Occurrence of PG-Lb, a leucine-rich small chondroitin/dermatan sulphate proteoglycan in mammalian epiphyseal cartilage: molecular cloning and sequence analysis of the mouse cDNA

Kazuhiro KURITA*†, Tamayuki SHINOMURA*, Minoru UJITA*, Masahiro ZAKO*, Daihei KIDA*†, Hisashi IWATA† and Koji KIMATA*‡

*Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-11, Japan and †Department of Orthopaedic Surgery, Nagoya University School of Medicine, Showa, Nagoya 466, Japan

PG-Lb is a chondroitin/dermatan sulphate proteoglycan first isolated from chick embryo limb cartilage. It had been assumed that osteoglycin represents its mammalian homologue. However, partial amino acid sequences of a novel proteoglycan from bovine epiphyseal cartilage showed high identity with those of chick PG-Lb (P. Neame, L. Rosenberg and M. Höök, personal communication). Reverse transcriptase PCR using degenerate oligonucleotide primers gave a cDNA fragment that might correspond to mouse PG-Lb. We isolated a clone from a cDNA library of newborn mouse epiphyseal cartilage using the cDNA fragment as a probe. The cloned cDNA was 1430 bp long and contained a 966 bp open reading frame which encoded the core protein consisting of 322 amino acid residues. The deduced amino acid sequence showed a high overall identity with chick PG-Lb (about 62 %, reaching about 80 % over the carboxyl two-thirds). In addition, the amino acid sequence contained a signal peptide, six cysteine residues at the invariant relative position to chick PG-Lb, six leucine-rich repeats at the carboxyl two-thirds, three possible glycosaminoglycan-attachment sites (two sites at the N-terminal side and one site at the C-terminus) and two possible Asn-glycosylation sites near the C-terminus. Northern-blot analysis demonstrated the specific expression of a 1.5 kb message in cartilage and testis. These structural features and the characteristic expression suggest that the cloned molecule is mouse PG-Lb.

INTRODUCTION

In the process of chick limb cartilage development, four distinct types of proteoglycan (PG-H, PG-Lb, PG-Lt and PG-M) are expressed in a well-defined temporal and spatial pattern [1–3]. PG-H is expressed at the cartilage primordia when cartilage differentiation begins and becomes one of the major cartilage matrix constituents involved in cartilage-specific functions. The expression of PG-Lt, a proteoglycan form of type-IX collagen, is also closely associated with cartilage differentiation and the molecule is found to be associated with type-II collagen fibrils [4]. PG-M is a large chondroitin/dermatan sulphate proteoglycan, and its expression is quite unique in that it appears preferentially in the precartilage mesenchymal condensation area and then disappears from the cartilage with the completion of differentiation [2,5]. PG-H, PG-Lt and PG-M are now known to be widely conserved in mammals and have been extensively investigated.

PG-Lb was first found in chick embryo cartilage [6]. This proteoglycan consists of a core protein of M_r 43000 and chondroitin/dermatan sulphate chains of M_r 52000, and has similar structural features to the mammalian small chondroitin/dermatan sulphate proteoglycans, decorin and biglycan [7]. Immunohistochemical analyses of these proteoglycans using specific antibodies have revealed that both proteoglycans are widely expressed in various tissues including developing limb cartilage [8]. We have previously shown that PG-Lb is expressed exclusively in the zones of flattened chondrocytes and the ossifying region in the limb buds of chick embryos (stage 19–33) [9]. Therefore PG-Lb appeared to be different from decorin and biglycan in this respect. We confirmed this by sequencing cDNA clones for chick PG-Lb [9]. The deduced primary structure was quite similar to that of osteoglycin which had been thought to have osteoinductive activity [10], and suggested that osteoglycin might be a mammalian equivalent of PG-Lb. The high identity of the amino acid sequence with human osteoglycin [about 50 % in the region containing leucine-rich repeats (LRRs)], the detection of six cysteine residues in both PG-Lb and osteoglycin with invariant relative positions, and the unique localization of chick PG-Lb at the ossifying area of the cartilage peripheral zone bordering on the bone marrow cavity at stage 38 all suggested possible participation in the ossification process. However, recent information on the amino acid sequences of the peptides generated by cleavage of a proteolgycan isolated from bovine epiphyseal cartilage suggested that the proteoglycan may correspond to chick PG-Lb and not osteoglycin (P. J. Neame, L. C. Rosenberg and M. Höök, personal communication), which suggested the existence of PG-Lb in mammals.

In this study, we determined the cDNA sequence of mouse PG-Lb in newborn mouse epiphyseal cartilage. This is the first demonstration of the presence of this proteoglycan in mammals at the message level and we discuss the primary structure in comparison with other molecules containing LRR sequence.

MATERIALS AND METHODS

Preparation of λgt11 cDNA library

Polyadenylated [poly(A)$^+$] RNA was isolated from epiphyseal cartilage of newborn mouse by the guanidinium isothiocyanate method [11] and purified by oligo(dT)-cellulose affinity column

Abbreviations used: PG-Lb, proteoglycan Lb; LRR, leucine-rich repeat; poly(A)$^+$, polyadenylated.

‡ To whom correspondence should be addressed.

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number D78274.

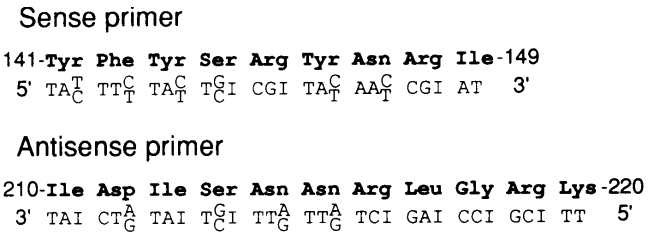

Figure 1 Design of PCR primers for the amplification of mouse PG-Lb cDNA

Sense and antisense primers were designed on the basis of the Tyr^{141}–Ile^{149} residues and on the Ile^{210}–Lys^{220} residues of chicken PG-Lb core protein respectively.

chromatography (Pharmacia Biotec Inc., Piscataway, NJ, U.S.A.). Some 5 μg of poly(A)$^+$ RNA was used to construct an oligo(dT)-primed cDNA library. cDNAs were synthesized using Time Saver cDNA Synthesis Kit (Pharmacia) as recommended by the manufacturer. The cDNA library was constructed in λgt11 using the Gigapack III Gold Packaging Extract (Stratagene, San Diego, CA, U.S.A.). The library thus obtained gave 3×10^7 independent clones when transfected into *Escherichia coli* Y-1088.

Preparation of DNA probe

Degenerate oligonucleotide primers for the sense and antisense directions were designed on the basis of the highly conserved amino acid sequences between chick PG-Lb core protein and the novel proteoglycan isolated from bovine epiphyseal cartilage (Figure 1). The sense primer corresponded to residues Tyr^{141}–Ile^{149}, and the antisense primer to Ile^{210}–Lys^{220} of chick PG-Lb [9]. To reduce the degeneracy of these primers, deoxyinosine was introduced at the ambiguous codon positions where more than two alternative deoxynucleotides were possible, and the first base of each triplet was taken from the deoxynucleotide of cDNA for chick PG-Lb. PCR amplification was performed using oligo(dT)-primed cDNAs prepared from epiphyseal cartilage of newborn mouse as templates. The reaction was carried out in a model PJ 9600 (Cetus Co., Emeryville, CA, U.S.A.) using AmpliTaq DNA polymerase (Perkin–Elmer, Norwalk, CT, U.S.A.) for 45 cycles at 95 °C for 1 min, 42 °C for 2 min, 72 °C for 3 min, and finally, 72 °C for 15 min. This PCR product was then cloned into pGEM-3zf(−) plasmid vector (Promega, Madison, WI, U.S.A.). The nucleotide sequence was determined by the dideoxy chain termination method using 7-DEAZA Sequencing Kit Ver.2.0 (Takara Shuzo, Otsu, Japan) [12] and [α-^{32}P]dCTP (Amersham International, Amersham, Bucks., U.K.) as a tracer. DNA sequences were determined for both strands using primers corresponding to the vector sequences. After the sequence was confirmed using the GENETYX-MAC computer software program (Software Development Co., Tokyo, Japan), this PCR product was used as a probe for cloning mouse PG-Lb cDNA [13].

Cloning of cDNA encoding mouse PG-Lb

The cDNA library without amplification was screened by plaque hybridization. First 6.25×10^5 plaques formed on *E. coli* Y-1088 were transferred to Hybond N$^+$ nylon membranes (Amersham) under alkaline blotting conditions. They were then hybridized with ^{32}P-labelled PCR product. Prehybridization was carried out for 24 h at 42 °C in solution containing 35% formamide, $5 \times$ SSPE, $5 \times$ Denhardt's solution, 40 μg/ml denatured salmon sperm DNA and 0.4 μg/ml of denatured *E. coli* DNA. Then hybridization was carried out for 24 h under the same conditions. Membranes were washed once for 20 min at 60 °C in $2 \times$ SSPE/0.1% SDS, three times for 20 min at 60 °C in $1 \times$ SSPE/0.1% SDS, and finally three times for 20 min at 60 °C in $0.55 \times$ SSPE/0.1% SDS. The membranes were exposed to X-ray films and positive clones were picked up. The composition of $20 \times$ SSPE and $100 \times$ Denhardt's solution was 3 M NaCl/0.2 M sodium phosphate (pH 7.4)/20 mM EDTA and 2% Ficoll 400 (Pharmacia)/2% polyvinylpyrrolidone/2% BSA (Sigma) respectively.

DNA sequencing and analysis

One positive clone was digested with restriction endonuclease to cleave out the 1.4 kb cDNA insert from recombinant phage DNA. The DNA was subcloned into pGEM-3zf(−) plasmid vector. The nucleotide sequence of this cDNA insert was determined by the dideoxy chain termination method using primers synthesized on the basis of the preceding sequences. The sequences were determined for both strands at all positions. The cDNA sequences obtained were compiled and analysed using a computer program. The deduced amino acid sequence was compared with chick PG-Lb and other protein sequences in the database compiled by the European Bioinformatics Institute in February, 1995.

Northern-blot analysis

Poly(A)$^+$ RNA (1 μg) isolated from newborn mouse epiphyseal cartilage and poly(A)$^+$ RNA (1.5 μg) isolated from lung, kidney, skeletal muscle, smooth muscle, brain, liver, testis and pancreas of adult mouse (Clontech laboratories, Palo Alto, CA, U.S.A.) were electrophoresed in a denaturing formaldehyde–agarose gel (1% gel for cartilage, 0.6% gel for others), and then transferred to Hybond N$^+$ membranes by the use of a vacuum blotter (VacuGene XL; Pharmacia) under alkaline blotting conditions as recommended by the manufacturer. The 1.4 kb cDNA fragment was ^{32}P-labelled using a random primer labelling kit (Takara) and was used as a probe. Prehybridization and hybridization were carried out at 42 °C in the presence of 50% formamide/10% dextran sulphate overnight. After hybridization, membranes were washed once for 10 min at 65 °C in $2 \times$ SSPE/0.1% SDS, three times for 20 min at 65 °C in $1 \times$ SSPE/0.1% SDS, and three times for 20 min at 65 °C in $0.1 \times$ SSPE/0.1% SDS. The hybridization was analysed by autoradiography.

RESULTS

Preparation of probe

PCR amplification with degenerate primers to amplify the part of the cDNA encoding mouse PG-Lb was performed. The cloned PCR product was 239 bp long containing the primer sequences. The nucleotide sequence (data not shown) showed a high identity (78%) with chick PG-Lb and the amino acid sequence encoded by this PCR product (79 residues) showed 82% identity with residues Tyr^{141}–Arg^{219} of the chick PG-Lb core protein. We therefore presumed that the cDNA encoding mouse PG-Lb was amplified, and used this PCR product as a probe to screen a cDNA library.

Isolation and analysis of cDNA clone

Positive clones were detected by screening a total of 6.25×10^5 independent λgt11 cDNA clones. The nucleotide sequence of one

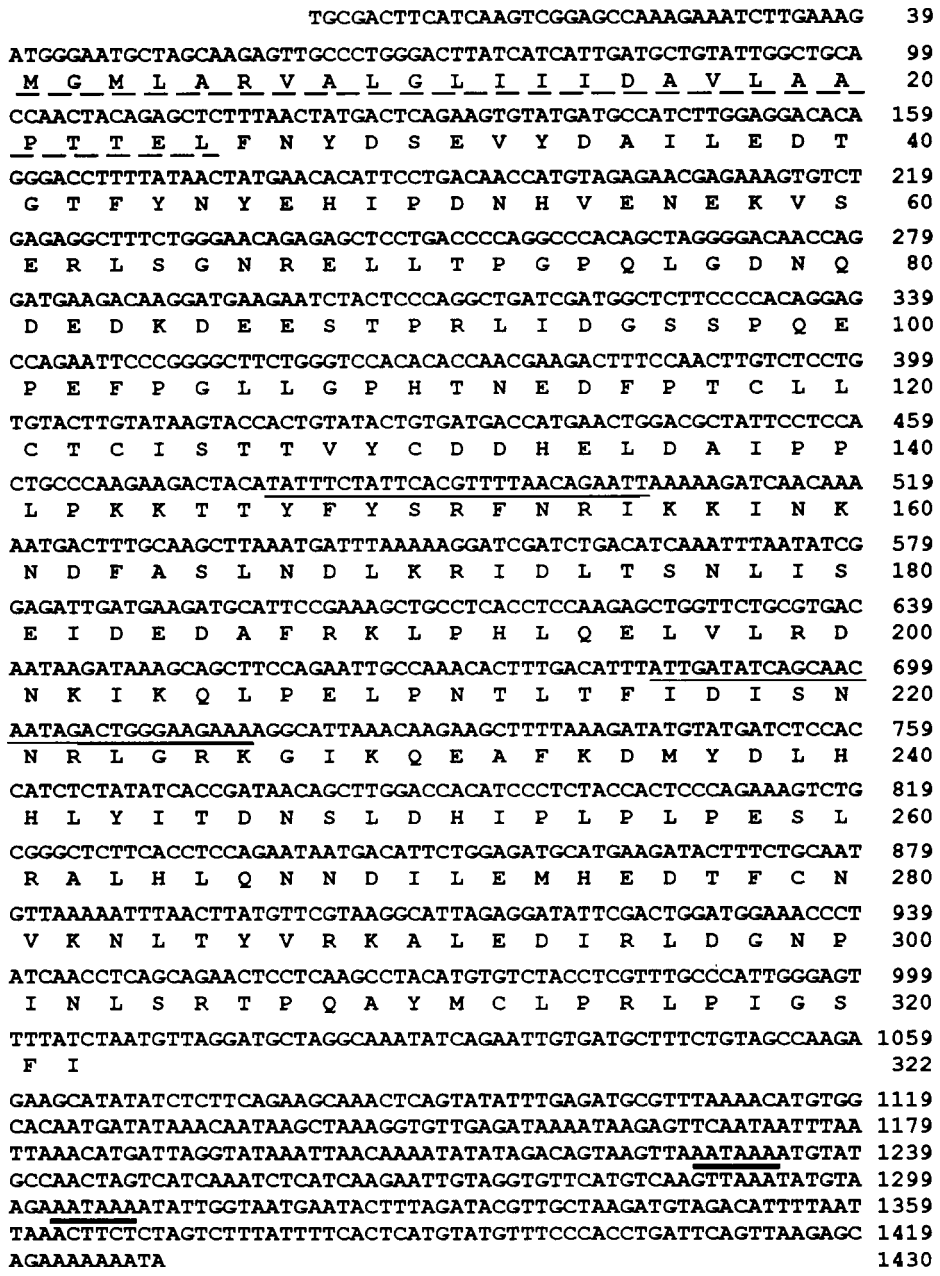

Figure 2 Nucleotide sequence of cloned cDNA for mouse PG-Lb core protein and the deduced amino acid sequence

A putative signal peptide is underlined with a broken line. Two thin underlines indicate the sequences corresponding to PCR primers. Poly(A)$^+$ signals (AATAAA sequences) are underlined with bold lines.

positive clone containing a 1.4 kb cDNA insert was determined (Figure 2). The cDNA is 1430 nucleotides long and contains a short 5′-untranslated region of 39 nucleotides, followed by a single open reading frame of 966 nucleotides which encodes 322 amino acid residues. The nucleotide sequence showed 63.2% identity with chick PG-Lb. The portions with nucleotide sequences similar to but not identical with those of the primers were found in the coding region. The 3′-untranslated region of 425 bp included two poly(A)$^+$ signals of AATAAA [14], but did not extend to a poly(A) tail. This region is unusually AT-rich and contains five TATT or ATTT(A) sequences that might contribute to the stability of the mRNA [15]. The nucleotide sequence upstream from the translational initiation site is in good agreement with Kozak's rule [16]. The second ATG codon for the methionine residue at position 3 does not fit Kozak's rule.

Analysis of deduced amino acid sequence

Computer-assisted amino acid sequence analysis revealed that the putative 322-amino acid sequence has 63.2% identity with chick PG-Lb and all six cysteine residues are completely conserved. Even higher identity (about 80%) was detected on the two-thirds of the putative core protein sequence at the C-terminal

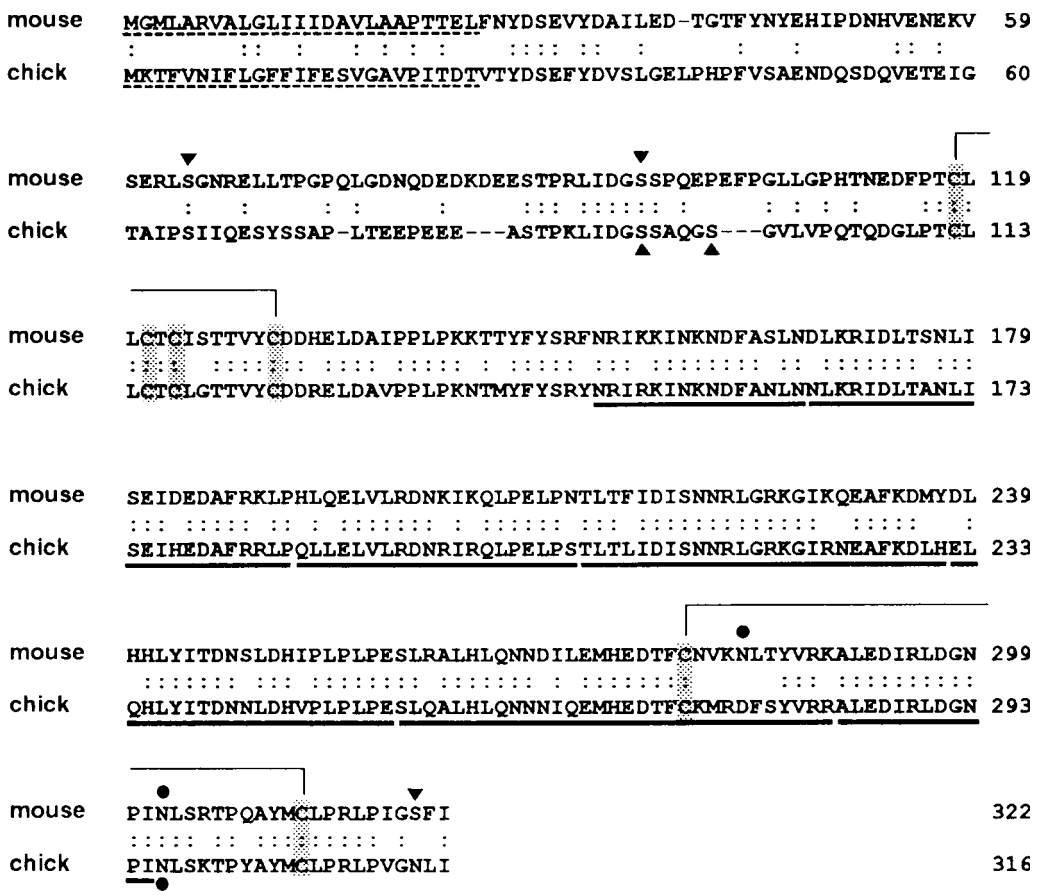

Figure 3 Comparison of the amino acid sequences of PG-Lb core proteins in mouse and chicken

Identical amino acids are marked by two dots (:). Cysteine residues are lightly shaded. The lines connecting cysteine residues indicate the expected disulphide bonds. Possible signal peptides are underlined with broken lines. Serine residues at the sites containing the minimal sequences required for dermatan/chondroitin sulphate attachment are marked by closed triangles. Possible Asn-linked glycosylation sites are marked by closed circles. Six XLXXLXLXXNXIXXIXXXXFXXLX sequences (X, any amino acid) are underlined with bold lines, although half of the first repeat unit is shifted to half of the seventh repeat unit.

side (residues 116–322) (Figure 3). The identity of mouse PG-Lb with mouse osteoglycin (42.5%) was significantly higher than its identity with other proteins but obviously lower than with chick PG-Lb. The structural features of the putative core protein are as follows. The start methionine is followed by a putative signal peptide composed of 25 amino acid residues. The amino acid sequence for a possible signal peptidase cleavage site is (−5)-Pro-Thr-Thr-Glu-Leu-Phe-(+1) and is consistent with von Heijne's rule except that the −1 residue is leucine instead of alanine, serine, glycine, cysteine, threonine or glutamine which are frequently found in eukaryotes [17]. The cleavage site was at the same position as in chick PG-Lb. There is one Ser-Gly sequence (residues 64–65) and two Gly-Ser sequences (residues 95–96 and 319–320) which are the minimal sequences required for attachment of glycosaminoglycan chains. The Gly^{95}–Ser^{96} site is conserved in chick PG-Lb (Figure 3) [18–20]. Two possible glycosylation sites for Asn-linked oligosaccharides [21] are present at residue 283 and residue 302 and Asn^{302} appears to be conserved in chick PG-Lb (Figure 3).

The putative core protein sequence also contained six LRRs in the middle region which consisted of consensus motifs with the XLXXLXLXXNXIXXIXXXXFXXLX sequence proposed by Kobe and Deisenhofer [22], although there was some divergence in the amino acid residues among the motifs. The LRRs were highly conserved in chick PG-Lb (Figure 3). Furthermore, all of the six cysteine residues detected in both the sequenced protein and chick PG-Lb were localized at the N- and C-terminal sides of the LRRs, and their relative positions are completely identical with each other (Figure 3).

The high identity of the amino acid sequence and structural features with those of chick PG-Lb suggest that this cloned cDNA encodes mouse PG-Lb.

Northern-blot analysis of RNA

Northern-blot hybridization was performed to verify the size of mRNA encoding mouse PG-Lb core protein using the cloned 1.4 kb cDNA insert as a probe. A single transcript of 1.5 kb was detected in poly(A)$^+$ RNA preparations obtained from epiphyseal cartilage of newborn mouse (Figure 4). It was somewhat longer than expected from the cDNA shown in Figure 2. Since the cDNA sequence obtained had no poly(A) tail at the 3′ end, the unidentified sequences should be at the 3′-untranslated and/or 5′-untranslated regions of the 1.5 kb transcript. In order to investigate expression in other tissues, poly(A)$^+$ RNAs from various tissues were hybridized with the cloned cDNA insert. A single transcript of 1.5 kb was detected in poly(A)$^+$ RNA from testis, but no PG-Lb message was detected in lung, kidney,

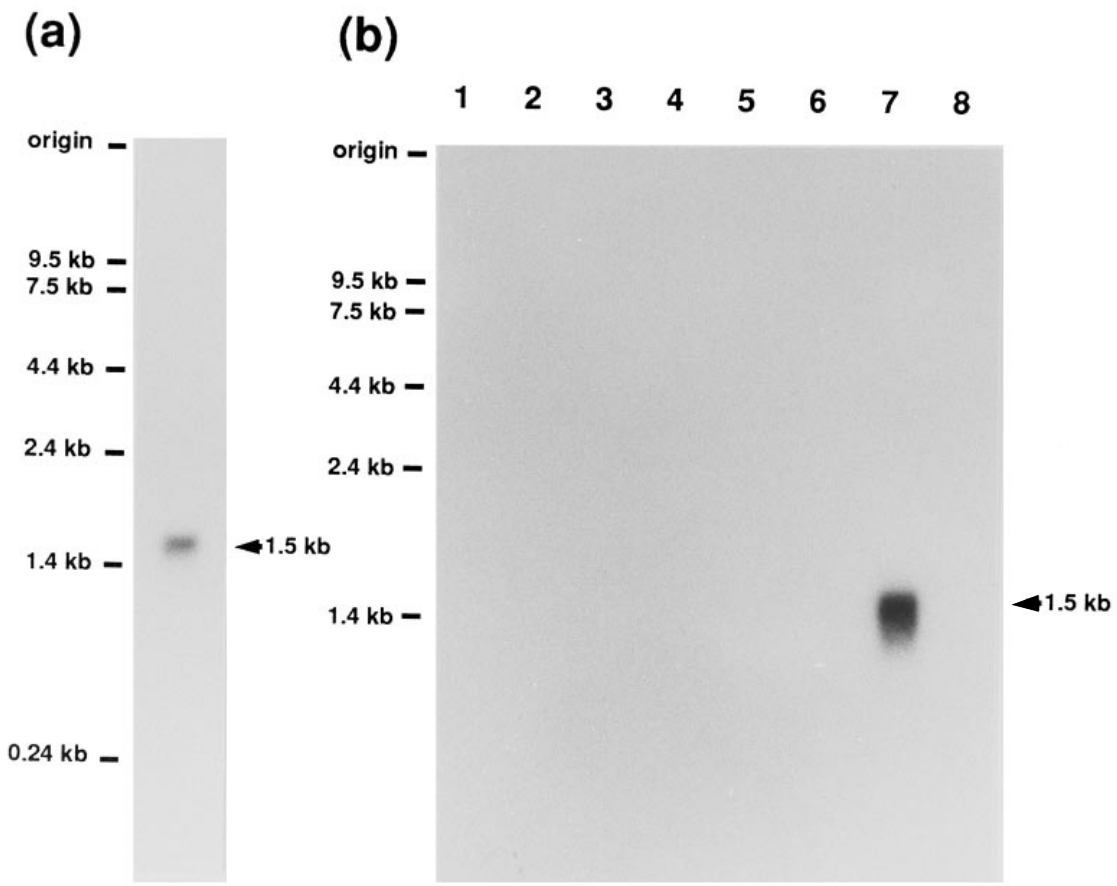

Figure 4 Northern-blot hybridization analysis of mouse PG-Lb

Poly(A)$^+$ RNA (1 μg) from newborn mouse epiphyseal cartilage (**a**) and poly(A)$^+$ RNA (1.5 μg) from lung (lane 1), kidney (lane 2), skeletal muscle (lane 3), smooth muscle (lane 4), brain (lane 5), liver (lane 6), testis (lane 7) and pancreas (lane 8) of mouse (**b**) were hybridized to a 1.4 kb cDNA fragment. Sizes of RNA species used for calibration are indicated on the left.

skeletal muscle, smooth muscle, brain, liver or pancreas (Figure 4).

DISCUSSION

We have isolated a clone from a cDNA library of newborn mouse epiphyseal cartilage which probably encodes PG-Lb. We previously isolated a cDNA for mouse osteoglycin [23] and assumed that it might be a mammalian variant of PG-Lb because of the high identity of the amino acid sequences. However, our present results strongly suggest that this cloned molecule corresponds to mouse PG-Lb.

In mouse PG-Lb, none of the three possible sites for glycosaminoglycan attachment (Ser-Gly or Gly-Ser) (Figure 3) fit the proposed consensus sequence (Ser-Gly-X-Gly) [18–20]. However, since the partial amino acid sequence neighbouring the Gly^{95}–Ser^{96} of mouse PG-Lb was completely identical with that next to the Gly^{92}–Ser^{93} of chick PG-Lb, which has been suggested to carry a dermatan/chondroitin sulphate chain [9], and, in other small dermatan/chondroitin sulphate proteoglycans such as biglycan and decorin, the attachment sites are commonly located at the N-terminal side, Ser^{96} of mouse PG-Lb may carry the glycosaminoglycan chain. Chondroitinase ABC digestion of the [^{35}S]methionine-labelled proteoglycan fraction from the epiphyseal cartilage extract of 19-day embryo mice gave a core protein which was smaller in size than that derived from decorin (K. Kurita, T. Shinomura, M. Ujita, M. Zako, D. Kida, H. Iwata and K. Kimata, unpublished work), suggesting that mouse PG-Lb exists in a proteoglycan form. However, there is no direct evidence for this at present.

The deduced amino acid sequence of mouse PG-Lb shows that this protein is an extracellular molecule that contains six LRRs, each unit of which consists of 24 residues with some divergence in sequence and length (Figure 5a). Such divergence among the repeating units is also observed in several other extracellular LRR-containing proteins [23–30]. In addition, the number of repeating units varies from molecule to molecule. Biglycan, decorin, fibromodulin, chondroadherin, lumican and PRELP contain a longer repeat region (made up of 10 or 11 repeating units) than PG-Lb (Figure 5b) [24–30]. Osteoglycin contains only six repeating units in the LRRs [23].

Recent X-ray-crystallographic structure analysis of porcine RNase inhibitor has revealed the crystal structure of LRRs consisting of 29 or 28 amino acid residues (A-type or B-type respectively) where each repeating unit is composed of a region with β-sheet and α-helix structures so that the repeats form a parallel β-sheet with one surface exposed to the solvent. In this structure, the protein acquires a non-globular horseshoe-like shape that may function as a binding motif [31,32]. Similar structures and functions have been suggested for other molecules containing LRRs [22,32]. Decorin and fibromodulin are reported to bind to collagen type I and II via their core proteins [33–35]

(a) Leucine-rich repeats of mouse PG-Lb

144–167	KTTYFYSRF**N**R**I** **KK**I**N**K**N**D**F**A**S**L**N**
168–191	**D**L**K**R**I**D**L**T**S**N**L**I **SE**I**D**E**D**A**F**R**K**L**P**
192–211	**H**L**Q**E**L**V**L**R**D**N**K**I **KQ**L**P**E**L**P**N**
212–237	**T**L**T**F**I**D**I**S**N**N**R**L**G**R**K**G**I**K**Q**E**A**F**K**D**M**Y**
238–258	**D**L**H**H**L**Y**I**T**D**N**S**L **DH**I**P**L**P**L**P**E**
259–289	**S**L**R**A**L**H**L**Q**N**N**D**I **LE**M**H**E**D**T**F**C**N**V**K**N**L**T**Y**V**R**K**
290–322	**A**L**E**D**I**R**L**D**G**N**P**I**N**L**S**R**T**P**Q**A**Y**M**C**L**P**R**L**P**I**G**S**F**I**
consensus	**-L--L-L--N-I --I----F--L-**

(b) Consensus sequences of leucine-rich repeats of related proteins

mouse PG-Lb	-L--L-L--N-I--I----F--L-
mouse osteoglycin	-L--L-L--N-I--L----F--L-
mouse biglycan	-L--L-L--N-I--I----F--L-
mouse decorin	-L--L-L--N-I--I----F--L-
bovine fibromodulin	-L--L-L--N-I--V--aa---a-
bovine lumican	-L--L-L--N-I--a----F----
bovine chondroadherin	-L--L-L--N-L--L--GAF--a-
human PRELP	NL--L-L--N-L--aP--------

Figure 5 Alignment of LRRs in mouse PG-Lb (a) and comparison with other extracellular matrix molecules containing LRRs (b)

(**a**) LRR of mouse PG-Lb is arranged according to the consensus sequence for LRRs of porcine RNase inhibitor [22]. Consensus amino acid residues are lightly shaded and the regions containing these consensus residues are in bold. (**b**) Consensus sequences of LRRs are aligned. However, since these molecules consist of repeating units that vary in length from about 20 to 25 amino acids, consensus sequences shown here are taken from typical units consisting of 24 amino acid residues. A letter 'a' in the consensus sequence represents aliphatic residues; leucine residues are sometimes replaced by isoleucine or other aliphatic residues.

and it has further been shown that binding sites for type-I collagen on decorin are mainly located in LRRs 4–5 [36]. In addition, decorin, biglycan and fibromodulin are reported to bind to transforming growth factor β via their core protein and this is also thought to be via LRRs [37].

It has been shown that biglycan and decorin have two intramolecular disulphide bonds between the first and fourth cysteine residues at the N-terminal regions and between the two cysteine residues near the C-terminus [38]. By analogy, cysteine residues in PG-Lb are likely to form similar disulphide bonds, as shown in Figure 3. These structures may be essential for the correct packing of the LRRs.

Northern-blot analysis showed that mouse PG-Lb is expressed as a single 1.5 kb message, whereas, in 12-day chick embryonic epiphyseal cartilage, two messages, 1.6 and 2.0 kb long, were detected, which might be the result of alternative splicing in the 5′-untranslated region [9]. Northern-blot analysis also showed that expression of this molecule is specific to cartilage, except for the detection of a single 1.5 kb message in testis. Further investigation of the expression of PG-Lb in this tissue is required.

Overall, it is likely that the cloned molecule corresponds to mouse PG-Lb. However, there is little information on the functions of PG-Lb at the moment. Considering the common structures and functions of LRR-containing extracellular matrix molecules, it is plausible to assume that, by binding to other extracellular molecules, PG-Lb might function by decorating their structures or regulating their activities during chondrocyte differentiation and osteogenesis.

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