Small leucine rich repeat proteoglycans (SLRPs) in the human sclera: Identification of abundant levels of PRELP

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Purpose: The small leucine rich proteoglycan (SLRP) family is made up of several members which are thought to guide matrix assembly and organization through protein:protein and/or protein:carbohydrate interactions. In order to better characterize the composition of the scleral extracellular matrix, gene and protein expression of several members of the SLRP family were evaluated in the human sclera from donors aged 2-93 years of age.

Methods: Semi-quantitative and quantitative RT-PCR analyses were performed on RNA isolated from human donor sclera using primers for decorin, fibromodulin, PRELP (proline arginine rich end leucine-rich protein), biglycan, chondroadherin, and lumican. Additionally, the protein expression and distribution of the SLRP family member, PRELP, was determined in the human sclera through western blot detection and immunohistochemistry.

Results: Semi-quantitative and quantitative PCR analysis showed that all six SLRPs were expressed in the human sclera, with PRELP exhibiting the highest steady state mRNA levels, relative to that of the other SLRPs (p<0.001, ANOVA). Further analysis of PRELP in the human sclera by western blot analysis indicated that PRELP contained a 45 kDa core protein with short unsulfated keratan sulfate side chains and appeared in greatest abundance in sclera during the fourth decade of life.

Conclusions: These results suggest that several SLRP proteoglycans are expressed in the human sclera and provide the first description of the PRELP protein in the human sclera. The relative abundance of PRELP mRNA and protein in the human sclera, and the observed age-related variation in scleral PRELP expression suggests that PRELP may play a critical role in regulating the biomechanical properties of scleral extracellular matrix.

In mammals, the sclera is the tough fibrous outer layer of the eye, composed largely of collagenous bundles that run parallel to the scleral surface in irregularly arranged lamellae. Interspersed among the collagen fibers are tissue-specific glycoproteins and proteoglycans (PG) and a network of elastic fibers. Flat, elongated, fibroblasts populate the sclera and are responsible for the production and secretion of the proteins and carbohydrates that make up the extracellular matrix (ECM) of the scleral tissue [1].

The sclera has been shown to contain the sulfated PGs, aggrecan, decorin, and biglycan [2]. Decorin and biglycan are both members of the Small Leucine Rich Repeat Proteoglycan (SLRP) family of proteoglycans. The SLRP family is categorized by the presence of several conserved regions found within their core proteins. These conserved regions include a central leucine rich repeat domain, four cysteine residues within the NH2-terminus, and 2 cysteine residues within the COOH-terminus. The family totals nine members including decorin, biglycan, lumican, fibromodulin, PRELP (proline arginine end leucine rich proteoglycan), keratocan, osteoglycin, chondroadherin, and epiphycan [3].

A great deal of literature has focused on several members of the SLRP family, including decorin, fibromodulin, lumican, and biglycan for their ability to bind a variety of growth factors and ECM components via their core proteins, including type I collagen [4-7], where they are thought to guide matrix assembly and organization through protein:protein and/or protein:carbohydrate interactions. Experimental analysis of the interaction between type I collagen and SLRPs has demonstrated that SLRP binding to the collagen molecule affects fibril and/or fiber diameter, organization, and stabilization. Decorin, fibromodulin, and lumican have all been shown to interact in vitro with fibrillar collagens, resulting in alterations in fibril size by slowing the rate of fibril formation and final collagen fibril diameter [4-6]. In addition to these in vitro studies, decorin, fibromodulin, keratocan and lumican-deficient mice exhibit numerous abnormalities in the arrangement and structure of collagen fibrils in skin, tendon, cornea, and sclera [8-11]. Moreover, mutations in the keratocan gene (KERA) have been shown to cause a severe recessively inherited form of cornea plana in humans, a condition characterized by corneal flattening and reduction of refractive power of the cornea [12]. Structural modeling predicts that the substitution mutation in the KERA gene associated with this form of cornea plana will induce a conformational change in the LRR domain and may affect collagen binding, which in turn, may result in alterations in the diameter or in the spacing of collagen fibrils and alter corneal shape [13].
cDNA synthesis and semiquantitative PCR analysis: cDNA was synthesized from total RNA using random decamer primers (Ambion, Austin, TX) and M-Mul reverse transcriptase (Roche, Indianapolis, IN) according to standard protocols. Primers specific for decorin, fibromodulin, chondroadherin, PRELP, osteoglycin, and lumican were designed to amplify cDNA of approximately 300 bp within the coding region of each gene (Table 1). All primer sets were designed using MacVector 4.1 (Eastman Kodak Co., Rochester, NY) and purchased through Sigma-Genosys (Haverhill, UK; Table 1). After amplification parameters for each primer were optimized, PCR was performed on the cDNA generated from RNA pools A, B, and C using the primers specific for each SLRP analyzed according to standard protocols. Each reaction underwent 25-35 amplification cycles consisting of denaturation at 94 °C for 30 s, annealing for 30 s at various primer specific annealing temperatures, and extension for 30 s at 72 °C. Aliquots of each PCR reaction were electrophoresed on a 1% agarose gel, and stained with ethidium bromide. PCR products were quantified on digitized images of DNA gels using NIH Image version 1.62.

The number of PCR cycles was determined for each SLRP and G3PDH which would maintain exponential amplification in the semi-quantitative PCR analysis. To quantify relative SLRP expression, PCR analysis was carried out for each SLPR in triplicate using 30 or 31 PCR cycles. Each analyses series included the amplification of G3PDH as a housekeeping control gene. PCR products were electrophoresed on a 1% agarose gel and analyzed using NIH Image version 1.62.

Digitized images were used to determine the average relative expression for the SLRPs and the G3PDH control. The pixel density of each individual SLRP PCR reaction was normalized to the average pixel density for G3PDH or lumican obtained for the corresponding RNA pool. The SLRP/G3PDH and SLPR/lumican density ratios were averaged for each SLRP in each RNA pool. The relative expression of SLRPs was analyzed over all three RNA pools by averaging the SLRP/G3PDH or SLRP/lumican density ratio averages obtained for each of the RNA pools described above.

Competitive PCR analysis: Competitive PCR experiments were performed using known amounts of internal standards to “compete” with SLRP cDNAs for primers. Internal standard DNA fragments used in the competitive PCR analysis were generated through PCR amplification using pbluebacHis2B DNA (Invitrogen, Carlsbad, CA) as a template. Primers were designed to contain a 16 nucleotide sequence of pbluebacHis2B plasmid DNA (nucleotide 83-98) positioned at the 3’ end of the forward decorin or PRELP primer used in the previous semiquantitative analysis and an 18 nucleotide antisense sequence of the plasmid DNA (nucleotide 175-192) positioned at the 3’ end of the reverse decorin or PRELP primer. The 110 bp internal standard DNA fragments were generated by PCR using the modified PRELP/pbluebacHis2B or decorin/pbluebacHis2B primers on pbluebacHis2B plasmid DNA together with 0.025 U/µl DNA Taq Polymerase using standard PCR protocols. PCR products were gel purified using gel purification kit (QiaQuick, Qiagen, Valencia, CA) and concentr-
Concentrations of each PCR product were determined using UV spectrophotometry, and subsequently used as internal PCR competitors.

For quantification of decorin expression from scleral cDNA, decorin competitor DNA was added in decreasing concentrations from 0.25-0.0005 pg to PCR samples containing 1 µl of cDNA from RNA of a 35-year-old human sclera. For quantification of PRELP expression from scleral cDNA, PRELP internal standard competitor DNA was added in decreasing concentrations from 0.5-0.0025 pg to competitive PCR samples containing 1 µl of cDNA amplified from RNA extracted from the same 35-year-old human sclera. PCR reactions containing cDNA for GOI and the internal standard PCR products were electrophoresed on a 1% agarose gel and assessed by scanning densitometry with NIH Image. The concentration of the PCR amplified cDNA for the gene of interest (for convenience “GOI”) was determined by comparing the log of the GOI pixel density/internal standard pixel density ratio with the log of the internal standard pixel density. When the pixel density for the internal standard and the pixel density for the GOI were equal, the log of their ratios was equal to zero. Therefore, the point on the X-axis that corresponded with the Y-value of zero represented the log of the initial concentration of the gene of interest in the scleral RNA sample.

Protein extraction from human sclera and cornea: Human eyes from donors aged 2, 19, 35, 46, 54, 84, and 93 years were obtained from the National Disease Research Interchange 48 h or less postmortem and stored at -80 °C. Donor tissue was handled according to the tenets of the Declaration of Helsinki and the research was approved by the University of

Figure 1. RT-PCR analysis of SLRPs. SLRPs were amplified from cDNA Pool A (A), Pool B (B), and Pool C (C) by reverse transcription PCR, using primers from Table 1. Pool A RNA was extracted from of a 35-year-old woman, pool B RNA was extracted from three eyes of donors aged 40-60 years of undetermined gender, and pool C RNA was extracted from a 58-year-old male. cDNA was electrophoresed on 1% agarose gels, digitized, and analyzed in NIH Image, version 1.62. In the image, PRELP=proline/arginine end leucine-rich proteoglycan; Osteo=osteoglycin; Lum=lumican; G3PDH=glyceraldehyde 3-phosphate dehydrogenase; Dec=decorin; Chon=chondroadherin.
North Dakota’s institutional review board. In preparation for experimental analysis, human donor tissue was thawed on ice and, if present, the cornea was removed and minced in a weigh boat on ice into fine (<2 mm³) pieces with a razor blade. The remaining tissue was dissected to remove adherent muscle, fat, lamina cribrosa, and the optic nerve head from the sclera. The sclera was then divided into anterior, equatorial, and posterior regions and minced in a weigh boat on ice into fine (<2 mm³) pieces with a razor blade according to the dissection parameters previously described [20]. The minced cornea and sclera samples were weighed and total protein was extracted using 4 M guanidine HCl containing 0.01 M sodium acetate, 0.01 M ε-amino-n-caproic acid at 4 °C as described previously [2]. The extracted protein was then dialyzed exhaustively against distilled deionized water and lyophilized. Each lyophilized scleral and corneal sample was reconstituted in a volume of dH₂O proportional to the starting wet weight (675 µl/gram wet weight). The samples were then centrifuged at 4 °C for 15 min at 2000 rpm, supernatants were removed, assayed for total protein using the Bradford protein assay (BioRad, Hercules, CA), and stored at -20 °C.

SDS/PAGE and western blot analysis: Protein extracts from each scleral region and cornea were subjected to western blot analyses using antisera raised against human PRELP (a gift from Peter Roughley, Joint Diseases Laboratory, McGill University, Montreal, Quebec, Canada). Prior to SDS/PAGE, aliquots of some scleral protein extracts were digested with endo-β-galactosidase, keratinase I, or keratinase II (Seikagaku America, Falmouth, MA) as well as N-glycosidase F (Boehringer Mannheim, Mannheim, Germany) as described previously in the literature [2,21] and were electrophoresed, along with undigested samples on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Following electrophoresis the proteins were transferred to a nitrocellulose membrane (Osmonic™, Minnetonka, MN) at 100 volts for 1 h. The immobilized protein was incubated with antisera generated against human PRELP followed by a second incubation with alkaline phosphatase conjugated anti-rabbit IgG developed in goat (Sigma, St. Louis, MO). Specificity of the immune reaction was previously established [22]. Antibody reactivity was detected through chemiluminescence (Western Star; Tropix, Bedford, MA).

Figure 2. Relative expression of SLRPs in the human sclera using semi-quantitative RT-PCR. Digitized images from Figure 1 were used to determine the average relative expression for 4 or 5 SLRPs and the housekeeping gene G3PDH in each of the 3 scleral RNA pools (Pool A, Pool B, and Pool C). The relative expression of SLRPs was averaged over the three scleral pools by determining the average SLRP/G3PDH pixel density for each SLRP in the three scleral pools (Average). Additionally, SLRP expression was normalized to the average expression of lumican for each cDNA pool, and the averages for the three cDNA pools were plotted for comparison (Average/LUM). In the image, Chon=chondroadherin; dec=decorin; G3PDH=glyceraldehyde-3-phosphate dehydrogenase; lum=lumican; ost=osteoglycin; PRELP=proline/arginine end leucine-rich proteoglycan.
Immunohistochemistry: The anterior sclera of a 36-year-old male donor was embedded and frozen in OCT compound (Tissue-Tek, Elkhart, IN), sectioned using a cryostat, and 10 µm frozen sections were mounted on gelatin-coated slides. Immunostaining was carried out on frozen sections using PRELP antiserum or non-immune rabbit sera as a control, followed by incubation with biotinylated anti-rabbit secondary antibody and avidin:biotinylated horse radish peroxidase complex (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA). PRELP protein distribution was visualized following incubation with diaminobenzidine (DAB)/Tris-HCl solution (50 mM Tris-HCl, 0.02% DAB, 0.0045% H₂O₂) prepared just prior to use. The DAB reaction was stopped after 15 min on all tissue sections including the control samples with 2 washes of 50 mM Tris-HCl (pH 7.4) for 20 min each. The tissue slides were then dehydrated, dried, and mounted in Permount (Fisher, Pittsburgh, PA).

RESULTS

gMacropart# Semi-quantitative RT-PCR analysis: The exponential linear range for PCR amplification of the SLRPs was determined to be 31 cycles for RNA pools A and B and 30 cycles for RNA pool C. Therefore, semi-quantitative PCR analysis was carried out in triplicate using 31 cycles for pools A and B and 30 cycles for pool C, in order to maintain exponential amplification of all SLRPs for each RNA pool. Additionally, the housekeeping gene, G3PDH, was amplified in order to normalize gene amplification across the separate RNA pools. Analysis of the semi-quantitative results revealed the presence of mRNA for decorin, lumican (as a doublet in pool A only), osteoglycin, and PRELP in the human sclera in all three RNA pools (Figure 1). Additionally, chondroadherin was amplified from Pool C RNA (Figure 1C). Low levels of fibromodulin were detected in all RNA pools, but required 34 cycles of PCR to visualize the product. The number of cycles required to visualize fibromodulin was out of the linear range determined for the other SLRPs examined and therefore fibromodulin was not included in this study (data not shown). Analysis of Pool A RNA suggests that of the 5 SLRP messages analyzed in the human sclera, PRELP message was most abundant, followed by G3PDH, osteoglycin, decorin, and lumican. When normalized to G3PDH levels and compared to the levels of the other SLRP PCR products, the PRELP PCR product was found to be 1.5 to 24 fold higher than that of the other SLRPs (Figure 2, pool A). According to the average pixel densities obtained for RNA pool B, PRELP mRNA was found in the highest concentration. PRELP mRNA levels were followed, in decreasing order, by lumican, decorin, osteoglycin, and G3PDH. Analysis of the pixel densities indicated that PRELP steady state mRNA levels were 4.3 to 43 times higher in the human sclera than the steady mRNA levels of the other SLRPs and the G3PDH control (Figure 2, pool B). Analysis of SLRP expression in Pool C RNA demonstrated highest levels for PRELP followed by osteoglycin, lumican, G3PDH, chondroadherin, and decorin, listed in decreasing order of abundance and PRELP levels were found to be 2.7 to 47 fold higher than the steady state mRNA levels of all other SLRPs and the G3PDH control (Figure 2, pool C). An overall comparison of the means for all 6 SLRPs and the G3PDH control in all RNA pools examined established that the steady state mRNA level of PRELP was 5 to 43 fold higher than the steady state mRNA levels of all other SLRPs and the G3PDH control.

Figure 3. Quantification of scleral PRELP and decorin by competitive PCR analysis. Decreasing amounts of decorin and PRELP internal standard were added to the scleral cDNA prepared from RNA of a 35-year-old human sclera. Absolute levels of decorin and PRELP in total scleral RNA were determined at the point of cross over between the amplification of decorin (A) or PRELP (B) and the competitive internal standard DNA. At point 0 on the y-axis (C), the concentration of decorin (A) and PRELP (B) equal the anti-log of the internal standard (plotted on the x-axis). From this comparison it was determined that 0.02 pg of decorin and 0.11 pg of PRELP mRNA were present in the scleral RNA pool. 
in the human sclera (Figure 2, average). Since G3PDH expression exhibited variability in expression across the three RNA pools (Figure 1), SLRP expression was also normalized to the average level of expression for lumican (LUM) for each RNA pool, and the average SLRP expression/LUM was similarly calculated for the three RNA pools (Figure 2, average/LUM). When expressed relative to LUM expression, the PRELP mRNA level was 2.5 to 10.5 fold higher than the mRNA levels of the other SLRPs (Figure 2, average/LUM).

Competitive PCR analysis: Because semi-quantitative RT-PCR is subject to variation in amplification efficiencies between different target templates, competitive PCR was performed on a fourth cDNA pool amplified from RNA extracted from a separate 35-year-old donor to verify the high steady state mRNA level of PRELP relative to the steady state mRNA levels of other SLRP family members in the sclera observed in the semi-quantitative analysis. Results of the competitive PCR analysis indicated that PRELP steady state mRNA levels were greater than 5 fold higher than the steady state mRNA levels of decorin in this mRNA pool extracted from human sclera (0.11 pg PRELP [1.389x10^8 copies] compared with 0.02 pg [2.535x10^7 copies] decorin mRNA; Figure 3).

PRELP protein expression in the human sclera: Based upon the relatively high steady state mRNA expression levels of PRELP found in the human sclera, western blot was used to characterize PRELP protein expression in the human sclera and cornea. In undigested samples, PRELP could be seen as a light-staining smear which migrated at a molecular weight of 55-60 kDa in scleral samples and 60-116 kDa in cornea samples, suggesting that PRELP is present in the human cornea and sclera with some degree of glycosylation. Digestion with endo-β-galactosidase, which cleaves keratan sulfate glycosaminoglycans (GAGs) or shorter oligosaccharides with a low degree of sulfation, resulted in the appearance of an about 50 kDa band in scleral samples and 50 and 45 kDa bands in cornea samples. Digestion with N-glycosidase F, which removes GAGs or oligosaccharides at the glycosylamine linkage of the core protein [23], resulted in a further shift in migration to a molecular weight of scleral PRELP to approximately 45 kDa and the appearance of 45 and 42 kDa bands in cornea samples (Figure 4). Bands at 55-60 kDa and 50 kDa represent partially deglycosylated PRELP in the cornea. Digestion with keratanase I and II, which cleaves highly sulfated keratan sulfate GAGs, did not alter the molecular weight of PRELP in either cornea or scleral samples (data not shown). According to these results, PRELP was present in the human sclera as a glycoprotein, with a 45 kDa core protein substituted with short keratan sulfate GAG chains with a very low degree of sulfation. Our results also suggest PRELP is present in the cornea with core proteins of 45 and 42 kDa, both of which are substituted with low-sulfated keratan sulfate GAGs.

To determine whether the protein expression of PRELP is altered during postnatal growth and aging, total protein was extracted with 4 M guanidine HCl from the anterior, equatorial, and posterior regions of human donor sclera ages 2, 19, 35, 54, 84, and 93 years old, digested with endo-β-galactosidase, and detected with PRELP antiserum using western detection. Equal amounts of total protein (46 µg) of each scleral sample were electrophoresed on a 10% SDS-PAGE gel. Western analysis indicated that PRELP was expressed in human sclera at all ages examined. However, the expression of PRELP appeared in greatest concentration at ages 19 and 35 years. Beyond the fourth decade of life, PRELP expression dropped to significantly lower levels, with the exception of the equatorial region of the 84-year-old individual (Figure 5).
**Immunohistochemistry:** The distribution of PRELP protein in the human sclera was determined through immunohistochemical analysis of frozen scleral tissue using PRELP antisera. Based upon the results of the immunohistochemical staining, PRELP protein was found throughout the scleral stroma, with intense staining along the inner or retinal side of the scleral tissue (Figure 6).

**DISCUSSION**

Proteoglycans (PGs), including several of the small leucine rich proteoglycan (SLRP) family members, have been shown to influence ECM organization in a variety of tissues [24]. We hypothesized that SLRPs are important in the organization of the scleral ECM as well. However, little information is available regarding the SLRP composition of the human sclera. To characterize the SLRPs present in human sclera, we have analyzed and compared the steady state mRNA levels of a variety of SLRPs by amplifying cDNA obtained from total RNA isolated from human donor sclera with primers specific for decorin, biglycan, chondroadherin, lumican, osteoglycin, and PRELP.

The semi-quantification studies used to characterize SLRP steady state mRNA levels in the human sclera revealed high expression levels for the SLRP family member, PRELP, as compared to the expression levels for the other SLRPs tested and the G3PDH control. While there was variability between the mRNA levels for each pool of RNA, an overall analysis of all three semiquantitative analyses, illustrated the amplification for decorin, chondroadherin, lumican, osteoglycin, and PRELP in the human sclera in one or more RNA pools. The appearance of a lower (about 500 bp) band for lumican PCR amplified products was observed only in RNA pool A. This lower band could represent a previously uncharacterized splice variant but that scenario is doubtful as the primers were designed to amplify a 546 bp fragment from nucleotides 258-804 within the central and largest exon of the lumican gene. Because this band was absent in the other two pools of RNA, only the top correct size band was used for quantification analysis. Semi-quantitative RT-PCR can be subject to variations in amplification efficiencies between different target templates, therefore a more stringent assay was needed to verify the high steady state mRNA level observed for PRELP in the semiquantitative studies. The direct comparison of PRELP and decorin copy numbers within the human sclera achieved through competitive PCR analysis confirmed the findings of the semiquantitative analysis, indicating PRELP mRNA was expressed at levels at least five fold higher than the ubiquitously expressed decorin mRNA.

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**Figure 6.** Immunostaining of human sclera for PRELP. Immunohistochemical staining of frozen sclera obtained from the anterior region of a 36-year-old male donor with anti-PRELP using the Vectastain ABC kit. A: The negative control, where non-immune rabbit serum was substituted for anti-PRELP in the primary antibody incubation. B: Positive staining using the anti-PRELP antibody. The top of the slide represents the outer sclera. The bottom portion of the slide represents the inner, retinal side of the sclera (R). The scale bars represent 150 µm.
PRELP was first characterized in bovine articular cartilage [21] and until recently, significant expression of human PRELP was thought to be limited to cartilage tissue [19]. The findings in our quantitative studies are the first to show significant expression of PRELP in the human sclera in comparison to the expression of the other SLRP family members. The high concentration of PRELP mRNA found within the human sclera, suggests that PRELP may be important to the integrity of the highly collagenous scleral tissue.

Based upon the high expression levels of PRELP steady state mRNA in the human sclera, PRELP protein expression, glycosylation, and distribution were determined in human scleral and corneal tissue. The shift in PRELP migration, following digestion with endoβagalactosidase, which cleaves keratan sulfate GAGs of low sulfation, verified that the PRELP core protein is substituted with keratan sulfate GAG of low sulfation in both the human sclera and the human cornea. A second digest of scleral PRELP protein with N-glycanase F, which removes GAGs or oligosaccharides at the glycosylamine linkage of the core protein [23], resulted in yet another shift in molecular weight to approximately 45 kDa (the approximate size of the PRELP core protein) in scleral samples. The shift in molecular weight of PRELP induced by N-glycanase F digestion establishes the presence of a GAG side chain on the PRELP core protein. In a similar digest of corneal protein with N-glycanase F, PRELP migrated as a smear with the appearance of several bands between 66 and 42 kDa in size. In combination with the results from the N-glycanase F digest, the endoβ-galactosidase digest confirmed the presence of keratan sulfate GAG substitution of the PRELP core protein in both the human sclera and human cornea. The presence of multiple bands in the N-glycanase F digested corneal sample could represent the presence of O-linked keratan sulfate GAGs or other oligosaccharides of the PRELP core protein resistant to the N-glycanase F enzyme which cleaves N-linked residues only. In fact, along with the three potential N-linkage sites found within the core protein of human PRELP, there is evidence of a potential O-linkage on the Thr23 residue within the PRELP core protein [25].

Keratan sulfate substitution of PRELP has been the subject of speculation in a number of studies, but until now, the glycosylation of PRELP has evaded elucidation. In addition to keratan sulfate GAGs, PRELP may have O-linked oligosaccharide substitution as well. Carbohydrate linkage to the core protein is yet to be determined.

PRELP expression was examined from extracts of total protein from human donor sclera at a variety of ages. PRELP expression was abundant throughout the sclera of the 19- and 35-year-old donors, however, it was low or absent in all regions of the sclera of the 2-, 54-, and 93-year-old donors, as well as from the anterior region of the 84-year-old donor. This data is consistent with previous reports that PRELP protein levels increase with age in human articular cartilage, (based on western analysis) [22,26], and then appear to decline in older adults, (based on Northern analysis) [22]. These results mimicked a similar trend found within the protein expression of the SLRPs, decorin and biglycan, which are both components of the human sclera [20]. The increase of protein expression observed through the fourth decade of life for decorin, biglycan, and PRELP indicates that the SLRPs may play a role in the early development and subsequent growth of the rapidly elongating sclera of the child and adolescent. The loss of SLRPs beyond the fourth decade of life may, in part, contribute to the increased rigidity observed within the scleral stroma as aging occurs. In addition to the influence of PRELP protein on the normal development and growth of scleral and cartilaginous tissues, PRELP may also play a role in the pathogenesis associated with systemic diseases of joints, cartilage, and other connective tissues such as Hutchinson-Gilford progeria, where the onset of PRELP expression and theorized function are thought to coincide with the onset and progression of this particular disease [19].

Immunohistochemical analysis confirmed the presence of PRELP protein throughout the scleral stroma with intense staining along the choroidal border. The variation in PRELP distribution across the thickness of the sclera indicates that PRELP may function differently within different regions of the scleral ECM. A potential function for PRELP was theorized in a recent study where PRELP was shown to bind the glycosaminoglycan (GAG) heparan sulfate through its basic amino terminus [27]. Heparan sulfate can be associated with proteoglycans at the cell surface (e.g., syndecan) [28] and references therein, or can be found in the extracellular matrix in conjunction with proteoglycans (e.g., perlecan and agrin) [29,30]. Therefore, PRELP may act as a potential linkage between the cell and its surrounding matrix by simultaneously interacting with heparan sulfate at the cell surface through its basic amino terminus and with fibrillar collagen in the surrounding matrix through the protein’s leucine rich central domain. This type of interaction could facilitate a number of cellular processes such as cell attachment, migration, and anchorage of epithelial cell layers to the underlying basement membrane.

In addition to a protein link between the cell and the matrix, PRELP, like a number of other SLRP family members, may influence fibrillar collagen organization through protein/protein interactions as well. Binding affinity assays have recently demonstrated that PRELP binds to type I and type II collagen as well as the heparin sulfate chains of perlecan. Based on PRELP’s location in connective tissues and protein interactions, it has been suggested that PRELP may function as a bridging molecule that anchors the basement membrane to the underlying connective tissue [31]. We speculate that PRELP acts to stabilize and organize the collagenous ECM of the scleral stroma through its interaction with type I collagen, the major protein constituent of the sclera. PRELP interaction with procollagen molecules, which are found throughout the lamellae of the scleral stroma, could explain the uniform immunohistochemical staining of PRELP protein within the scleral stroma observed in the present study.

Based on previous studies of the corneal stroma [32] it is likely that the scleral stroma similarly contains trace amounts of the basement membrane-associated molecules, heparan sulfate proteoglycan (perlecan) and laminin. We speculate that
PRELP may participate in scleral assembly and organization, through binding interactions with pericellular heparan sulfate and fibrillar collagens within the scleral stroma.

The intense immunostaining observed at the choroidal surface of the scleral tissue may have evolutionary significance. In lower vertebrates, such as the amphibians and birds, the scleral stroma is divided into an inner cartilaginous layer and an outer fibrous layer [33]. This division of the sclera into a fibrous and cartilaginous layer is not observed within the mammalian eye, where the sclera appears uniformly fibrous [33]. However, the intense immunostaining of PRELP protein, which is also an abundant component of cartilage matrix, around the inner portion of the sclera may represent a residual scleral cartilaginous layer. Anatomically, the scleral region defined by the intense PRELP staining borders a transitional area located between the scleral stroma and the perichoroidal space, called the lamina fusca [34]. The lamina fusca is populated with a large number of pigment cells, which includes pigmented macrophages and melanocytes to name a few [34]. The high expression of PRELP protein at the scleral border of the lamina fusca may facilitate contact between the scleral stroma and choroid through cell/matrix interactions among the cells of the lamina fusca and the surrounding ECM of the stroma and perichoroidal space. The potential interaction between the cells of the lamina fusca and the surrounding matrix may prevent dissociation of the vascular choroid from the relatively avascular scleral stroma.

In summary, a variety of SLRPs are found in abundance throughout the human scleral and corneal tissue where they most likely act to modulate the assembly and organization of the ECM of these tissues. Furthermore, the relatively high concentration of steady state mRNA levels of PRELP in the human sclera, suggests that PRELP may be important to the integrity of the highly collagenous scleral tissue. High myopia in humans is characterized by vitreous chamber elongation, scleral thinning and ectasia at the posterior pole of the eye, as well as abnormal collagen fibril structure and arrangement within the posterior scleral stroma [35]. Based on the relatively abundant levels of PRELP in the human sclera, together with the known interactions of PRELP with collagen fibrils and perlecan [27,31], we speculate that alterations in PRELP could result in abnormalities in scleral structure, which could affect ocular shape and refraction. Although several myopia-associated loci have been identified in the human genome [36-42], the genetic locus for PRELP (1q31) has not been shown to be associated with inherited myopia in humans. Additional studies using transgenic or knockout mice to over- or underexpress PRELP are needed to directly assess the role of PRELP in scleral collagen assembly, scleral biomechanics and ocular growth.

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