The Amino-terminal Part of PRELP Binds to Heparin and Heparan Sulfate*

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PRELP (proline, arginine-rich end leucine-rich repeat protein) is an extracellular matrix leucine-rich repeat protein. The amino-terminal region of PRELP differs from that of other leucine-rich repeat proteins in containing a high number of proline and arginine residues. The clustered proline and basic residues are conserved in rat, bovine, and human PRELP. Although the function of PRELP is not yet known, the clustered arginine residues suggest a heparan sulfate/heparin-binding capacity. We show here that PRELP indeed binds heparin and heparan sulfate. Truncated PRELP without the amino-terminal region does not bind heparin. The dissociation constant for the interaction of PRELP with heparin was determined by an in solution binding assay and by surface plasmon resonance analysis to be in the range of 10–30 nM. A 6-mer heparin oligosaccharide was the smallest size showing binding to PRELP. The binding increased with increasing length up to an 18-mer and depended on the degree of sulfation of heparin as well as heparan sulfate. Sulfate groups at all positions were shown to be of importance for the binding. Fibroblasts bind PRELP, and this interaction is inhibited with heparin, suggesting a function for PRELP as a linker between the matrix and cell surface proteoglycans.

The leucine-rich repeat (LRR)† proteins in connective tissues are likely to function in regulating the assembly of the matrix and to provide linkages between the major structural constituents (for reviews, see Refs. 1 and 2). Several of the LRR proteins (e.g. biglycan, decorin, lumican, and fibromodulin) are known to bind to collagen and affect fibril formation (3–7).

Most of the members of the LRR proteins present in the extracellular matrix contain 10 or 11 LRRs flanked by four and two cysteine residues at the amino- and carboxyl-terminal side, respectively. Proteoglycan-Lhb/epiphycan and the osteoinductive factor are smaller proteins with fewer repeats but with the same cysteine pattern (8, 9). Chondroadherin diverges at its carboxyl terminus with two disulfide bridges formed by four cysteines (10).

All matrix LRR proteins, except chondroadherin (10), contain an N-terminal extension domain that differs considerably. This domain may therefore be important for the specific functions of the individual proteins. Decorin and biglycan carry one and two glycosaminoglycan chains, respectively, at the amino terminus (11, 12). Fibromodulin (13), lumican (14), keratocan (15), and osteoadherin (16) have consensus sequences for tyrosine sulfation, which in the case of fibromodulin (17) and osteoadherin² have been shown to be sulfated. The sulfated tyrosine residues and the glycosaminoglycan chains contribute polyanionic domains to the proteins. PRELP, however, is unique in having a basic amino-terminal domain rich in arginine and proline residues (18). The proline residues are likely to give the region an extended structure, whereas the clusters of arginine residues in the amino-terminal region may provide a heparin-binding site (19).

Heparin is a highly sulfated glycosaminoglycan produced by connective tissue mast cells and structurally closely related to heparan sulfate, which is found in most mammalian cells. Glycosaminoglycans are synthesized using a core protein as a primer. Heparin and heparan sulfate are composed of alternating uronic acid and glucosamine units, where extensive modifications occur after the initial polymer synthesis. A portion of the glucosamine units are N-deacetylated and subsequently N-sulfated, which is followed by epimerization of some of the glucuronic acid units converting them to L-iduronic acid. Following these first steps of polymer modification, additional complexity in structure is introduced by variable O-sulfation of both units. Due to the more extended modification, heparin contains more L-iduronic acid and is more extensively sulfated than heparan sulfate, whereas in heparan sulfate blocks of less sulfated or even nonsulfated sequences are interspersed by highly sulfated sequences (20). Several types of heparan sulfate proteoglycans are found on cell surfaces and in the extracellular matrix. They are known to bind, via their negatively charged glycosaminoglycan chains, a variety of proteins such as growth factors, cytokines, matrix proteins, enzymes, and enzyme inhibitors (for a review, see Ref. 21).

Based on its structure, PRELP may function as a linker molecule in the matrix, interacting with other extracellular matrix proteins through its LRRs and with glycosaminoglycans through its amino-terminal domain. Since most cells contain heparan sulfate on the cell surface, PRELP could also function as a linker between the matrix and cell surface proteoglycans.
as a link between the cells and the matrix. The territorial localization of PRELP in cartilage (22) and its cell binding capacity (23) support the latter idea. In addition to cartilage, PRELP has been found in a number of other tissues such as kidney, skin, and tendon (22).

We now show that PRELP indeed binds heparin and heparan sulfate and that this interaction is mediated through its amino-terminal region to highly sulfated sequences of heparin and heparan sulfate. We also show that fibroblasts bind to PRELP and that heparin inhibits PRELP-fibroblast interaction.

**EXPERIMENTAL PROCEDURES**

The rat chondrosarcoma cell Agt11 cDNA library (24) and the cDNA fragment of bovine PRELP (18) were described previously. Total RNA was extracted from bovine articular cartilage essentially according to the method described by Adams et al. (25). The expression vector pCEP/Pu/Ac7c was a kind gift from Dr. Ulrike Mayer (Max Planck Institute, Germany) (26, 27). Bovine PRELP was extracted from nasal cartilage with guanidine hydrochloride as described (22), and bovine albumin (fraction V) was purchased from Serva. Bovine lung heparin (a gift from Dr. The Upjohn Co.) was purified as described (28), porcine intestinal mucosa heparin was purchased from Sigma, and heparan sulfate from Bovogen. We also show that PRELP has been found in a number of other tissues such as kidney, skin, and tendon (22).

Full-length human PRELP was amplified with primers (5'-CCCAAGCTTACACCCGAAAGCAGCA-3' and 5'-ATACAGCTGCGGGGTGCGGACG-3') corresponding to nucleotides 189–203 and 1248–1297 (18) with flanking HindIII and BamHI sites and ligated into the pCEP4 vector digested likewise. To obtain a histidine tag and an enterokinase site (29), pCEP/Pu/Ac7 using oligonucleotides 112-base pair fragment digested with KpnI and PstI sites, respectively. The 112-base pair fragment was digested with KpnI and PstI and cloned into the pCEP4 vector digested likewise. Limited nitrous acid cleavage at pH 1.5, followed by radiolabeling with NaBH4. The DNA Sequencing Kit was obtained from Applied Biosystems, PerkinElmer Life Sciences. The Lasergene program package was from DNASTAR Inc. The pCEP4 vector and 293-EBNA cells were purchased from Invitrogen. Superscript II reverse transcriptase, Life Technologies, Inc., whereas soybean trypsin inhibitor was from BAB Industries. Hygromycin B was from DUCHEN, Nis-NTA Superflow was from QIAGEN, and enterokinase and EKapture agarose were from Novagen. The BIAcore 2000 System, sensor chip CM5, and ChemMatrix P20 were obtained from BIAcore AB (Uppsala, Sweden), whereas soybean trypsin inhibitor was from Pharmacia, and trypsin were all purchased from Prozyme, Boston, MA.

**Fluorescence Spectroscopy**—To evaluate recombinant protein folding, we determined fluorescence emission peak shifts upon guanidine hydrochloride denaturation, as described (35). Recombinant PRELP solutions were adjusted to 20 mM Hepes (pH 7.5), 150 mM NaCl with or without guanidine hydrochloride. In parallel, native and denatured PRELP were found to absorb equally at 285.4 nm. Two emission spectra were recorded for each native and denatured sample or buffer blank at 25 °C using a 400-µl cuvette in a Jasco FP-777 spectrofluorimeter with excitation bandwidth of 3 nm and emission bandwidth of 10 nm. The excitation wavelengths were 285 nm for Trp excitation and 285.4 nm for simultaneous Tyr and Trp excitation.

**Construction of Expression Vectors**—DNA corresponding to a part of the 5'-untranslated region, the signal peptide, and two N-terminal amino acid residues for BM-40 was amplified from the expression vector pCEP/Pu/Ac7 using primers (5'-CCGGATCCGCTCGCCGCTCTGTT-3' and 5'-ATACAGCTGCGGGGTGCGGACG-3') corresponding to nucleotides 116–133 and 1268–1285 of the human PRELP sequence.

The obtained 1.2-kb fragment was phosphorylated at the 5'-ends and cloned into the pCEP4 vector digested likewise. A cDNA fragment of bovine PRELP was amplified from the expression vector pCEP4-BM40-hisEK. Truncated human PRELP was constructed in the same way but was amplified with primers (5'-CCCAAGCTTACACCCGAAAGCAGCA-3' and 5'-CCGGATCCATCCGGTGGCGGAGG-3') corresponding to nucleotides 255–269 and 1248–1297 (18), resulting in a fragment lacking DNA sequence coding for the first 22 amino acid residues of the mature protein.
Nitrocellulose filters, 0.45-

then trapped together with the protein by vacuum filtration through

becco's modified Eagle's medium with 10% FBS and 100

regression analysis using the GraphPad Prism 3 program. The alignment of rat, bovine, and human PRELP sequences. Rat PRELP and bovine PRELP were aligned to the sequence of human PRELP (Fig. 1). The putative cleavage site of the signal sequences is indicated by a filled triangle, the conserved cysteines are indicated by C, N-glycosylation sites are indicated by vertical arrows, and the starting points of the LRRs are marked with numbers and horizontal arrows. Nonidentical residues in the sequences are boxed.

**RESULTS**

Conserved Amino Acid Sequences in Rat, Bovine, and Human PRELP—To clone the rat PRELP, we screened a cDNA library from rat chondrosarcoma cells with a bovine cDNA fragment (18), which gave several positive clones. One clone was characterized by digestion with restriction enzymes and sequencing and contained the cDNA sequence for full-length PRELP with signal peptide, stop codon, and poly(A)-tail (GenBank accession number AF163569). The orientation of the fragments was confirmed by polymerase chain reaction on the 5' and 3' ends of human PRELP (18). The translated amino acid sequences of rat and bovine PRELP were aligned to the sequence of human PRELP (Fig. 1). The sequences show a 90% identity except for the basic amino-terminal domain (residue 1–22 of the human sequence), with

then resuspended in medium containing 1 mg/ml albumin. Cell number was counted in a Bürker chamber, and the suspension was diluted to approximately 700,000 cells/ml. Polystyrene 96-well plates were coated with 10 μg/ml PRELP purified from bovine cartilage or albumin in PBS in a humid chamber at room temperature overnight. The wells were washed three times with 200 μl of PBS and blocked with 100 μl of 1 mg/ml albumin in PBS for 3 h. After washing of the wells, 100 μl of heparin (from porcine intestinal mucosa) or chondroitin 6-sulfate at concentrations 0, 0.2, 0.6, 2, 6, and 20 μg/ml in Dulbecco's modified Eagle's medium containing 1 mg/ml albumin were added and incubated for 30 min, after which 100 μl of resuspended cells were added to each well. After incubation in a cell incubator for 2 h, the nonadherent cells were removed with three washes of 200 μl of PBS. The number of adhered cells was quantified by measuring lysosomal hexosaminidase activity (38). In brief, 60 μl/well of 7.5 mM p-nitrophenyl N-acetyl-p-
glycosaminidase buffer (pH 5.0) or 25% Triton X-100 was added, and the wells were incubated for 1 h at 37 °C. The reaction was stopped by adding 100 μl/well 50 mM glycine, 5 mM EDTA (pH 10.4), and absorbance of released p-nitrophenol was read at 405 nm.

**RESULTS**

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Especially the putative signal peptide (39, 40), cysteine residues, N-glycosylation sites, and LRRs conserved. The amino terminus, however, showed only 40% identity, although the motifs rich in positively charged and proline residues are conserved in all three amino-terminal sequences.

**PRELP Binds Heparin via Its Basic Amino Terminus**—To determine whether PRELP binds heparin, as suggested by the presence of basic amino acids (18), 3H-acetylated heparin was incubated at different concentrations with bovine PRELP in solution. The protein-bound labeled heparin chains were trapped by filtration through a nitrocellulose filter (36). As shown in Fig. 2, heparin bound to PRELP in a saturable fashion, and a nonlinear regression analysis of the data showed a $K_d$ of 33 nM, indicating a strong binding capacity for heparin.

To determine whether the basic amino terminus was involved in heparin binding, we produced recombinant human PRELP with and without the amino-terminal 22 amino acids of the mature protein. For this purpose, a mammalian expression plasmid based on the pCEP4 vector was constructed containing a signal peptide, a histidine tag, and an enterokinase cleavage site (Fig. 3A). The full-length as well as a truncated human PRELP lacking the first N-terminal 22 amino acids, i.e. the proline- and arginine-rich stretch (Fig. 3B), were cloned into the plasmid at the 3′-end of the enterokinase cleavage site. The proteins were expressed in human kidney 293-EBNA cells and purified from conditioned medium by binding of the histidine-tagged proteins on Ni$^{2+}$-NTA agarose. The histidine tag was cleaved off from the proteins with enterokinase and separated from PRELP by another purification on Ni$^{2+}$-NTA beads. The purity of the proteins was confirmed by SDS-polyacrylamide gel electrophoresis showing a band of 59 and 53 kDa for the full-length and truncated form, respectively (Fig. 3C). The fluorescence emission peaks. When excited at 295 nm to measure Trp fluorescence, the emission peak was shifted from 333 to 352 nm, indicating that the Trp residue became exposed to the aqueous surrounding in the guanidine hydrochloride buffer. At 285.4 nm, where both Tyr and Trp residues are excited, a single emission peak at 330 nm was observed for the native protein, suggesting energy transfer from the Tyr residues to the Trp residue. Under denaturing conditions, two emission peaks were observed at 310 and 347 nm, representing Tyr and Trp residues in an aqueous surrounding, respectively. Taken together, these data clearly demonstrate folding of the recombinant PRELP proteins and that the complete and truncated human PRELP were similarly folded.

To characterize the heparin binding capacity of the two forms of recombinant human PRELP, their interaction with the carbohydrate chain was monitored by surface plasmon resonance. Heparin was immobilized to a dextran surface, and different concentrations of full-length and truncated human PRELP were applied at a constant flow over the surface. Full-length human PRELP showed clear binding to heparin with characteristic association and dissociation phases evident in the surface plasmon resonance curves. The truncated protein, in contrast, showed no measurable binding activity to the immobilized heparin. The bulk shift in response during protein injection is due to the different refractive index of the protein solution (Fig. 5, A and B). This absence of binding for the truncated form clearly demonstrates that the human PRELP heparin-binding domain is confined to the N-terminal sequence of the protein. The binding of the full-length protein was evaluated by a Langmuir 1:1 model for binding kinetics using local fitting. The $K_d$ varied between 15 and 24 nM for the different concentrations with a mean value of 20 nM. A global analysis using all concentrations simultaneously did not result in an acceptable fit, which could be due to the heterogeneity in heparin structure.

To compare the binding strengths to heparin of PRELP from different species, we used the surface plasmon resonance technique. Both bovine and rat PRELP bound to heparin on a dextran surface (Fig. 5, C and D) with a $K_d$ similar to human PRELP. The $K_d$ varied between 5 and 14 nM and between 1 and 17 nM for bovine and rat PRELP, respectively, and the mean

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**Fig. 2. Binding of 3H-labeled heparin to PRELP in solution.** 3H-Acetylated heparin at 0–500,000 dpm/ml, corresponding to 0–2.4 μM, was incubated with bovine PRELP (0.2 μM) and assayed with a nitrocellulose binding assay. Bound heparin was plotted against added heparin, and the $K_d$ was calculated to be 33 nM using nonlinear regression analysis ($R^2 = 0.91$).

**Fig. 3. Recombinant full-length and truncated PRELP.** A, cloning site of pCEP4-BM40-hisEK expression vector with signal peptide from BM-40 (long dashed line), histidine tag (solid line), and enterokinase cleavage site (short dashed line). Signal peptide and enterokinase cleavage sites are indicated by arrows. B, human PRELP amino-terminal heparin-binding sequence, removed in truncated PRELP. The amino acid numbers are indicated above. Positively charged amino acids are marked with a plus sign. C, recombinant full-length (F) and truncated (T) PRELP after cleavage with enterokinase and purification were analyzed on an 8% SDS-polyacrylamide gel and silver-stained.
performed as a competition assay. [3H]heparin was allowed to bind to bovine PRELP in the presence of unlabeled heparin fragments added as competitors. Also in this second case, 6-mer fragments showed some capacity to partially inhibit the binding of PRELP to [3H]heparin (Fig. 6D). This capacity was doubled with the 8-mer and gradually increased with oligosaccharide length up to 14-mer.

**Importance of Heparin Sulfation for the Interaction with PRELP**—To investigate if a specific sulfation pattern of heparin is important for binding to PRELP, we used chemically desulfated heparin (31) (Table I). Concentrations ranging from 0 to 500 µg/ml (corresponding to 0 to ~48 µM) of unlabeled desulfated heparin or native heparin were used to inhibit the interaction of bovine PRELP with [3H]-acetylated native heparin in the nitrocellulose binding assay (Fig. 7). Inhibition with native heparin resulted in a half-maximal inhibition concentration of 0.5 µg/ml or ~48 nM native heparin. This is in good agreement with the calculated dissociation constants obtained by either bovine PRELP binding to heparin in solution (33 nM), the biosensor technique (10 nM), or with human PRELP binding to heparin and measured by the biosensor technique (20 nM). Heparin either selectively 2-O-desulfated on the hexuronic acid or 6-O- or N-desulfated on the glucosamine showed a drastically reduced inhibition capacity with a half-maximal inhibition at approximately 400-fold higher concentrations than native heparin. Desulfation at two or all positions simultaneously resulted in an essentially complete elimination of binding. No significant difference in inhibition was seen between preparations with different sulfate groups removed, indicating an equal and strong importance of all sulfate groups.

**PRELP Binding to Heparan Sulfate**—To determine if PRELP is able to interact with glycosaminoglycans other than heparin, nitrocellulose binding assays with bovine PRELP were performed with heparan sulfate from bovine kidney (Fig. 8) and aorta (data not shown). The interaction with bovine kidney heparan sulfate showed a saturable binding with an approximate affinity of 1.4 µM, whereas the binding to a much less sulfated bovine aorta heparan sulfate preparation (29) was considerably weaker (data not shown), supporting the requirement for highly sulfated domains of heparan sulfate.

**PRELP Binding to Fibroblasts Is Inhibited with Heparin**—PRELP has previously been shown to bind chondrocytes (23). An initial experiment indicated that PRELP also bound rat skin fibroblasts; therefore, we tested if this interaction was dependent on heparin/heparan sulfate. Fibroblasts bound to PRELP, but not to albumin, coated on a plastic surface. The interaction was inhibited with low concentrations of heparin (0.1 µg/ml) and totally abolished with 0.3 µg/ml heparin, whereas chondroitin 6-sulfate had no effect even at a 100-fold higher concentration (10 µg/ml) (Fig. 9).

**DISCUSSION**

The LRR proteins in the extracellular matrix of connective tissues are known to interact with other proteins present either in the matrix or at the cell surface. One role therefore may be as linkers in the extracellular matrix or between the cell and the matrix. The specificity of the interactions is probably determined by the nonconserved residues between the LRRs and/or by the amino-terminal domain of these proteins. The amino-terminal domains differ markedly, dividing the proteins into different groups. PRELP is so far the only member of the family, which carries basic residues at the amino terminus. Based on sequence analysis, we therefore proposed that the protein might bind to heparin (18).

A comparison of the rat and bovine PRELP sequences with the human sequence (18) shows a high degree of conservation between these species. Compared with the overall sequence,
the amino-terminal part is less conserved, although all of the sequences contain two proline and basic amino acid clusters (Fig. 1). The human and bovine PRELP sequences contain one of the proposed heparin binding consensus sequences (XB-BXBX, where B is a basic amino acid) (19). However, many heparin-binding proteins do not follow this pattern (41). The amino-terminal region of rat PRELP does not contain the proposed consensus sequences, but clusters of basic residues are still present. As demonstrated both by an in solution assay and by surface plasmon resonance analysis, human, bovine, and rat PRELP indeed bind all to heparin, whereas truncated recombinant human PRELP without the amino-terminal region does not bind (Fig. 5). These results confine the heparin-binding domain to the N-terminal sequence. Although it cannot be excluded that the remaining parts of the protein may also participate in the interaction, their contribution is too small to be measured by the sensitive biosensor assay. The affinity of PRELP for full-length heparin is very high as determined in solution or with heparin immobilized to a sensor matrix and showed binding strength of the same order of magnitude, a $K_d$ of 33 nM for the in solution assay (Fig. 2) and 6–20 nM for the biosensor measurements (Fig. 5). However, the association curve of rat PRELP to heparin was different from that of bovine PRELP-heparin interaction. Rat PRELP showed a faster association rate and reached saturation earlier than bovine PRELP. In addition, the response of bovine PRELP to heparin in the biosensor measurement is considerably lower compared with rat PRELP at the same concentration. The differences in the association phases suggest that rat PRELP may bind to a shorter heparan sulfate/heparin sequence and as a result more easily finds heparan sulfate/heparin domains with a high sulfation degree. Indeed, the amino-terminal region of rat PRELP sequence is slightly shorter than bovine and human PRELP and differs in the pattern of positively charged residues interspersed by prolines. A comparison of the amino-terminal sequences shows that PRELPs of bovine and human origin only contain positively charged arginine residues, whereas rat PRELP has both arginines and lysines. This could also influence heparin binding, since the branched arginine residues are more bulky than the lysines and may have less accessibility to the sulfate groups in heparin.

The minimal oligosaccharide length of heparin required for efficient binding to bovine PRELP is approximately a 6-mer. The binding strength is markedly increased even upon adding only a disaccharide to an 8-mer, as estimated by inhibition concentrations needed to eliminate binding of PRELP to $[^3H]$heparin (Fig. 6). Binding changed only marginally with length over 14-mer. This correlation of inhibition capacity to increasing fragment length suggests that the protein binds to a sequence commonly found in heparin and that the chance of hitting a binding site is increasing with increasing fragment length. These results also indicate that the interaction rather is dependent on a highly sulfated domain as found in heparin and N-sulfated blocks of heparan sulfate than on a highly specific.
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TABLE I
Composition of modified heparins

| Preparationa | Sulfate groups/disaccharide unitb | Major disaccharidec |
|--------------|----------------------------------|---------------------|
| 1. Native    | 2.7                              | -4idoA/2-OSO3/1-4GlcNSO3(6-OSO3)α1- |
| 2. N-Desulfated/N-Acetylated | 1.6                              | -4idoA/2-OSO3/1-4GlcNSO3(6-OSO3)α1- |
| 3. 2-O-Desulfated | 1.9                              | -4idoA/2-OSO3/1-4GlcNSO3(6-OSO3)α1- |
| 4. Pref. 6-O-Desulfated | 1.7                              | -4idoA/2-OSO3/1-4GlcNSO3(6-OSO3)α1- |
| 5. N-Desulfated/N-Acetylated; 2-O-Desulfated | 1.1                              | -4idoA/2-OSO3/1-4GlcNSO3(6-OSO3)α1- |
| 6. N-Desulfated/N-Acetylated; 6-O-Desulfated | 0.7                              | -4idoA/2-OSO3/1-4GlcNSO3(6-OSO3)α1- |
| 7. Extensively 2-/6-O-desulfated | 1.3                              | -4idoA/2-OSO3/1-4GlcNSO3(6-OSO3)α1- |
| 8. N'-Desulfated/N-Acetylated; 2-/6-O-Desulfated | 0.3                              | -4idoA/2-OSO3/1-4GlcNSO3(6-OSO3)α1- |

a Bovine lung heparin was selectively modified as described by Spillmann et al. (31).
b Average number of sulfate groups per single disaccharide unit as calculated from compositional analysis.
c IdoA, iduronic acid; GlcN, glucosamine; 2-OSO3, 2-sulfate; 6-OSO3, 6-sulfate; NAc, N-acetyl; NSO3, N-sulfate.

FIG. 7. Competition of PRELP-native heparin interaction with partially desulfated heparin. Native 3H-acetylated heparin (0.2 μM) was incubated with bovine PRELP (0.2 μM) in the presence of nonlabeled native heparin or chemically desulfated heparin with different sulfate groups removed (0–500 μg/ml). Bound heparin was analyzed as described in the legend to Fig. 2. The various desulfated positions in heparin are indicated in the figure and are as follows: native heparin (preparation 1 in Table I, filled squares), N-desulfated/N-acetylated (preparation 2, open squares), 2-O-desulfated (preparation 3, filled circles), 6-O-desulfated (preparation 4, open triangles), N-Desulfated/N-Acetylated; 2-O-desulfated (preparation 5, open inverted triangles), N-Desulfated/N-Acetylated; 6-O-desulfated (preparation 6, filled diamonds), 2-/6-O-desulfated (preparation 7, filled triangles), N-Desulfated/N-Acetylated; 2-/6-O-desulfated (preparation 8, open diamonds).

FIG. 8. Binding of 3H-labeled heparan sulfate from bovine kidney to PRELP in solution. 3H-Acetylated heparan sulfate at 0–100,000 dpm/ml, corresponding to 6–33 μg/ml, was assayed with bovine PRELP (0.2 μM) as described in the legend to Fig. 2. Bound heparan sulfate (HS) was plotted against added heparan sulfate, and the Kd value was calculated to be 1.4 μM using nonlinear regression analysis (R² = 0.98).

sulfation pattern, contrasting to that seen with e.g. antithrombin-3. The antithrombin-3 interaction with heparin requires a specific pentasaccharide, and binding is not highly increased with longer saccharides (36) due to the fact that the specific antithrombin sequence is occurring only in an average of one per three chains of commercial heparin (42). The importance of the degree of sulfation is also clearly seen in studies of inhibition of the PRELP-heparin interaction with modified heparin desulfated at different positions. Whereas native heparin gave a half-maximum inhibition value at the concentration 0.5 μg/ml, corresponding to ~48 nM, heparin preparations desulfated at either position 2-O, 6-O, or N were approximately 400-fold less efficient inhibitors (IC50 = 200 μg/ml). No difference was seen in regard to which position was desulfated. Likewise, heparin desulfated at two positions in different combinations showed almost no binding at a 1000-fold higher concentration (Fig. 7). The half-maximum value of inhibition with heparin, 48 nM, is in good agreement with Kd values calculated in the other analytical systems (10–30 nM). These properties resemble the heparin binding characteristics of other proteins, which are less sequence-dependent than antithrombin-3 but predominantly electrostatic as platelet factor 4, interleukin-8, or fibronectin (31, 36).

Some proteins, e.g. platelet factor 4 and interleukin 8, are known to bind with a high affinity to heparin/heparan sulfate as oligomers, whereas the monomeric forms have a much lower affinity (31, 43, 44). In this context, it is interesting that PRELPs affinity to heparin fragments is much lower compared with native heparin. The IC50 value for competition with native heparin is 48 nM, whereas with 12-mer heparin we see a 44% inhibition with 14 μM (50 μg/ml). The difference in affinity for the full-length heparin chain versus short fragments may indicate a polymerization of the protein along the native chains, whereas the short fragments do not provide this possibility.

Presumably, PRELP is not interacting with heparin in the
tissue but rather with heparan sulfate found in the same locations as the protein. Heparan sulfate is present on the cell surface of most cells either on transmembrane proteoglycans, e.g. syndecans, or on glycosylphosphatidylinositol-anchored proteoglycans such as glypicans (45). In basement membranes, heparan sulfate is present linked to the core protein of perlecan and other proteoglycans (46). When tested for binding to different glycosaminoglycans, bovine kidney heparan sulfate shows a saturable binding to PRELP, although at a lower affinity than measured for heparin. Even weaker than the kidney heparan sulfate was aorta heparan sulfate, which is found to contain a low degree of modification by sulfation (29). In conclusion, all of these findings indicate a predominantly electrostatic binding of PRELP to highly sulfated domains in heparan sulfate or heparin, possibly enhanced by multimerization of the protein on the carbohydrate chain.

It has previously been shown that PRELP is found in the territorial region in cartilage (22) and mediates cell attachment to chondrocytes (23) in vitro. Here we show for the first time that PRELP interacts with skin fibroblasts and that this interaction is most likely mediated by cell surface heparan sulfate proteoglycans, since the interaction was inhibited with heparin at a low concentration but not with chondroitin 6-sulfate. A proteoglycan, such as glypicans (45). In basement membranes, such as skin, cornea, kidney, and tendon (22). Therefore, it is likely that PRELP may function as a link between the matrix and the cell not only in cartilage but also in other connective tissues.

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