mouse and rat PL-I synthesized by CHO cells (5, 9). We hypothesize that the difference in mobility between native and recombinant Rcho-1 PL-I proteins is attributable to the glycosylating properties of Rcho-1 trophoblast cells vs. HRP-1 cells. HII cells (HRP-1 cells transfected with the PL-II cDNA expression vector) synthesized and secreted immunoreactive PL-II protein possessing an electrophoretic mobility similar to native Rcho-1 PL-II (Fig. 4).

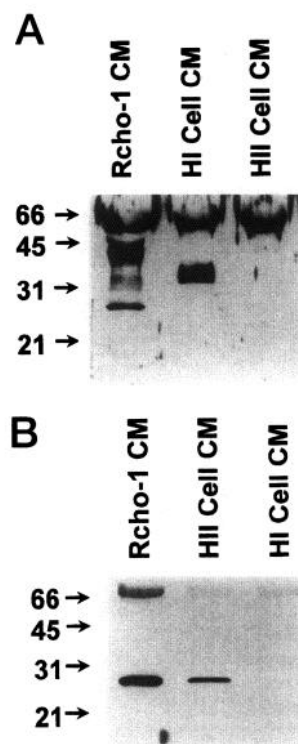


FIG. 4. Heterologous expression of Rcho-1 PL-I and PL-II in HRP-1 cells. Concentrated medium conditioned by the HI (PL-I expressing) and HII (PL-II expressing) cell lines were analyzed for PL-I (top panel) and PL-II (bottom panel) by Western blot analysis. Samples were separated by PAGE in 12.5% gels under reducing conditions. Proteins from the gels were electrophoretically transferred to nitrocellulose and probed with polyclonal antibodies specific to PL-I or PL-II. Immune complexes were detected using the enhanced chemiluminescence system as previously described. Please note the bands in each blot migrating near the 66  $M_r$  standard are nonspecific.  $M_r$  standards ( $\times 10^{-3}$ ) are shown.

TABLE 1. Cosegregation of the rat PL-I gene and chromosome 17 in mouse x rat cell hybrids

Hybrids	Rat PL-I gene <sup>a</sup>	Rat chromosomes <sup>b</sup>																			
		x	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
LB20	+	+	-	(+)	(+)	-	-	(-)	+	-	-	-	+	+	-	-	+	(+)	+	+	-
LB150-1	+	+	-	-	+	+	-	-	+	-	+	(+)	+	+	+	-	(+)	(+)	+	+	-
LB161	+	+	-	+	+	+	+	+	+	+	+	-	(+)	+	+	+	+	+	+	+	(+)
LB210-I	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-
LB251	+	+	+	+	-	+	-	(+)	+	-	-	+	-	+	+	-	-	-	+	-	+
LB330	+	+	-	+	+	+	+	+	-	-	-	+	-	+	-	-	-	-	+	-	-
LB360B	+	+	-	-	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+
LB510-6	+	+	-	+	+	+	-	-	+	-	-	-	+	+	+	+	+	+	+	+	-
LB600	-	+	+	+	+	+	+	(+)	+	-	(-)	+	+	+	+	+	+	+	-	+	+
LB630	-	+	(-)	-	+	+	(+)	+	+	-	+	-	+	+	+	(+)	+	+	-	+	(-)
LB780	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	-	-	-	+	+	+
LB810	+	+	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+
LB860	+	+	-	+	+	+	-	-	+	-	+	-	+	+	+	-	+	+	+	+	(+)
LB1040	-	+	-	-	+	+	(-)	+	+	-	-	+	+	+	-	-	+	+	-	+	+
Independent discordant clones <sup>c</sup>		4	10	3	4	4	9	8	4	8	8	5	8	4	4	9	8	6	0	7	6

<sup>a</sup> + and - = presence or absence of the rat gene, respectively.

<sup>b</sup> + = rat chromosome present in more than 55% of the metaphases; (+) = rat chromosome present in 25–55% of the metaphases; (-) = rat chromosome present in less than 25% of the metaphases; - = rat chromosome absent.

<sup>c</sup> When a chromosome was present in less than 25% of the metaphases (- *in parentheses*), the hybrid in question was not taken in account to establish the number of discordancies for that particular chromosome.

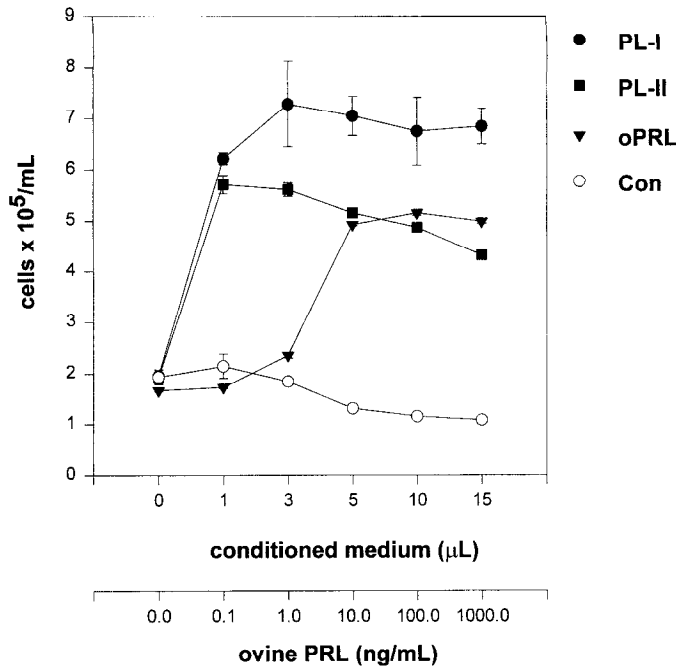


FIG. 5. Nb2 lymphoma cell proliferation in response to recombinant PL-I and PL-II PRL-like biological activities were assessed through the use of the rat Nb2 lymphoma cell proliferation assay. At the initiation of the assay cells were washed and aliquoted into 16-mm wells (100,000 cells/ml-well) of a 24-well culture plate. Concentrated conditioned medium containing PL-I (HI cells), PL-II (HII cells), and control (Con; HRP-1 cell), or ovine PRL were added at various concentrations and incubated for an additional 72 h. Samples of treated cells were collected and counted in a Sysmex Microcell counter (model CC-110, TOA Medical Electronics). Treatments were performed in triplicate. Values for PL-I and PL-II treatments were significantly greater than controls  $P < 0.01$ .

#### Biological actions of recombinant Rcho-1 PL-I and PL-II

Concentrated conditioned medium enriched for either recombinant Rcho-1 PL-I or PL-II significantly stimulated the proliferation of lactogen-dependent rat Nb2 lymphoma cells, whereas conditioned medium generated from parent HRP-1 cells did not significantly modify the growth pattern of the Nb2 lymphoma cells (Fig. 5). The actions of recombinant PL-I and PL-II were dose dependent and closely mimicked the action of ovine PRL (Fig. 5). Concentrated conditioned medium containing either recombinant Rcho-1 PL-I or PL-II also significantly stimulated the proliferation of mouse mammary epithelial cells (Fig. 6). Parent HRP-1 cell conditioned medium did not significantly influence the behavior of the mouse mammary epithelial cells (Fig. 6). The actions of recombinant Rcho-1 PL-I and PL-II were dose dependent and also synergistic with the addition of progesterone (Fig. 6). Mammary epithelial cell responses to recombinant Rcho-1 PL-I and PL-II were similar to those previously reported for mammary epithelial cell responses to pituitary PRL (55).

#### Discussion

Rcho-1 trophoblast cells have developed into a valuable model system for studying trophoblast cell biology. In this report, we have specifically identified and characterized PLs produced by Rcho-1 trophoblast cells. PL expression in

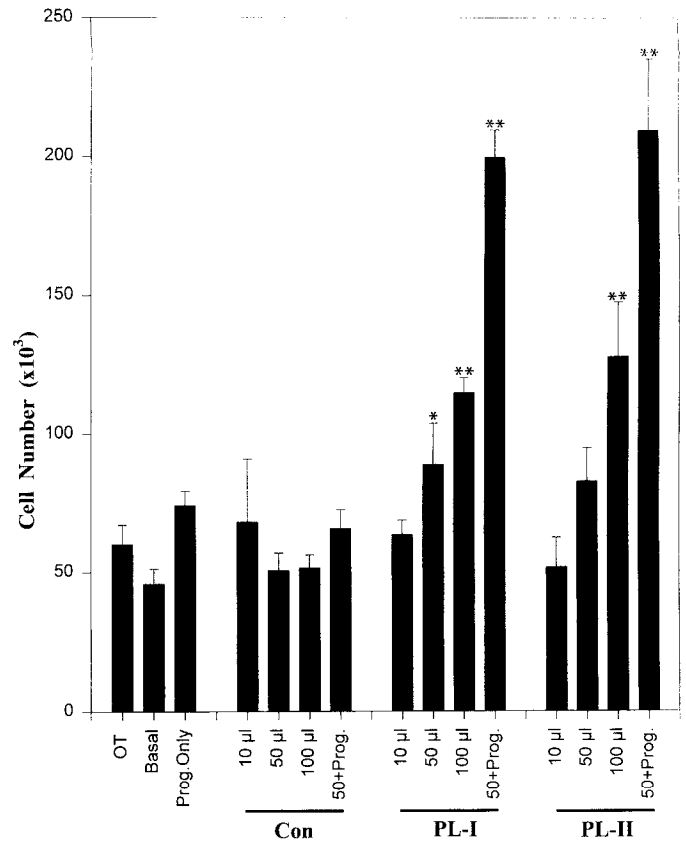


FIG. 6. Effects of recombinant PL-I and PL-II on mouse mammary epithelial cell proliferation. Epithelial cells were obtained by enzymatic dissociation of minced glands from virgin Balb/c mice. Cells were cultured for 10 days in serum-free medium containing insulin (10 μg/ml) plus the test substances [Con (control medium conditioned by the HRP-1 parent cell line), PL-I (medium conditioned by HI cells), and PL-II (medium conditioned by HII cells)]. Some cultures were also treated with progesterone (prog; 50 ng/ml). At the termination of culture proliferation was assessed by DNA fluorometric assay using cell standards. Statistical comparisons between control cultures and PL-I or PL-II treated cultures are shown (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). OT, Original cell count at the beginning of the culture; Basal, Cell number at the end of the culture in the absence of any treatments.

Rcho-1 trophoblast cells is differentiation dependent and is represented by three different members of the placental PRL gene family.

Two constituents of the PL-I subfamily and PL-II are expressed by differentiating Rcho-1 trophoblast cells. Rcho-1 PL-Iv and PL-II were found to be identical to their previously characterized placental counterparts (3, 11–13; present study). Rcho-1 PL-I was distinctly different than the earlier reported PL-I (10) but was identical to PL-Im throughout its coding sequence (28). Thus, Rcho-1 PL-I appears to be equivalent to PL-Im. Upon further analysis, Rcho-1 PL-I/PL-Im was readily identified in normal developing placental tissues and a portion of its corresponding gene was amplified from a rat genomic library by PCR. In contrast, we were not successful in identifying the originally identified PL-I (10) from Rcho-1 trophoblast cells, midgestation placental tissue, the Rcho-1 cDNA library, or from a rat genomic library using various combinations of two different sets of PCR primers spanning distinct regions of the original PL-I sequence

(present study). We anticipated that each of these sources should have been positive for the originally identified PL-I (10). Additionally, Rcho-1/PL-Im possesses PRL-like biological actions similar to those previously reported for PL-I (5, 9; present study). These results prompted a reexamination of the original PL-I cDNA. Following PCR and nucleotide sequence analyses, we conclude that the original PL-I, Rcho-1 PL-I (present study), and PL-Im (28) each encode the same protein. The appropriate PL-I sequence is represented by the Rcho-1 PL-I/PL-Im sequence (28, present study). Thus, there are only two members of the PL-I subfamily (PL-I and PL-Iv) and PL-I is equivalent to Rcho-1 PL-I/PL-Im.

All of the presently identified genes corresponding to members of the rat PRL family (PRL, PL-Iv, PL-II, PLP-A, PLP-B, PLP-C, PLP-C variant, and decidual/trophoblast PRL-related protein) and now PL-I have been assigned to chromosome 17 of the rat genome (2, 13, 42, 43, 58, 59). Human chromosome 6 and mouse chromosome 13 show conservation of synteny with rat chromosome 17 and contain human and mouse PRL family genes, respectively (60, 61). Specific linkage arrangements of the PRL family genes on their respective chromosomes have not been reported.

Recombinant rat PL-I and PL-II proteins were produced in the rat HRP-1 trophoendodermal stem cell line (present study). HRP-1 cells were originally selected as a host for the PL-I and PL-II expression vectors because they do not endogenously express PL-I, PL-II, or any other lactogenic hormone and are readily transplantable (50). Unfortunately, PL-I and PL-II expression did not continue following transplantation of the transfected HRP-1 cell populations beneath the kidney capsule (unpublished observations). Nonetheless, under *in vitro* conditions, the proteins were expressed and exhibited biochemical, immunochemical, and biological characteristics consistent with previously published work on rat PL-I and PL-II (9, 26, 53). Processing of PL-I by HRP-1 cells was somewhat different than the processing of native PL-I by trophoblast giant cells. HRP-1 cell-derived recombinant PL-I exhibits a faster electrophoretic mobility than native PL-I. Similar differences have been reported for CHO cell-recombinant PL-I *vs.* native PL-I and are likely attributable to cell-specific glycosylation patterns (5). Trophoblast giant cell-specific carbohydrate modifications may relate, at least in part, to the longevity of PL-I in circulation. PL-I has an extended half-life (62), which compensates for the paucity of available trophoblast giant cells during early phases of pregnancy. As gestation progresses, PL-II expression is activated and PL-I expression is terminated (7). PL-II is not glycosylated, possesses a short half-life in circulation, and increases in expression as the placenta grows in size (3, 6, 53).

Both recombinant PL-I and PL-II generated in HRP-1 cells possessed biological activities indicative of their functional relatedness to PRL. The recombinant hormones effectively stimulated the proliferation of rat Nb2 lymphoma cells consistent with previous reports for recombinant rat PL-I and native rat PL-II (9, 26; present study). Mammatrophic actions of both recombinant PL-I and PL-II were also demonstrated for the first time and closely resembled mitogenic effects of PRL on mammary epithelial cells (55; present study). Pregnancy-specific control of mammary epithelial cell proliferation is thus dependent upon the coordinated expression of

PRL, PL-I, and PL-II by the pituitary and placenta. These three PRL family members also affect mammary gland milk protein synthesis and IGFBP expression (17, 24, 27). Most interestingly, the mitogenic actions of PRL, PL-I, and PL-II on mammary epithelial cells are significantly augmented by progesterone (55, present study). This synergism is particularly noteworthy in that PRL, PL-I, and PL-II are also prominent regulators of progesterone biosynthesis by the corpus luteum (19).

In summary, we have provided new insights into the biology of PLs expressed by the Rcho-1 trophoblast cell line. The information further validates the use of this important *in vitro* model for studies on the trophoblast giant cell lineage and the biology of members of the PRL gene family.

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