The Leucine-rich Repeat Protein PRELP Binds Perlecan and Collagens and May Function as a Basement Membrane Anchor*

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PRELP (proline arginine-rich end leucine-rich repeat protein) is a heparin-binding leucine-rich repeat protein in connective tissue extracellular matrix. In search of natural ligands and biological functions of this molecule, we found that PRELP binds the basement membrane heparan sulfate proteoglycan perlecan. Also, recombinant perlecan domains I and V carrying heparan sulfate bound PRELP, whereas other domains without glycosaminoglycan substitution did not. Heparin, but not chondroitin sulfate, inhibited the interactions. Glycosaminoglycan-free recombinant perlecan domain V and mutated domain I did not bind PRELP. The dissociation constants of the PRELP-perlecan interactions were in the range of 3–18 nM as determined by surface plasmon resonance. As expected, truncated PRELP, without the heparin-binding domain, did not bind perlecan. Confocal immunohistochemistry showed that PRELP outlines basement membranes with a location adjacent to perlecan. We also found that PRELP binds collagen type I and type II through its leucine-rich repeat domain. Electron microscopy visualized a complex with PRELP binding simultaneously to the triple helical region of procollagen I and the heparan sulfate chains of perlecan. Based on the location of PRELP and its interaction with perlecan heparan sulfate chains and collagen, we propose a function of PRELP as a molecule anchoring basement membranes to the underlying connective tissue.

Connective tissue is ubiquitous in the body of vertebrates. The relative proportion of connective tissue varies between organ systems, constituting the entire structure in cartilage or contributing a minor element in muscle tissue. The common denominator is a predominant extracellular matrix. This is composed of collagens, non-collagenous glycoproteins, and proteoglycans, the latter of which are proteins carrying glycosaminoglycan (GAG)1 substituents. Among the constituents, leucine-rich repeat (LRR) proteins constitute a family of small proteoglycans and proteins. They contain a central, main portion of 10–11 LRRs flanked by disulfide-linked loops on both sides. Several of the LRR proteins have been shown to bind to collagen through this domain. In contrast to the fairly uniform LRR regions, the amino-terminal domains are quite variable. Most of these proteins have an amino-terminal region with acidic properties. In decorin and biglycan, there are one and two chondroitin/dermatan sulfate chains, respectively. In fibromodulin, osteoadherin, lumican, and keratocan, sulfated tyrosine residues contribute the anionic properties (Ref. 1 and the references therein). In asporin, an extended stretch of aspartic acid residues provides a cluster of negative charge (2). PRELP is the only member of this family with a basic amino-terminal region that is rich in proline and has clusters of arginine residues (3). This domain has been shown to bind heparin and heparan sulfate (HS) (4). HS chains are very variable in fine structure. They are components of a set of proteoglycans, where syndecans and glypicans occur at the cell surface, and perlecan is found in the extracellular matrix. It is not clear whether PRELP will bind HS chains on all these proteoglycans. LRRs are believed to facilitate protein-protein interactions (5), but no ligands for this part of the PRELP molecule are known. However, in view of the described interactions of several other proteins within the family, collagen is a likely candidate (reviewed in Ref. 1).

PRELP was originally purified as a component of bovine articular cartilage with a molecular mass of 58 kDa. It is predominantly found in the territorial matrix (6). However, radioimmunoassays demonstrated its presence also in kidney, aorta, sclera, liver, skeletal muscle, cornea, skin, and tendon (6). Preliminary experiments indicate that PRELP is present in or close to several basement membranes. This structure is found as a thin sheet of extracellular matrix separating epithelial cells from the underlying connective tissue. It functions as a barrier for molecules and cells (Ref. 7 and the references therein). Moreover, basement membranes provide an anchor to cells modulating their phenotypes. Structurally, basement membranes are built up by networks of collagen type IV and laminin, respectively, connected to one another via nidogen (reviewed in Ref. 8). There are a number of other proteins that contribute to the complex structure of basement membranes. One of the most abundant is the proteoglycan perlecan. This molecule consists of five domains with three GAG chains attached to domain I. In some cases, additional chains are found at domain V (9–11). The perlecan core protein is known to interact with the extracellular matrix proteins nidogen (12, 13), fibulin-2 (14), and fibronectin (9, 15). It also binds platelet-derived growth factor (16), the cell surface molecule α-dystroglycan (17), and the fibroblast growth factor-binding protein...
PRELP Links Perlecan and Fibrillar Collagens

(18). The perlecan HS chains have been shown to bind fibroblast growth factor 2 (19, 20), collagen type IV, fibronectin, and laminin-1 (12, 21).

The discovery of PRELP close to basement membranes prompted us to search for potential interaction partners. We show here that PRELP binds to the HS chains of perlecan and also, to some extent, to other basement membrane proteins. In addition, PRELP is shown to interact with triple helical collagens type I and II. By binding to perlecan HS chains in the basement membrane via its amino-terminal part and to collagens in the connective tissue via its LRR domain, PRELP is a likely candidate as one of the anchoring molecules at basement membrane-connective tissue junctions.

EXPERIMENTAL PROCEDURES

Sources of Proteins and GAGs—Perlecan and complexes of laminin-nidogen were purified from mouse Engelbreth-Holm-Swarm tumor (22, 23). Fibronectin from human plasma (a kind gift from Behringwerke AG, Marburg, Lahn, Germany) was further purified by heparin-Sepharose chromatography. Mouse nidogen-1 (24), fibulin-1C (25), fibulin-2 (26), and human BM-40 (27) were prepared in recombinant form using human embryonic kidney 293 cells. Recombinant mouse perlecan domain II was produced as a mixture of HS and chondroitin sulfate (CS) (28), or as a mutated form without GAGs (21), domain II (29), domain III (1-1, 3-2, and III-3 (30, 31), domain IV-2 (32), and domain V, with and without GAG chains (9), was produced in 293-EBNA or 293Cs cells. Bovine PRELP was extracted from nasal cartilage under dissociative conditions (6). Recombinant human PRELP, both the full-length form and the truncated form without its amino-terminal basic region, was expressed in 293-EBNA cells (4). Bovine tracheal CSA (containing ~70% 4-O-sulfate and ~30% 6-O-sulfate, according to the manufacturer) and heparin were from Sigma and Hoffmann-La Roche, respectively. Pepsin-solubilized bovine dermal collagen type I (Vitrogen 100) was from Collagen Research Laboratories, Inc. (Denver, CO). The Texas Red dye-conjugated anti-rabbit IgG and fluorescein isothiocyanate-conjugated anti-rat IgG were visualized with a Bio-Rad 1024 confocal laser scanning microscope. The images were obtained using pseudocolored imaging with Adobe Photoshop 6.0 without any other modifications, and the final figure was assembled using Macromedia FreeHand 8.0. Control experiments included secondary antibodies alone, as well as inhibition of anti-PRELP antibody binding with some 10 μg/ml bovine protein.

Electron Microscopy—Glycerol spraying/rotary shadowing, negative staining, and evaluation of the data from electron micrographs was carried out as described previously (35). For negative staining, 5-μl samples of different complexes between procollagen, PRELP, and perlecan (typical concentrations of about 5 μg/ml in TBS) were adsorbed onto 400 mesh carbon-coated copper grids, washed briefly with water, and stained on two drops of freshly prepared 0.75% uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. In some experiments, the PRELP was labeled with 5 nm colloidal gold (36). For rotary shadowing, 30 μl of perlecan or PRELP/perlecan samples (typical concentrations of about 20 μg/ml) were dialyzed overnight at 4 °C against 0.2 M ammonium hydrogen carbonate, pH 7.9. They were mixed with equal volumes of 80% glycerol and sprayed onto freshly cleaved mica discs with a nebulizer designed for small volumes. They were dried in a high vacuum for 2 h and shadowed under rotation with 2 nm-platinum/carbon at a 9° angle, followed by coating with a stabilizing 10 nm carbon film. Specimens were observed in a Jeol 1200 EX transmission electron microscope operated at 60 kV accelerating voltage. Images were recorded on Kodak SO-163 plates without preirradiation at a dose of typically 2000 electrons/nm². The negatives were scanned, cropped, and assembled into the final figure using Adobe Photoshop 6.0.

RESULTS

PRELP Interacts with Basement Membrane Components—Because preliminary results indicated that PRELP was located in or close to several basement membranes, e.g. in skin, testes, and Bowman’s capsule of the kidney, we tested various basement membrane proteins for binding to the protein. In a microtiter solid phase assay, perlecan clearly bound bovine PRELP (Fig. 1). Other basement membrane proteins such as collagen, fibulin-2, and BM-40 also interacted with PRELP, but with lower affinities compared with perlecan. Fibronectin and fibulin-1C showed little or no binding (Fig. 1).

To determine the binding constants, bovine PRELP was immobilized to carboxyl groups on a BIAcore sensor chip, and the binding was analyzed by surface plasmon resonance (Table I). As expected from the microtiter assay, PRELP interaction with perlecan was the strongest, with a steady-state dissociation constant (Keq) of ~3 nM. Binding of the other basement membrane components nidogen-1, fibulin-2, and BM-40 to PRELP showed Keq values of ~60–180 nM.

PRELP Interacts with the HS Chains of Perlecan—The domains of perlecan that bind PRELP were identified using recombinant fragments of perlecan (Fig. 2). Together, these cover...
the full length of the molecule, and all were examined, except for domain IV-1. GAG-substituted perlecan domain I and domain V both bound PRELP in solid phase assay (Fig. 3), whereas fragments containing other perlecan domains (II, III-1, III-2, III-3, and IV-2; data not shown) did not bind PRELP. To elucidate whether the interaction was mediated via the core protein or the GAG side chains, perlecan domain I with and without GAGs was tested. Perlecan domain I with HS chains interacted with PRELP to a much higher extent than a preparation of the same domain substituted with both HS and CS chains. A mutated form of perlecan domain I containing no GAG chains did not bind PRELP (Fig. 3B). Similarly, perlecan domain V with GAG chains present bound to PRELP, whereas perlecan domain V lacking GAGs did not bind to PRELP (Fig. 3C).

The interactions of the different fragments were also analyzed by surface plasmon resonance, confirming the result of the solid phase assay. Perlecan fragments IA, IB, and Vc with GAG chains all bound to bovine PRELP, whereas mutated perlecan domain I and perlecan domain V without GAGs did not interact with PRELP. The dissociation constants of PRELP-perlecan IA (18 nM) and PRELP-Vc (4 nM) were similar to the $K_D$ calculated for the interaction between PRELP and full-length perlecan (3 nM). Attempts to calculate the dissociation constant for the interaction of PRELP with perlecan domain I with both HS and CS chains did not result in an acceptable curve fit, but binding was observed.

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To further show that PRELP interacts with HS chains of
perlecan domain I and V, we performed competition experiments with isolated heparin and CS chains. Indeed, low concentrations of heparin (0.1–0.01 μg/ml) efficiently inhibited the interactions, whereas addition of CS only resulted in a small reduction in binding, even at a high concentration (100 μg/ml) (Fig. 4). Also, the PRELP binding of perlecan domain I carrying a mix of HS and CS chains was inhibited by heparin, whereas the addition of CS had a very limited effect (1000–10,000-fold difference). Because fraction IB contains both HS and CS, binding probably reflects PRELP interaction with HS or possibly reflects PRELP interaction with a highly sulfated form of CS.

**The Amino-terminal Part of PRELP Interacts with Perlecan GAG Chains**—The amino-terminal part of PRELP is known to interact with heparin (4). In view of the shared structures between heparin and heparan sulfate, it is reasonable to assume that PRELP can bind to perlecan via its amino-terminal domain. To confirm this, full-length PRELP and truncated human PRELP (lacking the amino-terminal domain) were immobilized to a carboxymethylated chip, and interactions with perlecan were determined by surface plasmon resonance. As expected, full-length PRELP bound perlecan domain V substituted with GAG chains and perlecan domain I with HS chains, whereas truncated PRELP did not do so (Fig. 5).

**PRELP Is Located Adjacent to Basement Membranes in Connective Tissue**—Confocal immunohistochemistry was used to determine the localization of PRELP at the basement membrane. Human skin and bovine testis and kidney were double-stained with antibodies to PRELP and perlecan domain IV, respectively. Bound antibodies were detected with Texas Red- or fluorescein isothiocyanate-conjugated secondary antibodies. In kidney, PRELP was present mainly around Bowman’s capsule, whereas the perlecan antibody stained Bowman’s capsule as well as glomeruli and tubuli. However, the PRELP staining in Bowman’s capsule did not overlap with but was adjacent to the perlecan staining (Fig. 6A). In testis, perlecan was detected in the basement membranes surrounding the seminiferous tubules and blood vessels. PRELP was localized adjacent to the basement membrane in the seminiferous tubules of testis (Fig. 6B), which was evident at a higher magnification (Fig. 6C). A similar pattern was seen in human skin, where PRELP was detected as a gradient in the dermal tissue, with the most intense staining just underneath the basement membrane (Fig. 6D). The faint staining of the epidermis in skin with PRELP antibodies was also seen in control sections incubated with only the secondary antibodies (data not shown). The specificity of the anti-PRELP antibodies was confirmed by inhibition with added PRELP protein (data not shown).

**PRELP Binds to Collagens**—PRELP is closely related to fibromodulin and several other LRR proteins known to bind to collagen (37). Therefore, it is possible that PRELP also interacts with collagen. Indeed, surface plasmon resonance assays showed that PRELP binds to collagen. PRELP extracted from bovine cartilage and recombinant human full-length PRELP and truncated PRELP (i.e. without the HS binding amino-terminal) were all immobilized to carboxyl groups on the BIAcore surface chip. Interaction was studied by injection of collagen type I or II over the surface (Fig. 7). All three forms of PRELP, including truncated PRELP, were shown to interact with both types of collagen. It is apparent that binding is mediated by the central domain, probably the LRR region, rather than through the amino-terminal part. In addition, the binding curves of collagen to full-length and truncated PRELP, respectively, had a similar appearance, suggesting that the interactions have similar affinities. Because both full-length PRELP and truncated PRELP bind to collagen, it was also...
confirmed that recombinant truncated PRELP is active and thus apparently correctly folded. Comparison of the binding curves of type I and II collagens disclosed higher association rates and lower dissociation rates for type I collagen, showing a higher affinity for this collagen. Attempts to calculate the affinities of the interactions using different models did not result in acceptable curve fits. This could be due to the presence of several binding sites for PRELP on the collagen molecule (see below) or possibly to the self-interaction of collagen, once bound to the PRELP surface.

Electron Microscopy of Combinations of PRELP, Perlecan, and Procollagen—To visualize the complexes between PRELP, perlecan, and procollagen I, we performed electron microscopy after glycerol spraying/rotary shadowing and negative staining. The carboxyl-terminal globular propeptide of procollagen was used to indicate the polarity of the collagen helix. In negative staining electron microscopy of complexes, PRELP was identified at two distinct sites at the triple helical region of the procollagen molecule. Bound PRELP molecules were located either 33 ± 8 nm from the carboxyl-terminal end or 98 ± 9 nm from the amino-terminal end of the 300 ± 24-nm-long procollagen molecule (Fig. 8D). Perlecan, on the other hand, bound to the ends of the procollagen molecule, to both the amino-terminal (Fig. 8B) and carboxyl-terminal domains (Fig. 8A). Occasionally, procollagen molecules were observed with perlecan bound to both the amino- and carboxyl-terminal domains (Fig. 8C).

As shown by rotary shadowing of complexes, PRELP interacts with the HS chains of perlecan of both domains I and V. Several PRELP molecules were able to bind to different positions on either different HS chains or the same HS chain (Fig. 8E).

The ternary complex between PRELP, perlecan, and procollagen I was reconstituted in vitro. Electron microscopy after rotary shadowing revealed that the HS chains of perlecan were occasionally found to extend from the amino- or carboxyl-terminal domain of procollagen, to which perlecan was bound. These chains looped back to the position on the triple helix to which PRELP was bound. Such arrangements were predominantly found in the middle of sprayed drops, with presumably high local salt concentration. Under these conditions, it was possible to directly visualize the ternary complex between PRELP, the perlecan HS chains, and procollagen I (Fig. 8, F and G).

**DISCUSSION**

In the present study, we demonstrated that PRELP bound the HS chains of perlecan. The interaction was mediated through the basic amino-terminal region of PRELP because full-length PRELP, but not truncated PRELP lacking the amino-terminal domain, bound perlecan. We also showed that both full-length PRELP and truncated PRELP bound collagen type I and II, an interaction apparently involving the LRR-containing domain. Because the collagen binding ability was retained, truncation of PRELP had not disturbed the protein conformation. Further evidence for correct folding was obtained by flu-
orescence spectroscopy, where full-length and truncated PRELP yielded identical spectra (4).

Whether PRELP binding requires a specific perlecan HS sequence motif remains unknown. This study was performed with perlecan from the EHS basement membrane tumor, which has a 50% ratio of N-sulfate groups/total N-substituents, and these sulfate groups comprised 80% of the total sulfation (38). This suggests that the PRELP interaction with perlecan does not require highly sulfated HS chains. Contrary to this, we found in a previous study that a high degree of sulfation is important for a PRELP-heparin interaction (4). This seemingly conflicting result could have several explanations. The perlecan HS chains, although showing a low total sulfation pattern, may contain highly sulfated regions, albeit with a sparse occurrence. In addition, the PRELP-heparin study does not exclude that PRELP binds with a high affinity to a specific sulfated heparin/HS sequence, which does not require a high overall degree of sulfation in the GAG chains. It may well be that the EHS perlecan HS chains differ from those in basement membranes in kidney and skin. In fact, perlecan from Reichert’s membrane, an extraembryonic basement membrane, showed a different sulfation pattern than EHS perlecan, with a higher proportion of O-sulfation and antithrombin binding sequences (38). Although no publications thus far have been presented describing the HS chain structure of perlecan from basement membranes of skin, testis, or kidney, we have shown in a previous study that PRELP is able to interact with HS from bovine kidney (4), thus indicating that PRELP can interact with the HS chains of perlecan extracted from the EHS tumor.

Originally, PRELP was purified as a component of cartilage, but the protein is also present in other connective tissues, e.g. in kidney and skin, as demonstrated by radioimmunoassays (6). Here we present data, using confocal immunohistochemistry, showing that PRELP outlined the basement membranes in skin and in the seminiferous tubules in testis. The protein was also present adjacent to perlecan in Bowman’s capsule in the kidney. Perlecan, however, also was present in other kidney structures, i.e. the tubules and glomeruli. Interestingly, Bowman’s capsule in rat contains HS chains with a different structure than those of tubuli or glomeruli, as shown by immunostaining with anti-HS antibodies (39). It is therefore likely that the HS composition of perlecan varies in the different parts of kidney and that PRELP may react only with a subpopulation. In addition, PRELP may interact with HS chains of other basement membrane proteoglycans such as agrin and collagen XVIII. Agrin is the major HS proteoglycan in neuromuscular junctions but is also present in Bowman’s capsule (40–42). Collagen XVIII is found in the basement membrane in a number of tissues (43).

The biological role of PRELP is not yet known. However, based on its location in connective tissue and its interaction with the HS chains of perlecan, PRELP may function as a bridging molecule, anchoring the basement membranes to the underlying connective tissue. Perlecan is present in the basement membrane, where it binds to the major structural components laminin and collagen type IV via its HS chains (12). The core protein of perlecan interacts with nidogen-1, which connects the networks of laminin and collagen type IV (12). PRELP, on the other hand, binds perlecan via its heparan sulfate chains and mediates binding to collagen I, which is present in most connective tissues. This collagen interaction does not involve the amino terminus of PRELP because binding
PRELP Links Perlecan and Fibrillar Collagens

The finding of PRELP and perlecan immunostaining next to each other at the basement membrane/stromal junction suggests a direct interaction. However, one can argue that such an interaction should result in overlapping immunofluorescence stainings. One possible explanation for the lack of overlap is that perlecan is visualized using an antibody recognizing the core protein, whereas PRELP binds to the HS chains of perlecan, extending out into the matrix. The HS chains in various basement membranes are likely to be of varying length, and the HS chains of perlecan extracted from EHS tumor are about 100-nm long (23). In addition, the fixation of the tissue could lead to disruption of the basement membrane from the connective tissue, creating a broader gap than normally present. However, PRELP's appearance adjacent to basement membranes, which are known to contain a high amount of HS proteoglycans, points to a possible in vivo interaction between PRELP and perlecan and/or other HS proteoglycans in basement membranes such as agrin and collagen XVIII.

The broad distribution of PRELP in skin indicates that the protein has other functions in the matrix in addition to binding perlecan. Considering its interaction with collagen type I, PRELP could contribute to the intricate structure of the extracellular matrix either by holding the structure together or by regulating the formation of collagen fibers. In addition, PRELP is known to bind fibroblasts through cell surface HS proteoglycans (4). A third function of PRELP in connective tissue could therefore be as a molecule anchoring cells to the surrounding matrix via collagen.

PRELP is also present in the territorial matrix of cartilage (6). As demonstrated above, PRELP interacted with collagen type II (Fig. 7), the major collagen in cartilage. However, other collagens, e.g. type IX, X, and XI, are known to be present in cartilage and may also be interacting with PRELP. Cartilage has a matrix rich in glycosaminoglycans, primarily chondroitin/dermatan, keratan sulfate, and hyaluronic acid, present in the aggrecan-hyaluronan network. The sulfated GAGs are also present on the small LRR proteoglycans in cartilage, whereas HS is usually considered as a GAG found on cell surfaces and in basement membranes. Interestingly, perlecan has been found in the extracellular matrix of nasal cartilage, the articular cartilage, and the growth plate. The core protein of the cartilage perlecan variant was shown to be substituted with both HS and CS chains (47). Indeed, mice lacking perlecan develop a severe cartilage phenotype (chondrodysplasia), characterized by a reduced collagen network and shorter collagen fibrils, implying an important role for perlecan in the cartilage structure (48, 49). Human perlecan deficiencies have been found in chondrodysplasia, e.g. dyssegmental dysplasia of the Silverman-Handmaker type (50) or Schwartz-Jampel syndrome (51). Perlecan is found by immunohistochemistry in the territorial matrix with highest intensity in the pericellular region in rat chondrosarcoma and nasal cartilage (47). A function of PRELP as a linker between the matrix close to the chondrocyte and the more distant matrix via perlecan is indicated by the capacity of the protein to simultaneously bind collagen and perlecan. In further support of this argument, PRELP is localized in the territorial matrix, and perlecan is localized closer to the chondrocytes in cartilage. Alternatively, PRELP may have the capacity to interact directly with cell surface HS chains on syndecans.

In this study we also observed interactions of PRELP with BM-40, nidogen, and fibulin-2, although the affinity of these interactions are 20–60 times weaker than that of perlecan-PRELP. Overall, the present data provide support for the hypothesis that PRELP represents an anchoring molecule of basement membranes and in connective tissues. The domain of

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**FIG. 8. Electron microscopy of the complex between procollagen type I, PRELP, and perlecan after negative staining (A–D) or rotary shadowing (E–G).** Complexes between procollagen I and perlecan (A–C) or PRELP (D) were visualized by negative staining. Perlecan (P) bound to the amino-terminal (A) or carboxy-terminal (B) domain on the collagen or to both ends (C). In B, but not in A, the globular propeptide is visible at the carboxy-terminal on procollagen I. D, PRELP is found at two distinct binding sites on the procollagen triple helix (arrows). The interaction of PRELP with the procollagen occasionally leads to the formation of lasso-like structures (D, last picture). Complexes between perlecan and PRELP (E) or all three components (F and G) were visualized by glycerol spraying/rotary shadowing. PRELP interacts with the perlecan HS chains at different sites (E, arrow). The ternary complex consists of perlecan (P) bound to the procollagen amino- or carboxy-terminal, and the perlecan HS chains localized close to the collagen triple helix (asterisk), sometimes detaching (arrowheads) but specifically looping back to gold-labeled PRELP molecules (arrow), which are bound to the triple helix. The letter indicating the panels belonging to a particular section is shown only on the first picture. Scale bar, 100 nm (A–F) and 50 nm (G).
PRELP involved in the weaker interactions has not been identified, and it is not clear whether they represent a structural feature or reflect a role in the regulation of matrix assembly.

In conclusion, we propose that the high affinity interaction of PRELP and the HS chains of perlecan has an important function in vitro, both as a connection between the basement membrane and underlying connective tissue and as a link between different structures in the matrix or to cells in other tissues such as cartilage.

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