The Primary Structure of a Basic Leucine-rich Repeat Protein, PRELP, Found in Connective Tissues*

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We have determined the primary structure of a connective tissue matrix protein from the nucleotide sequence of a clone isolated from a human articular chondrocyte cDNA library. The major part of the amino acid sequence has also been determined by direct protein sequencing. The translated primary sequence corresponds to 382 amino acid residues, including a 20-residue signal peptide. The molecular mass of the mature protein is 41,646 Da. The main part of the protein consists of 10 leucine-rich repeats ranging in length from 20 to 26 residues, with asparagine at position 10 (B-type). The N-terminal part is unusual in that it is basic and rich in arginine and proline. There are four potential N-linked glycosylation sites present. In three of these sites, post-translational modifications are likely to be present since Asn was not found by direct protein sequencing. The amino- and carboxyl-terminal parts contain four and two cysteine residues, respectively, probably forming disulfide bonds by analogy with the other members of this family. The protein shows highest identity (36%) to fibromodulin and 33% to bovine lumican, two other leucine-rich repeat connective tissue proteins. Northern blot analysis showed the presence of an ~3–8-kilobase mRNA in different types of bovine cartilage and cultured osteoblasts, whereas RNAs isolated from bovine kidney, skin, spleen, thymus, and trabecular bone and rat calvaria were negative. Human articular chondrocyte cultured osteoblasts, whereas RNAs isolated from bovine kilobase mRNA in different types of bovine cartilage and also in non-cartilage connective tissues. The extracellular matrix is also rich in smaller proteoglycans and other non-collagenous proteins. A few years ago, it was shown that one of the first sequenced smaller proteoglycans, decorin (1, 2), contains leucine-rich repeats (LRRs).1 Since the number of identified primary sequences for LRR proteins in connective tissues has increased. Connective tissue proteins of the extracellular matrix with LRRs so far known are biglycan (3), decorin and fibromodulin (4), lumican (5), chondroadherin (6), proteoglycan-Lb (7), and osteoinductive factor (8). Except for chondroadherin, all of them are proteoglycans with or without glycosaminoglycan chains, and several of these molecules have been shown to bind components of the extracellular matrix, e.g. collagen, bone growth factors (10), and cells (11). Intra- and extracellular LRR-containing proteins are found in almost any system studied, e.g. in mammalian and plant cells, yeast, and prokaryotes (12). The number of residues in a given LRR is between 20 and 29, and the consensus sequence derived from all known LRR proteins contains leucine or other aliphatic residues at positions 2, 5, 7, 12, 16, 21, and 24 and asparagine (B-type repeat), cysteine (A-type repeat), or threonine at position 10. Recently, the three dimensional structure of the LRR-containing porcine ribonuclease inhibitor was determined first for the protein and then for its complex with ribonuclease (13). It is likely that other LRR proteins may have a similar structure in their repeat region. The three-dimensional structure shows that each LRR is composed of a β-sheet and an α-helix. In the ribonuclease inhibitor, the β-sheets that form the consensus part of the LRR are arrayed on one face of the protein, while the less conserved helices are arrayed on the opposite face. LRR-containing proteins are known to participate in protein-protein interactions. The specificity and the diversity of the protein-protein interactions probably arise from the non-consensus residues.

The protein described here was originally purified as a prominent component of bovine articular cartilage with a molecular mass of 58 kDa (14). The amino acid composition of the 58-kDa protein was similar to that of fibromodulin, with a high content of leucine and aspartic acid/asparagine residues. The 58-kDa protein was also rich in proline. The proteins showed different ionic properties, with only fibromodulin binding to DEAE-cellulose. Studies of the distribution among tissues by radiomunoassays showed that the protein was present in many types of cartilage and also in non-cartilage connective tissues such as aorta, sclera, cornea, kidney, liver, skin, and tendon. It was not detected in bone extracts.

To further characterize the 58-kDa protein, we have determined its primary structure. This reveals that the protein belongs to the LRR family of connective tissue proteins with four potential N-linked glycosylation sites and, in contrast to those previously described, a rather basic N-terminal extension rich in arginine and proline. We therefore propose to refer to the protein as PRELP (proline arginine-rich end leucine-rich repeat protein).

MATERIALS AND METHODS

Protein Purification—PRELP was isolated from bovine articular cartilage according to the method described by Heinegård et al. (14).

*This work was supported by the Medical Faculty, Lund University, the Swedish Medical Research Council, FolkSaem Skistofse, Margaret and Axel Aescun Jonsson's Stiftelse, Greta och Johan Kock's Stiftelse, and Konung Gustaf V:s 80-Årfsfond (to D.H., Y.S., and E.B.) and by the Shriners of North America and National Institutes of Health Research Grant AR35322 (to P.J.N.). The costs of publication of this article were partly supported by the Medical Faculty, Lund University, P.O. Box 94, S-221 00 Lund, Sweden. Tel.: 46-46-222-95-87; Fax: 46-46-211-34-17; E-mail: yngve.sommarin@medkem.lu.se.
N-terminal Sequencing—N-terminal sequencing of PRELP indicated that the N terminus was blocked. Several attempts were made to remove the putative N-terminal pyrogallanic acid by digestion with pyrogallol-malononitrile (EC 3.4.19.3) using methods described by the supplier (Boehringer Mannheim). In no case were clear sequence data obtained, although there were indications that some cleavage had occurred in the N-terminal region, probably due to contamination with minor proteases. The possibility exists, therefore, that the protein is blocked in some other way, such as acylation.

Peptide isolation and Characterization—Peptides were isolated by digestion of purified protein with endoprotease Lys-C or endoprotease Glu-C (Boehringer Mannheim) at enzyme-substrate ratios of 1:25. Peptides were separated by reversed-phase high-performance liquid chromatography (HPLC) as described previously (6) with a gradient of 0–70% acetonitrile over 90 min. Peptides were sequenced on an Applied Biosystems 477A automated sequencer with on-line analysis of phenylthiohydantoin-derivatives on an Applied Biosystems 120A microbore HPLC apparatus.

Two-dimensional chromatography was performed on an endoprotease Lys-C digest by preceding the reversed-phase separation of peptides with a gel filtration step on Superdex 75 (Pharmacia Biotech Inc.) equilibrated in 4 M guanidine HCl, 25 mM phosphate, pH 6.5. Peptides were isolated from individual fractions by reversed-phase HPLC.

RNA Extraction—Chondrocytes from bovine tracheal cartilage were isolated by collagenase digestion (15). Total RNA was extracted from freshly isolated cells with guanidinium isothiocyanate essentially according to Adams et al. (16). Primary bovine osteoblasts were prepared according to Robey and Termine (17). Rat chondrosarcoma cells were provided by Dr. James Kimura (Henry Ford Hospital, Detroit, MI). Total RNAs from these cells were extracted by the same method. Various tissues from an 8-month-old rat were homogenized, and total RNA was extracted similarly. Total RNA from isolated human articular chondrocytes was provided by Dr. Michael Bayliss (Kemmy Institute of Rheumatology, London).

cDNA Synthesis and PCR—cDNA synthesis on total RNA isolated from bovine tracheal chondrocytes with both oligo(dT) and random hexamers as primers and the first PCR amplification with degenerate primers were performed essentially as described by Lee and Caskey (18). The resulting mixture was diluted 100 times and run in a second PCR with nested degenerate primers under the same conditions. The PCR was run on a 1% agarose gel and isolated with a QIAEX gel extraction kit (QIAGEN Inc.). The isolated cDNA was ligated into the pcR-Script SK (+) vector (Stratagene). The DNA sequence of the fragment was determined, and the resulting primary sequence was compared with the peptide sequences to verify that the amplified fragment represented a cDNA for PRELP.

Screening of cDNA Libraries and DNA Sequencing—The PCR fragment corresponding to bovine PRELP was used as a probe for screening of cDNA libraries. Approximately 700,000 plaque-forming combimants were prepared from a bovine articular cartilage (19) and 250,000 plaque-forming combimants were screened from a human articular cartilage ZAP-cDNA library (obtained through Dr. Michael Bayliss) (21) with several positive clones found. Approximately 250,000 plaque-forming recombinants were screened from a human articular cartilage ZAP-cDNA library with no positive clones found. The cDNA synthesis and PCR amplification was performed essentially according to Adams et al. (16). The resulting mixture was diluted 100 times and run in a second PCR with nested degenerate primers under the same conditions. The PCR was run on a 1% agarose gel and isolated with a QIAEX gel extraction kit (QIAGEN Inc.). The isolated cDNA was ligated into the pcR-Script SK (+) vector (Stratagene). The DNA sequence of the fragment was determined, and the resulting primary sequence was compared with the peptide sequences to verify that the amplified fragment represented a cDNA for PRELP.

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DNA Cloning and Sequencing—Screening of cDNA libraries from bovine tracheal and articular chondrocytes with the bovine cDNA fragment gave no positive results. Therefore, a ZAP-cDNA library from human articular chondrocytes was screened with the same probe. Three clones with 1.6-kb inserts and two containing 0.9-kb inserts were isolated. One of the longer clones was chosen for sequencing. The resulting DNA sequence and the translated primary structure are shown

Peptides from amino acid sequencing of PRELP after Lys-C digestion (Table I). Amino acids were derived from the N-terminal region of the protein (data not shown). The resulting primary structure of the fragment corresponded to peptide sequences and contained five LRRs with one potential N-linked glycosylation site (data not shown).

| Peptide | Amino acid sequence | Corresponding residues in the cloned sequence |
|---------|---------------------|---------------------------------------------|
| 58-1    | LSGVFKP9DTPQGLK     | 187–201                                    |
| 58-2    | NLQNLNLPLHNLK       | 202–215                                    |
| 58-4    | VPVIPSRHLXLMQNNIFTELPEEFK | 77–103                                  |
| 58-5    | LPSNFRHINIQHOLSDRLPK | 244–265                                    |
| 58-6    | GPEPFLYMEK          | 129–139                                    |
| 58-7    | NFLQEEVPAALPNNLEQRLSQINQSRIPPPVFKF | 140–173                                  |
| 58-8    | VPSALQYLDINRERAIQGYFK | 220–243                                  |
| 58-9    | VQDRVLEK           | 121–128                                    |
| 58-10   | LENLLDLQHNLK        | 174–186                                    |
| 58-11   | NSFVSXINLVLHLSNHRSEVFPAISX | 266–292                                |
| 58-12   | XATGLRWINILDNRR1XK  | 104–120                                    |
| 58-13   | IXPTQ1XPNIVAFHDPSXKXLEVXp | 306–331                                |

O-glycosidase (glycopeptide α-N-acetylgalactosaminidase, EC 3.2.1.17; bovine serum albumin-fucosidase, Boehringer Mannheim) in 50 mM Tris-HCl, pH 7.6, at 37°C for 3 h. The amount of protein was 10 μg in all digests. After digestion, the samples were run on an 8% SDS-polyacrylamide gel under reducing conditions using the procedure of Laemmli (22). The gel was stained with Coomassie Brilliant Blue-G 250 (23).

Northern Blots—10 μg of total RNAs from various tissues and species were run on a 1% formaldehyde-agarose gel. Northern blots were applied to a nitrocellulose filter (NitroPure, Micron Separations). The PCR-generated bovine cDNA fragment was random primer-labeled (Random Primed DNA Labeling kit, Boehringer Mannheim) with α-[32P]dCTP (Redivue™, Amersham Corp.). This probe was used for filters with bovine total RNA. A Styl fragment from the human cDNA of the 0.6-kb was used as probe for filters with rat or human total RNA. The Northern blot of human chondrocytes was washed in 0.2 SSC, 0.1% SDS at 65°C. The Northern blot with various bovine fetuses was washed in 0.1 × SSC, 0.1% SDS at 55°C. The other blots were washed in 0.2 × SSC, 0.1% SDS at 55°C prior to detection of radiolabel with the FUJI Bio Imaging BAS2000 analyzer.

RESULTS

Amino Acid Sequencing—The protein isolated from bovine articular cartilage was deaged with endoprotease Lys-C. The resulting peptides were purified by reversed-phase HPLC, and the amino acid sequences were determined (Table I). A data base search with the peptide sequences showed similarity to fibromodulin and biglycan. A number of the peptides could be aligned with similar regions of fibromodulin (data not shown). On the basis of the alignment of the peptides with fibromodulin, a set of nested degenerate PCR primers was designed (Table II).

Reverse Transcriptase-PCR—Total RNA from bovine tracheal chondrocytes was prepared and used for cDNA synthesis with both oligo(dT) and random hexamers as primers. The cDNA mixture was used for PCR amplification with degenerate primers, designed from the amino acid sequences. The resulting DNA fragment (0.39 kb) was isolated, ligated into pCR-Script, and sequenced. The translated primary structure of the fragment corresponded to peptide sequences and contained five LRRs with one potential N-linked glycosylation site (data not shown).

DNA sequence and the translated primary structure are shown
in Fig. 1 and represent 382 amino acid residues, including a 20-residue signal peptide. This gives a calculated molecular mass of 41,646 Da for the mature protein. A polyadenylation signal is not present, which indicates that it is not a full-length done and that the mRNA is further extended with noncoding sequence at the 3'-end.

Secretory Signal Sequence—A prediction of the eukaryotic secretory signal sequence by the PC/GENE program indicated a putative cleavage site between amino acid residues 20 and 21 (Fig. 1). This site is in good agreement with the predicting rules proposed by Kozak (24) and von Heijne (25).

Structure of LRRs—The translated sequence of PRELP contains 10 well conserved LRRs (Table III). These are ordered in a pattern with two longer repeats (24–26 amino acids) followed by a shorter repeat (20–21 amino acids), with some uncertainty regarding the length of the last repeat. The consensus sequences for the repeats are shown in Table III. All the LRRs are B-type, with an asparagine residue at position 10.

Glycosylation—The amino acid sequence contains four potential N-linked glycosylation sites (Fig. 1). After digestion with endoprotease Lys-C, three peptides (58-11, 58-12, and 58-13) (Table I) were found that contained putative N-glycosylation sites. All of these gave blanks on sequence analysis that were consistent with N-glycosylation sites. A peptide obtained by digestion with endoprotease Glu-C (V8) that contained a putative N-glycosylation site (HLYLNNNSI) did not have a blank cycle on the second Asn, indicating that Asn 300 is not substituted.

There is some evidence for a substituent, possibly an O-linked oligosaccharide, on Thr 252. A relatively high molecular mass peptide (10–15 kDa) could be isolated after digestion with endoprotease Lys-C. This peptide included the Thr residue and gave a blank on Edman degradation at this position. However, the background in the sequence data was high, and this peptide probably represented the blocked N-terminal peptide. A more detailed description of the N-terminal region of this protein will require further analysis.

Removal of N-linked oligosaccharides by N-glycosidase digestion resulted in a band of ~48 kDa in mobility (Fig. 2). Digestion with keratanase resulted in a small, but clearly visible shift on an 8% SDS-polyacrylamide gel, thus indicating the possible existence of keratan sulfate or polylactosamine on the protein (Fig. 2). O-Glycosidase digestion with and without prior digestion with neuraminidase resulted in no shift (data not shown).

| Primer | DNA sequence (IUPAC code) | Derived from amino acid sequence | Peptide |
|--------|---------------------------|---------------------------------|---------|
| 58-4-1 | 5'-CAR AAY AAY TTY ACN GA 3' | 5'-QNNFITE | 58-4 |
| 58-4-2 | 5'-CCN GTN GAR TTY AA | 5'-PVEEFK | 58-4 |
| 58-8-1 | 5'-TTR KCH TAD CHT CYN TAD | 5'-NRIIAE | 58-8 |
| 58-8-2 | 3'-CGN TAD GTR YAN ATR | 5'-AIHQLY | 58-8 |

**Table II**

Primer DNA sequence (IUPAC code) Derived from amino acid sequence Peptide

**Fig. 1.** The cDNA sequence and the translated amino acid sequence of PRELP. The putative cleavage site of the signal sequence is indicated ( ). Cysteine residues are encircled, and potential N-linked glycosylation sites are underlined.
**Basic Leucine-rich Repeat Connective Tissue Protein**

**Table III**

LRRs in PRELP and the consensus sequence for the repeats.

| LRR | Amino acid sequence | Residues | No. of residues |
|-----|---------------------|----------|-----------------|
| 1   | R I H Y E Q N F E T E P E S F Q N A T | 83–106   | 24              |
| 2   | G L R W N L D N N R E R K Y D Q R V L E K L P | 107–130  | 24              |
| 3   | G L V F L M E K N Q L E V P S A L P R | 131–150  | 21              |
| 4   | N E B Q L S Q N H S E K P P G V F S K L E | 151–175  | 24              |
| 5   | N L L L L L L Q H N R L S D G V F K P D T F I G L K | 176–201  | 26              |
| 6   | N L M Q L N L A H N I L R K M P R V P T | 202–222  | 21              |
| 7   | A I H Q L Y L D N K I E T E P N G Y F K S F P | 223–246  | 24              |
| 8   | N L A P L T N N K E I T D Q L P K N S F N I S | 247–271  | 25              |
| 9   | N L L V L S H N T F Y V P A T I N N | 272–290  | 20              |
| 10  | R L E H L Y E N N S I E K I G N T Q I C P N D L V | 291–317  | 26              |

Conserved residues (present in 50% or more of the repeats) are shown in boldface.

**Fig. 2.** Digestion of PRELP with keratan-sulfate 1,4-β-D-galacto-

**Fig. 3.** Dendrogram of the core regions of LRR-containing

**DISCUSSION**

The primary structure of mature human PRELP connective tissue protein represents 362 amino acid residues, which correspond to a calculated molecular mass of 41,646 Da. Laser desorption mass spectrometry indicates that the mass of the intact protein falls into a broad range of 52,000 ± 2500 Da. This difference in size compared with the protein isolated from articular cartilage is at least partly due to N-linked oligosaccharide modifications since N-glycosidase digestion of the protein resulted in an apparent molecular mass of 48 kDa. The discrepancy in size of the protein after N-glycosidase digestion compared with the translated amino acid sequence is likely to be caused by the presence of other post-translational modifications of the protein. An indication of other modifications was obtained from a Lys-C digest that showed that the threonine at position 23 could not be detected by Edman degradation, indicating the presence of an O-glycosidically linked carbohydrate moiety. Direct analysis for O-glycosidically linked oligosaccharides by enzyme digestion was, however, negative.

The main part of PRELP consists of 10 LRRs. The β-sheet forming part of the repeats is highly conserved, whereas residues from position 15 in the repeats to the end of the repeats are less well conserved. The length of the repeats ranges from 20 to 26 residues. They show a periodicity beginning with two 24–26-residue-long repeats followed by a shorter 20–21-residue-long repeat. The end of the last repeat is, however, difficult to predict. This periodicity of the last is also present in fibro-
modulin, lumican, decorin, and biglycan. In analogy with the other related proteins (6, 29), disulfide bridges are likely in the amino-terminal part between cysteine residues at positions 53 and 69 and in the carboxy-terminal part between cysteine residues at positions 312 and 353. Several other LRR-containing proteins show a similar pattern, with PRELP being most homologous to fibromodulin and lumican. Proteoglycan-Lb (7) and osteoinductive factor (8) are shorter proteins with fewer LRRs, but with the same conserved cysteine residues in the amino-terminal part and a pair of cysteines in the carboxy-terminal part. Chondroadherin diverges partly from this pattern of cysteines at its carboxy-terminal end, with four cysteines forming two disulfide bridges (6).

The post-translational modifications of the LRR-containing connective tissue proteins differ. All of them, except for chondroadherin, appear to have oligosaccharide substitutions, but with different content and in different numbers. Biglycan and decorin have two and one chondroitin/dermatan sulfate chains, respectively, close to the amino terminus (30). Fibromodulin and lumican have at least one and at the most four keratan sulfate chains at conserved positions in LRR-1, -3, -5, and -8 in both fibromodulin and lumican. In fibromodulin, the four sites are identified as hexosamine-rich, but whether they all contain keratan sulfate is uncertain. The sulfate substitutions show variations in different tissues (32). Arterial lumican appears to be unsulfated, whereas corneal lumican is highly sulfated. Fibromodulin contains sulfated tyrosine residues in the amino-terminal part (30), and lumican contains consensus sites for tyrosine sulfation. The sulfate substitutions of fibromodulin and lumican contribute acidic properties to the proteins. However, in addition, the primary sequences show low pl values. PRELP differs considerably from lumican and fibromodulin in that its basic amino-terminal region lacks consensus sites (33, 34) for tyrosine sulfation. Whether PRELP is a proteoglycan with keratan sulfate chains is not clear. Four potential N-linked glycosylation sites are present according to the consensus sequence Asn-Xaa-Ser/Thr. In three of the sites, post-translational modifications are likely to be present as amino acid sequence analysis gave blank cycles at these positions. Two of the N-linked oligosaccharide sites are situated in the same position in the LRRs as in fibromodulin and lumican (LRR-1 and -8), whereas the last substituted glycosylation site is positioned in the last repeat. The behavior on DEAE-cellulose chromatography shows that PRELP has basic properties. However, due to basic residues in the amino-terminal part, which give the protein's primary structure a higher pl than without (9.7 versus 8.3), a shorter keratan sulfate chain might be present. Keratanase digestion showed a small shift on a SDS-polyacrylamide gel, which might indicate either a keratan sulfate chain or a non-sulfated polylactosamine. Carbohydrate analysis (14) does not exclude keratan sulfate/polylactosamine substitutions. Attempts to identify keratan sulfate chains on the intact protein and on peptide fragments of the protein by the use of several monoclonal antibodies to keratan sulfate were not conclusive.

Residues 4–47 contain an arginine- and proline-rich segment followed by a proline-rich segment. Inserted between these is a short acidic segment. The proline-rich segment is reminiscent of three turns of an extended collagen-type helix and is therefore likely to form an extended structure. The arginine- and proline-rich segment is also likely to form an extended structure due to steric occlusion and/or charge repulsion of the side chains. The N terminus may, therefore, form an extended structure or loop back on itself in a hairpin, depending on whether the basic and acidic residues interact with each other.

![Image](Image1)

![Image](Image2)
The basic region of the amino-terminal part in PRELP contains two T/GRRPRP sequences. This sequence corresponds to the proposed sequence for protein-glycosaminoglycan interactions: X-B-B-X-B-X, where B denotes a basic residue (35). The consensus sequence was derived from 12 known heparin-binding sequences in vitronectin, apolipoproteins E and B-100, and platelet factor 4. It has also been suggested that the non-collagenous NC4 domain of collagen type IX, which is basic and has one consensus sequence at the N-terminal end, may interact with polyanionic glycosaminoglycan in cartilage (36). The existence of an interaction between PRELP and glycosaminoglycans, however, has to be experimentally verified.

Most of the LRR-containing proteins have been shown to participate in protein-protein interactions probably mediated through the LRR structure. Chondroadherin is cell binding (11). Fibromodulin and decorin bind to collagens I and II and affect fibril formation (9, 37, 38), and biglycan binds to collagen VI (2). Biglycan, decorin, and fibromodulin have all been shown to bind to transforming growth factor-β (10). Whether PRELP has any of these properties has yet to be determined.

In Northern blot analysis, PRELP seems to be synthesized in high amounts only in cartilage since in RNA isolated from tissues of a bovine fetus (liver, kidney, skin, spleen, and thymus), no mRNA corresponding to PRELP was detected. The detection of PRELP mRNA in cultured bovine osteoblasts may be the effect of up-regulated PRELP expression under culture conditions since the bovine trabecular bone and rat calvarial RNAs gave no positive signal. In the radioimmunonanalysis carried out by Heinegård et al. (14), no PRELP was detected in bone extracts. However, the radioimmunonanalysis indicated the presence of PRELP in bovine kidney, liver, and skin extracts. One possible explanation could be the age of the tissues. In the literature, a 55-kDa protein with similar properties to PRELP has been described (39). This protein appears to be identical to PRELP as partial peptide sequences of the 55-kDa protein are identical to PRELP at positions 138–149 and 217–228. The 55-kDa protein seems to be deficient in newborns and accumulates in cartilage with age rather than being destroyed and resynthesized by the chondrocytes.

Acknowledgments—We are grateful to Dr. Michael Bayliss for providing the human articular chondrocyte cDNA library and the total RNA from human articular chondrocytes, to Dr. Bruce Caterson and Dr. Clare Hughes (Division of Orthopedic Surgery, Chapel Hill, North Carolina) for providing different monoclonal antibodies against keratan sulfate, and to Dr. James Kimura for providing the rat chondrosarcoma cell line. We are also grateful to Ros-Mari Sandfalk for skillful technical work in the protein purification process.

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