A 1.9-kb cDNA clone to chick lumican (keratan sulfate proteoglycan) was isolated by screening an expressing vector library made from chick corneal RNA with antisemur to chick corneal lumican. The cDNA clone contained an open reading frame coding for a 343-amino acid protein, \( M_r = 38,640 \). Structural features of the deduced sequence include: a 18-amino acid signal peptide, cysteine residues at the N- and C-terminal regions, and a central leucine-rich region (comprising 62% of the protein) containing nine repeats of the sequence \( LXxLxLxxNxL/I \), where \( X \) represents any amino acid. Lumican contains three variations of this sequence that are tandemly linked to form a unit and three units tandemly linked to form the leucine-rich region. The sequential arrangement of these repeats and their spacing suggest that this region arose by duplication. The deduced sequence shows five potential N-linked glycosylation sites, four of which are in the leucine-rich region. These sites are also potential keratan sulfate attachment sites. The cDNA clone to lumican hybridizes to a 2.0-kb mRNA found in tissues other than cornea, predominantly muscle and intestine. Radiolabeling and immunoprecipitation studies show that lumican core protein is also synthesized by these tissues. The primary structure of lumican is similar to fibromodulin, decorin, and biglycan, which indicates it belongs to the small interstitial proteoglycan gene family. The expression of lumican in tissues other than cornea indicates a broader role for lumican besides contributing to corneal transparency.

The corneal stroma is a transparent connective tissue. It consists primarily of collagen types I and V, with lesser amounts of types III and VI. The stroma also contains proteoglycans with chondroitin/dermatan sulfate side chains and with keratan sulfate side chains. The transparency of the corneal stroma can be attributed in part to the ordered array of collagen fibrils of uniformly small diameter and regular interfibrillar spacing (1, 2). Several lines of evidence suggest that the corneal keratan sulfate proteoglycan plays an important role in corneal transparency. It appears in the cornea during the acquisition of transparency during embryonic development (3-6). Furthermore, in a rabbit corneal wound-healing model, opaque corneal scars lack both the keratan sulfate proteoglycan and uniform collagen fibril spacing. The restoration of transparency in this model is accompanied by the return of both the keratan sulfate proteoglycans and uniform collagen fibril spacing (7, 8).

The chondroitin/dermatan and keratan sulfate proteoglycans of the corneal stroma are similar in size, and their core proteins have a similar amino acid composition (9). In the chick, the chondroitin/dermatan sulfate proteoglycan is synthesized from a \( M_r = 40,000 \) precursor protein estimated to contain two N-linked oligosaccharides, while the keratan sulfate proteoglycan is synthesized from a larger precursor protein (\( M_r = 51,000 \)) estimated to contain five to six N-linked oligosaccharides (10, 11). The keratan sulfate side chains on the corneal keratan sulfate proteoglycan are N-linked (12) and that is different from chondroitin/dermatan sulfate, which is O-linked (13). Structural analysis has shown that the linkage region for corneal keratan sulfate is a complex type N-linked oligosaccharide (14, 15) while the N-linked oligosaccharides on the corneal keratan sulfate proteoglycan are the high mannose type (15). Thus, some of the N-linked oligosaccharides on the corneal keratan sulfate proteoglycan precursor protein go on to be processed into the high mannose-type while others are processed into the complex type and receive keratan sulfate side chains (10, 15).

Decorin is a chondroitin/dermatan sulfate proteoglycan that was originally cloned from a fibroblast cell line (16). A subsequent study shows that a cDNA clone to bovine decorin hybridized to RNA isolated from bovine corneas (17). In addition, antibodies to decorin reacted with the core protein of the chondroitin/dermatan sulfate proteoglycan isolated from bovine corneas (17). A cDNA close to bovine biglycan, a proteoglycan related to decorin, did not hybridize to bovine corneal RNA (17). This indicates that the chondroitin/dermatan sulfate proteoglycan found in cornea is decorin.

In addition to the differences in carbohydrate between corneal decorin and corneal keratan sulfate proteoglycan, antibodies to the keratan sulfate proteoglycan indicate its core protein is different from decorin (11, 18). Here we used antisemur to the core protein of chick corneal keratan sulfate proteoglycan to isolate cDNA clones to this proteoglycan from an expression vector library. We propose to name this proteoglycan "lumican," for the important role this proteoglycan plays in the acquisition and maintenance of corneal transparency. The deduced sequence of the clone to lumican reveals a homology to the small interstitial proteoglycan gene family consisting of fibromodulin, biglycan, and decorin.
Chick Corneal Proteoglycan Structure and Expression

MATERIALS AND METHODS

**Purification and Sequencing of Lumican—**Chick lumican (corneal keratan sulfate proteoglycan) was isolated as previously described (11). Briefly, adult chicken corneas were excised from adult chickens obtained from a local slaughterhouse, extracted in 4 M guanidine and then chromatographed on DEAE in 7 M urea to isolate the proteoglycans. The proteoglycans were digested with keratanase (Proteus vulgaris, Seikagaku Kogyo Co.) which removed the glycosaminoglycan side chains from lumican. The resulting core protein was purified by fast protein liquid chromatography and was used as an antigen to raise antisera in rabbits following a protocol from Ribi Immunogen Research Inc.

Sequencing from the N terminus of lumican was not successful. Consequently, lumican core protein was cleaved with CNBr and the resulting peptide fragments separated by reversed-phase chromatography (PepRPC; Pharmacia LKB Biotechnology Inc.) using a 5–50% acetonitrile gradient containing 0.1% trifluoroacetic acid. The isolated peptides were subject to Edman degradation on a Beckman model 890M or a Protein model 2008R Instrument. Phenylthiocyanid-3-olyl derivatives were identified by HPLC using a sodium acetate (containing 5.5% tetrahydrofuran) acetonitrile gradient system. One peptide yielded the following sequence: Tyr-Cys-Asp-Asn-Leu-Lys-Leu-Lys-Thr-Ile-Pro-Ile-Val-Pro-Ser-Gly-Ile-Lys-Tyr-Leu-Tyr-Leu-Xaa-Asn.

**Construction and Screening of a Chick Cornea-derived cDNA Library—**3-day-old White Leghorn chicks were decapitated and their corneas removed by excision. The corneas were frozen immediately in liquid nitrogen and stored at −80 °C. RNA was extracted from the corneas using isothiocyanate-phenol-chloroform procedure (19). Poly(A)+ mRNA was isolated by affinity chromatography on oligo(dT)-cellulose (Pharmacia) and used to construct a Uni-ZAP XR cDNA library (Stratagene custom library section). Approximately 200,000 plaque-forming recombinants from this library were transduced into Escherichia coli PLK-F7 plates and plated on agar dishes. Durulose membranes (Stratagene) containing isopropanol-0-thio-galactopyranoside oligonucleotides. The sequencing reactions were loaded onto a 482-bp fragment from the 5' end of a clone (KSPG 4) which was sequenced using a synthetic oligonucleotide from the N terminus of lumican was not successful. Consequently, lumican core protein was cleaved with CNBr and the resulting peptide fragments separated by reversed-phase chromatography (PepRPC; Pharmacia LKB Biotechnology Inc.) using a 5–50% acetonitrile gradient containing 0.1% trifluoroacetic acid. The isolated peptides were subject to Edman degradation on a Beckman model 890M or a Protein model 2008R Instrument. Phenylthiocyanid-3-olyl derivatives were identified by HPLC using a sodium acetate (containing 5.5% tetrahydrofuran) acetonitrile gradient system. One peptide yielded the following sequence: Tyr-Cys-Asp-Asn-Leu-Lys-Leu-Lys-Thr-Ile-Pro-Ile-Val-Pro-Ser-Gly-Ile-Lys-Tyr-Leu-Tyr-Leu-Xaa-Asn.

**DNA Sequencing of cDNAs—**The pBluescript plasmid containing the cDNA insert was rescued from Uni-ZAP XR by coelectrophoresing the Uni-ZAP XR vector with FI Helper Phage 1408 into E. coli XL1-Blue cells. The rescued phagemid from these clones were then propagated in XL1-Blue cells following a protocol from Stratagene, and purified. The inserts on the plasmids were partially or completely sequenced via the dideoxy chain-termination method (20). Sequencing was done at the 5' end of the insert. BamHI and BglII.

**Northern Blot Analysis—**Total RNA was isolated (19) from day 15 embryonic chick brain, cornea, whole eyes (less cornea), heart, aorta, liver, muscle, sciera, and from chick corneal fibroblasts grown in culture for 7 days (21). 15 µg of total RNA from each of the above tissue types were electrophoresed through a 1% agarose gel containing formaldehyde and transferred to GeneScreen (Du Pont-New England Nuclear) overnight. Following prehybridization, the blot was hybridized with the 32P-labeled probe, (prepared as described above) at 42 °C, 32 h. After high stringency washes were performed according to protocol from Du Pont-New England Nuclear, the washed blot was exposed to X-Omat AR film (Kodak) with an intensifying screen at −80 °C.

**RESULTS**

Screening 200,000 recombinants of the Uni-ZAP XR cDNA library with antisem to lumican yielded 20 positive clones that were rescreened to purity. These clones were tested for their ability to hybridize to a degenerate oligonucleotide probe ATOTA(C/T)TG(C/T)GAA(C/T)C(T/T) based on the sequence obtained from the CNBr peptide fragment isolated from the lumican core protein (see "Materials and Methods"). This oligonucleotide hybridized to 15 of the 20 immunopositives. The cDNA inserts on five of the strongest immunopositive clones that also hybridized with the oligonucleotide probe were characterized using restriction enzyme digestion and agarose gel electrophoresis. The cDNAs were originally constructed with a 5' EcoRI site and a 3' XhoI site so that the cDNA could be unidirectionally inserted into the Uni-ZAP vector. The results showed that four of the five clones had lost their XhoI site, one had lost its EcoRI site, and all had an internal EcoRI site. The 5' end of the clone with the smallest insert (KSPG 4) was sequenced using a synthetic oligonucleotide complementary to the plasmid near the 5' end of the insert. The deduced sequence contained an exact match with the 24 amino acid sequence obtained from the CNBr peptide fragment, thereby establishing the authenticity of the clone.

None of the immunopositive clones contained the entire open reading frame, so the cDNA library (375,000 recombinants) was then rescreened using a 482-bp fragment extending from the 5' end of KSPG 4 to the internal EcoRI site. Twenty-nine positives were obtained and purified. Since none of the clones may have lost their EcoRI site at the 5' end, the clone containing the longest insert was identified by digesting with BamHI and BglII. BamHI cuts at a site in the pBluescript plasmid which is 19-bp upstream from the EcoRI site located at the 5' end of the insert. BglII cuts the lumican cDNA insert at nucleotide 806 (Fig. 1). The insert size was the examined via agarose gel electrophoresis. The cDNA from the clone containing the largest insert (KSPG(07)) as well as from the original confirmed clone were sequenced from both strands using oligonucleotide primers. This produced a series of overlapping sequences where every region had been read at least two times.
The 1944-bp cDNA to lumican has a poly(A) sequence at the 3' end which begins 15 nucleotides after the putative polyadenylation signal, AATAAA (Fig. 1). The nucleotide sequence contains an open reading frame encoding 434 amino acids (theoretical mass = 38,640 Da). A 24-amino acid sequence obtained from a cyanogen bromide-generated fragment of lumican matched the deduced amino acid sequence (double-underlined region), confirming the identity of the clone. The helical underlined region is likely the signal peptide since it contains the characteristics of a signal sequence. In addition, application of the "(−3,−1)-rule" predicts the signal sequence cleavage site to be between Cys (amino acid 18) and Gln (amino acid 19) (23). The deduced sequence contains five potential N-linked oligosaccharide attachment sites (denoted by *). There are 6 cysteine residues (denoted by *) in the protein, excluding the cysteine in the proposed signal peptide. Four of the cysteines are located near the N terminus and two are near the C terminus.

Lumican also possesses multiple leucine-rich repeats which contain the following structure: LXXLXLXXN/XL/I, where X represents any amino acid. Nine leucine-rich repeats are located between amino acids 67 and 277, comprising 62% of the protein. Sequence analysis of the lumican region shows that there are three basic variations, or motifs, of the repeat (Fig. 2). Motif 1 has the structure LPXXLXXLYLXNNXI. The leucine motifs are arranged in a distinct pattern. Motifs within a unit are separated by 24 (motifs 1 and 2) or 25-26 (motifs 2 and 3) amino acids. This results in a composite spacing pattern of: 24, 26, 21, 24, 25, 21, 24, 25. The spacing of the units and leucine motifs along with the cysteine residues and potential N-linked oligosaccharide sites, is shown on the structural model of the proteoglycan (Fig. 3).
corresponding to the upper part of the broad band, and in whole eyes (less cornea) the probe hybridized to the region in the middle. The N- and C-terminal positions of these terminus, and the presence of the multiple leucine-rich repeats in the middle. The distance from the asparagine (amino acid 289) in the first leucine sequence is 28 amino acids. In addition, the two sequences do not match any of the three defined motifs.

The amino acid sequence of lumican was compared to the other small interstitial proteoglycans: fibromodulin (24), decorin (16), and biglycan (17) (Fig. 4). The boxed areas contain the residues which are identical to lumican. All share the same basic structure: the alignment of the 4 cysteine residues at the N terminus, the alignment of the 2 cysteine residues at the C terminus, and the presence of the multiple leucine-rich repeats in the middle. The N- and C-terminal positions of these sequences show the greatest variation. The overall percentage of identity of lumican with fibromodulin is 50%, with decorin 32%, and with biglycan 32%, not including positions containing gaps.

The presence of lumican mRNA in total RNA isolated from different tissues was measured by Northern blot using the 482-bp fragment from the 5' end of clone KSPG 4. The probe hybridized to a 1.8-2.0-kb message in RNA isolated from cornea (Fig. 5, lane 1). A similar-sized message was also found in decreasing amounts in the following tissues: corneal fibroblasts (lane 8), breast muscle (lane 3), intestine (lane 5) > whole eyes (less cornea) (lane 7) > heart (lane 6) > liver (lane 4) > sclera (lane 2). No message was detected in brain (lane 9). The mRNA appeared as a broad band in cornea, corneal fibroblasts, intestine, and muscle. In contrast, in liver and whole eyes (less cornea) the probe hybridized to the region corresponding to the upper part of the broad band, and in heart to a region corresponding to the lower part of the broad band.

Examination of the lumican sequence (Fig. 1) reveals two more possible leucine-rich sequences near the C terminus. One sequence (amino acid 281-291) has the structure LPXXLXXLXXNXL. The second sequence (amino acid 311-324) has the structure IXXLXLXXNXL. We did not include the residues which are identical to lumican. All share the same basic structure: the alignment of the 4 cysteine residues at the N terminus, the alignment of the 2 cysteine residues at the C terminus, and the presence of the multiple leucine-rich repeats in the middle. The N- and C-terminal positions of these sequences show the greatest variation. The overall percentage of identity of lumican with fibromodulin is 50%, with decorin 32%, and with biglycan 32%, not including positions containing gaps.

The synthesis of lumican precursor protein was examined in six different tissues by pulsing tissues in organ culture with [35S]methionine, immunoprecipitating the precursor protein with antibodies to lumican, and detecting the precursor protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis autoradiography. The precursor protein to lumican produced by corneas appears as a broad band at Mr = 51,000 (11) (Fig. 6, lane 12). The band at Mr = 40,000 is the precursor protein to decorin that coprecipitates with this antisera.

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tissues was loaded onto the gel as follows: derived from day 15 chick embryos and cultured corneal fibroblasts; equal amounts of incorporated radiolabel from each tissue type. The antilumican antiserum. The various tissue samples were loaded onto polyacrylamide gel electrophoresis then exposed to X-Omat film. The preimmune serum, and the samples in the lanes were immunoprecipitated with antilumican antiserum. Immunoprecipitations were done from various tissues. Tissues were labeled in culture with ["SI methionine and homogenized. Immunoprecipitations were done from equal amounts of incorporated radiolabel from each tissue type. The immunoprecipitates were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis then exposed to X-Omat film. The samples in the odd-numbered lanes were immunoprecipitated with preimmune serum, and the samples in the even-numbered lanes with antilumican antiserum. The various tissue samples were loaded onto the gel as follows: Lanes 1, 2, liver; lanes 3, 4, intestine; lanes 5, 6, heart; lanes 7, 8, breast muscle; lanes 9, 10, whole eyes (less cornea); lanes 11, 12, cornea.

**DISCUSSION**

The deduced amino acid sequence of lumican shows that this protein is a member of the small interstitial proteoglycan gene family consisting of: decorin (16), biglycan (17), and fibromodulin (24) (Fig. 4). Key features of this gene family, such as leucine-rich repeats and the number and positioning of the cysteine residues, are also present in lumican. Lumican contains three variations of the leucine-rich repeat: motifs type 1, 2, and 3 (Fig. 2). These three motifs are tandemly linked to form a unit and three units tandemly linked to form the leucine-rich repeat region of the core protein. Furthermore, the distance in amino acids between asparagines from one motif to the next was found to be 24, 26, 21, 24, 25, 21, 24, 25, and 24, 26, 21, 24, 25, and this periodicity supports the three motifs per unit arrangement with 21 amino acids only between motifs which are in adjacent units. This arrangement suggests that for lumican the original leucine motif was duplicated twice to form a unit of three motifs, and then this unit was duplicated twice to produce a total of nine leucine-rich motifs. This organization of leucine-rich motifs has not been reported for other proteoglycan members of this gene family, but analysis of their sequence indicates they all contain a similar arrangement of leucine-rich motifs. The spacing of the nine comparable leucine-rich repeats in decorin (16) is 24, 24, 21, 24, 26, 21, 24, 24. In biglycan (17) the spacing of repeats is 25, 24, 21, 24, 25, 21, 24, 24, and in fibromodulin (24) the spacing is 24, 26, 21, 24, 23, 21, 24, 25.

Among the members of the interstitial proteoglycan gene family, lumican is most similar to fibromodulin (24) with 50% identity at the amino acid level. The distance between asparagines in all the leucine-rich repeats in fibromodulin is the same as for lumican except for the distance between repeats 5 and 6 where the distance is 25 amino acids for lumican and 23 amino acids for fibromodulin. Lumican and fibromodulin both have five potential N-glycosylation sites. Four of these sites occur in the leucine-rich region and these sites are in alignment (lumican residue 91, 130, 164, 257). Their fifth potential N-glycosylation sites lie outside of the leucine-rich repeat region and are not in alignment. (Fig. 5) The fifth site of lumican occurs at residue 320. The fifth site of fibromodulin aligns with lumican residue 307. Previous work shows that the first four N-glycosylation sites (starting from the N terminus) of fibromodulin can be glycosylated and receive keratan sulfate chains (25).

There are, however, several distinguishing differences between lumican and fibromodulin. Fibromodulin has a tyrosine-rich 36-amino acid region near the N terminus that is absent in lumican and, as a consequence, the molecular mass of fibromodulin (42,200 Da) is larger than lumican (38,640 Da). The first leucine-rich repeat in fibromodulin is poorly conserved. It has a methionine, a valine and a phenyalanine in place of the leucines and it was not originally (24) designated as a repeat. All of the amino acids substituting for leucine in the repeat are nonpolar amino acids, like leucine, and the position of the asparagine relative to the nonpolar amino acids is conserved. Finally, this report shows RNA from chick sclera, which is cartilage and would presumably contain mRNA for fibromodulin, does not hybridize with the lumican clone in a Northern blot. These observations indicate that lumican and fibromodulin are related but separate genes.

It has been estimated that chick lumican contains two to three N-linked oligosaccharides with keratan sulfate side chains and an additional two to three N-linked oligosaccharides that do not receive keratan sulfate (10, 15). This suggests that all five of the potential N-glycosylation sites on lumican may be glycosylated, and that only two or three of these will receive keratan sulfate side chains. The immunoprecipitated lumican precursor protein was determined to have a Mr of 51,000 (11). The N-linked oligosaccharides on the precursor protein would be unprocessed and each oligosaccharide would have a Mr of 2,600. The predicted relative molecular mass of lumican without the 18-amino acid signal peptide but con-
taining five unprocessed N-linked oligosaccharides is 49,800 Da. The similarity between the predicted and determined sizes of lumican precursor protein strongly supports N-glycosylation of all five sites.

The leucine-rich repeat is found in at least 19 other proteins. It may also be due to alternative splicing of the lumican transcript. The core protein of decorin has been shown to bind to collagen with the other U2 snRNP proteins (28).

In support of this, antibodies to lumican have been shown to immunoprecipitate a doublet of 38,000 and 39,000 from corneal fibroblasts, and intestine. This broad band could be intermediate with the other U2 snRNP proteins (28).

The similarity between the predicted and determined acid spacing between some of its repeats and distances close to the levels for the tissues tested. This data confirm and extend a previous study which detected lumican in extracts of various tissues from bovine using polyclonal antibodies to the core protein in an enzyme-linked immunosorbent assay (43). It may be that as in the cornea, lumican functions in other tissues to regulate collagen fibril growth and organization in the extracellular matrix.

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