Mimecan, the 25-kDa Corneal Keratan Sulfate Proteoglycan, Is a Product of the Gene Producing Osteoglycin*

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Bovine cornea contains three unique keratan sulfate proteoglycans (KSPGs), of which two (lumican and keratocan) have been characterized using molecular cloning. The gene for the third protein (KSPG25) has not been identified. This study examined the relationship between the KSPG25 protein and the gene for osteoglycin, a 12-kDa bone glycoprotein. The N-terminal amino acid sequence of KSPG25 occurs in osteoglycin cDNA cloned from bovine cornea. The osteoglycin amino acid sequence makes up the C-terminal 47% of the deduced sequence of the KSPG25 protein. Antibodies to osteoglycin reacted with intact corneal KSPG, with KSPG25 protein, and with a 36-kDa protein, distinct from lumican and keratocan. KSPG25-related proteins, not modified with keratan sulfate, were also detected in several connective tissues. Northern blot analysis showed mRNA transcripts of 2.4, 2.5, and 2.6 kilobases in numerous tissues with the 2.4-kilobase transcript enriched in ocular tissues. Ribonuclease protection analysis detected several protected KSPG25 mRNA fragments, suggesting alternate splicing of KSPG25 transcripts. We conclude that the full-length translation product of the gene producing osteoglycin is a corneal keratan sulfate proteoglycan, also present in many non-corneal tissues without keratan sulfate chains. The multiple size protein products of this gene appear to result from in situ proteolytic processing and/or alternative splicing of mRNA. The name mimecan is proposed for this gene and its products.

The corneal stroma of vertebrate organisms contains a unique class of molecules, corneal keratan sulfate proteoglycans (KSPGs), consisting of several related proteins each bearing keratan sulfate. The specialized nature of corneal proteoglycans was recognized almost 60 years ago with the initial description of keratan sulfate, the most abundant glycosaminoglycan in cornea (1). Corneal keratan sulfate is a highly sulfated, linear polymer of N-acetyllactosamine, linked to asparagine residues in the KSPG core proteins (2). The unusual abundance of keratan sulfate in the cornea and studies of heritable metabolic diseases suggest that the KSPGs are essential in maintenance of corneal transparency (3, 4). Understanding the role of the KSPGs in corneal transparency, their interactions with cells and other matrix molecules, and the tissue-specific nature of their biosynthesis requires complete knowledge of their structure. Although we have excellent information regarding the carbohydrate components of the KSPGs, the core proteins as representatives of the primary KSPG gene products are still not fully understood.

Research from our laboratory has shown that keratan sulfate is attached to three unique proteins in bovine cornea (5, 6). The cDNAs for two of these proteins, keratocan and lumican and lumican (originally designated 37A and 37B), have been cloned and sequenced (7, 8). A third 25-kDa KSPG core protein, KSPG25, was recognized as unique from lumican and keratocan, but its primary sequence is not yet known. The deduced amino acid sequences of lumican and keratocan identify them as members of a group of small leucine-rich proteoglycans (SLRPs) (7, 8). These proteins share a common β-pleated sheet tertiary structure generated by a series of characteristic leucine-rich repeats. Other members of this group are common components of the extracellular matrix of a number of tissues. They include another keratan sulfate-containing proteoglycan, fibromodulin, and the dermatan sulfate proteoglycans decorin biglycan, and epiphycan (9). A seventh member of the family, isolated from bone, was originally called osteoinductive factor and later renamed osteoglycin (10, 11). The deduced amino acid sequence obtained from the cDNA of this gene suggests it is potentially a proteoglycan and a member of the SLRP family (12). The only known protein product of this gene is osteoglycin, a 12-kDa protein representing the C-terminal 35% of the coded protein (13). Osteoglycin is thought to be a proteolytic fragment of a larger translation product; however, a full-length protein has not been identified. In this study, we identified the KSPG25 protein as a product of this gene, and found that the KSPG25 protein is secreted by keratocytes as a full-length translation product. We propose the name mimecan for this gene and its products.

**EXPERIMENTAL PROCEDURES**

**Materials**—Preparation of bovine corneal KSPG, endo-β-galactosidase, and antibody against KSPG proteins has been described previously (14–17). Antibodies recognizing the N terminus of the KSPG25 protein were affinity-purified from anti-KSPG γ-globulin by two rounds of adsorption to a 2-ml column of SulfoLink gel (Pierce), on which was immobilized 5 mg of a synthetic peptide matching the deduced N-terminal sequence of KSPG25 (NH2-DEGTPIPLLKKENDEMTPCOOH) using a procedure described previously (8). Monoclonal antibody against bovine osteoglycin was generously donated by Celtrix Biologicals (18). N-Glycanase was obtained from Genzyme Corp. (Cambridge, MA).

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Immunoblotting—Tissues from slaughter-aged steers were minced and extracted in 10 volumes of 4 M guanidine-HCl in the presence of protease inhibitors (17). The extracts were dialyzed to 6 M urea containing 0.1 M Tris phosphate, pH 6.8, and stored frozen at −20 °C. Before electrophoresis, the extracts were treated overnight at 4 °C with 0.015 units/ml affinity-purified endo-β-galactosidase (14). Proteins were separated by electrophoresis in a 7.5–15% gradient SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (16). Antigenic proteins were then detected by immunoblotting as described previously (8, 19).

**Generation of cDNA by Reverse Transcriptase - Polymerase Chain Reaction**—Total RNA was isolated from different bovine tissues and from cultured bovine keratocytes by extraction with guanidinium thiocyanate (8). First strand cDNA was generated from 5 μg of total RNA from cornea and keratocytes using cloned Moloney murine leukemia virus reverse transcriptase (Superscript I, Life Technologies, Inc.) using the buffers supplied by the manufacturer. The single-stranded cDNA was amplified by polymerase chain reaction using a pair of oligonucleotide primers designed from the published bovine osteoglycin cDNA sequence. Primer pairs BOG307 (AATATTGTTTGGATGCTTC, sense) and BOG2132 (AGCCTATCTGCTGGTCTGG, antisense) were used to generate a 926-bp amplified product. This product was gel-purified and used as a template for primers BOG 472 (CAGGACCACCATCTCGACG, sense), and BOG 1080 (CCAAATGAGGAGAACGAGG, antisense) to produce a 608-base pair amplified product. These fragments were cloned into the pGEM-T plasmid (Promega Corp., Middleton, WI). Sequence analysis confirmed that the two fragments represent a partial sequence of the bovine osteoglycin cDNA, with the shorter 608-bp fragment contained within the larger 926-bp fragment.

**Screening of a Bovine Corneal cDNA Library**—A Uni-ZAP XR cDNA library made from cultured bovine keratocytes as described previously (8) was screened using the 926-bp PCR fragment as probe. Initial screening was done at high plating density (20–30 × 10^6 plaques/150-mm plate) and indicated high abundance (about 0.08%) of clones for subsequent rescreening at a low plating density. After the third screening, two clones were excised from the phage vector and collected for subsequent rescreening at a low plating density. After the third screening, two clones were excised from the phage vector and subjected to sequence analysis. The clones with the largest insert (clone 60) was used for further studies. Full bi-directional DNA sequence of the insert DNA was obtained with a redundancy of >2 in each direction using automated sequencing with primers generated from the published sequence (8).

**Construction of Antisense Riboprobes**—The HindIII-BglII DNA fragment containing the bovine keratan cDNA (positions 592–961) was cloned into the HindIII-BamHI sites of the pGEM-1 plasmid (Promega). Antisense RNA for RNase protection was transcribed by T7 RNA polymerase from this plasmid linearized with HindIII. For lumican, a BglII-NruI DNA fragment of bovine lumican (positions 548–1099) was cloned into the BamHI-SmaI sites of pGEM-1. The lumican antisense RNA probe was transcribed by T7 RNA polymerase from this plasmid linearized with HindIII. For KSPG25, a 662-bp HindIII fragment (positions 298–960) was cloned into the HindIII site of pGEM-1. The orientation with respect to the vector’s polylinker was determined by restriction enzyme mapping. KSPG25 antisense RNA probe was generated by SP6 RNA polymerase from the plasmid linearized with T7 polymerase. In *in vitro* transcription reactions with SP6 and T7 RNA polymerases were performed as described previously (20) using [α-32P]UTP (NEN Life Science Products) at a specific activity of 2 × 10^6 cpm/mmol. Integrity of the RNA transcripts was examined by agarose gel electrophoresis.

**RNase Protection Analysis**—For RNase protection, 1 μg of total RNA and 1 × 10^6 cpm of each probe were dripped under vacuum and resuspended in 30 μl of hybridization buffer (80% deionized formamide, 0.4 M NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA). Samples were heated at 90 °C for 3 min, and hybridization was carried out at 60 °C for 14–16 h. RNase A/T1 mixture (Ambion, Austin, TX) was added to each sample and digestion was carried out for 30 min at 37 °C. Then 20 μl of 10% SDS and 10 μl 1 mg/ml proteinase K were added to each sample, and incubation was continued for 30 min at 37 °C. Samples were extracted with phenol/chloroform and precipitated with ethanol. The protected fragments were resolved on 6% denaturing polyacrylamide gel and detected by autoradiography of the dried gel. RNase protection was performed on two independent RNA preparations. Northern blotting and hybridization were carried out on 5 μg of total RNA from keratocyte cultures and various bovine tissues essentially as described previously (8).

**RESULTS**

We previously reported a 13-residue amino acid sequence obtained from the 25-kDa protein of bovine KSPG (5). A search of computer data bases of known sequences revealed a close similarity between this sequence and the open reading frame of a cDNA (GenBank accession number M37974) that codes for a protein originally named bovine osteoinductive factor, later renamed osteoglycin. As shown in the *top panel* of Fig. 1, the match occurs in the interior of the deduced sequence not at the N terminus of a protein, and differs from the experimentally determined KSPG25 sequence by at least 2 amino acids. The similarity, nevertheless, was at least 10 of 13 amino acids, strongly suggesting a relationship between KSPG25 and this gene. We searched for the presence of M37974-related mRNA in bovine cornea by reverse transcription-PCR using two sets of primers made from the published cDNA sequence. As shown in the *bottom panel* of Fig. 1, DNA of the predicted size was amplified by an initial set of primers from both cultured keratocyte and corneal cDNA. Re-amplification of this product with a inner set of primers also gave a DNA product of the size predicted from clone M37974. Cloning of the PCR product and subsequent sequencing (data not shown) confirmed that the cDNA amplified from corneal mRNA was closely related or identical to the M37974 sequence.

The DNA insert in the cloned PCR product was used as a probe to screen a λ Zap expression library made from cultured bovine keratocyte mRNA. A purified clone that hybridized with the PCR product was sequenced and found to be identical to the published bovine cDNA sequence coding for osteoglycin, with the exception of eight single-base locations. Of these differences, shown with asterisks in Fig. 2, the most significant occurs at base 665, in which replacement of adenosine in the published sequence with cytosine resulted in the alteration of the deduced amino acid sequence at position 79 from Asn to Thr. This change occurs at the observed mismatch between the published and experimental amino acid sequence (Fig. 1) resolving one of the discrepancies between the published and experimentally determined amino acid sequence.

As shown in Fig. 3, the experimentally determined KSPG25 sequence represents the N terminus of a 233-amino acid protein with a calculated molecular size of 25 kDa. The experi-
mentally determined sequence of osteoglycin comprises the C-terminal 105 amino acids of the KSPG25 protein. A full-length mature protein coded by this cDNA would have a calculated molecular mass of 34 kDa excluding an 18-amino acid signal peptide.

**Demonstration That the Osteoglycin Gene Product Is a KSPG**

We found previously that three unique core proteins can be separated from purified corneal KSPG by a combination of ion exchange chromatography and SDS-PAGE after enzymatic removal of the keratan sulfate chains (6). These proteins, separated as shown in Fig. 4A, were subjected to immunoblotting with two antibodies having specificity for the osteoglycin gene product. In Fig. 4B, proteins were detected using an antiserum specific for the amino acid sequence from the N-terminus of the KSPG25 protein (amino acids 77–95). In Fig. 4C, a monoclonal antibody raised to the bovine osteoglycin protein was also used. In both cases, the antibodies bound primarily to the 36-kDa band in lane 1, shown previously to be a glycosylated form of the KSPG25 protein (6). In longer exposures of these blots, weak interaction was also observed of both antibodies with a protein band in lane 2 in the 48–50-kDa size range (see below).

Immunoblotting of unfractionated corneal extract with either of these two KSPG25-specific antibodies showed immunoreactive compounds separated as a smear of molecules with large and heterogeneous molecular sizes (Fig. 4D, lane 1).
Digestion of the keratan sulfate in the extract using endo-β-galactosidase, an enzyme that hydrolyzes internal bonds of keratan sulfate chains, released the 35S-labeled antigens into two bands of 36 and 48 kDa (Fig. 4D, lane 2). Release of the osteoglycin-related proteins by endo-β-galactosidase confirms these proteins as core proteins of corneal keratan sulfate proteoglycans.

The nature of the two antigenically related proteins in the corneal extract was further investigated by ion exchange high performance liquid chromatography separation of KSPG proteins after treatment with endo-β-galactosidase, shown in Fig. 5A. The elution characteristics of these proteins under these conditions are dependent on their substitution with sialic acids (6). Distribution of lumican and keratan in the fractions was identified after SDS-PAGE by immunoblotting with antibodies specific for these two proteins (Fig. 5B). In Fig. 5C, immunoblotting of the separated proteins using the anti-osteoglycin monoclonal antibody is shown. The smaller (25 kDa) form of the KSPG25 protein co-elutes with keratan, but can be distinguished by its size. The larger protein reacting with the anti-osteoglycin monoclonal antibody eluted later than the 25-kDa form. Its elution position partially overlapped the elution position of lumican but was distinct from lumican. Lumican eluted in two peaks under these conditions, but the larger of KSPG25-related protein was detected only in a trailing shoulder of the first lumican peak. This pattern of reactivity shows that the osteoglycin antibody identified a unique KSPG25-related protein in these fractions and was not merely exhibiting cross-reactivity with lumican or keratan.

Multiple sizes of KSPG proteins have been observed previously in species other than bovine, suggesting that KSPG25 may not be limited to bovine cornea (16, 21). We examined purified KSPG preparations from mouse, human, bovine, and chicken with the monoclonal anti-osteoglycin antibody. As shown in Fig. 6A, treatment of human KSPG with endo-β-galactosidase released a protein, somewhat smaller than the bovine KSPG25 protein, that reacted strongly with the antibody. The other KSPG preparations did not show specific reactivity with this antibody (data not shown). These results indicate that the protein product of the gene producing osteoglycin is a KSPG core protein in human as well as bovine corneas.

The experimentally determined N terminus of the bovine 25-kDa corneal KSPG protein occurs at amino acid residue 77 of the deduced amino acid sequence, immediately preceded by a lysine, a site of potential cleavage by serine proteases. The smaller form of this protein may arise, therefore, from proteolytic cleavage of a larger form of this protein. In Fig. 6B, immune precipitation of pulse-labeled proteins in keratocyte cultures was carried out using anti-KSPG25 peptide antibody to examine potential precursor-product relationship of the large and small forms of the corneal KSPG25 proteins. After a 30-min labeling period with [35S]methionine and a 2-h chase in unlabeled medium, KSPG25 proteins were precipitated and treated with N-glycanase to deglycosylate the proteins fully, and then separated by SDS-PAGE. As shown in Fig. 6B, the major labeled component precipitated was a protein exhibiting a molecular mass estimated as 36 kDa, slightly larger than the 34-kDa mature protein predicted from the sequence in Fig. 2. Little intracellular KSPG25 protein was present at 4 and 8 h after the pulse, but a similar 36-kDa protein was immunoprecipitated in the culture medium after 4 h and was present at 8 and 16 h after the labeling. A smaller, 25-kDa form was not observed during this time.

In addition to cornea, a number of tissues were found to have proteins recognized by the antibodies to the KSPG25 protein. As shown in Fig. 6C, proteins reacting to the antibody were

![Figure 5](image-url)  
**FIG. 5.** Ion exchange high performance liquid chromatography analysis of KSPG core proteins. Proteins released from purified bovine KSPG with endo-β-galactosidase were separated by anion exchange chromatography using a NaCl gradient as described previously (15). Total protein, detected using the Bradford assay (27), is shown in A. In B, lumican (solid line) and keratan (dotted line) were detected by immunoblotting with specific antibodies following SDS-PAGE separation of proteins from each fraction. Values on the abscissa represent intensity of the immunostained bands from the blots. C shows a similar analysis of fractions with monoclonal antibody against osteoglycin. The inset is a photograph of the blot illustrating the different sizes of the two protein components reacting with this antibody.

![Figure 6](image-url)  
**FIG. 6.** Expression of KSPG25 proteins. A, purified KSPG preparations from bovine and human corneas treated (+) or untreated (−) with endo-β-galactosidase were separated by SDS-PAGE and KSPG25-related proteins detected with the osteoglycin monoclonal antibody as described under “Experimental Procedures.” B, cultures of keratocytes were pulse-labeled for 30 min with [35S]methionine, transferred to unlabeled culture medium, and incubated an additional 2, 4, 8, or 16 h before KSPG25 antigenic proteins were immune-precipitated from both the cell layer and the culture medium using the peptide antibody to the N terminus of the KSPG25 proteins as described under “Experimental Procedures.” The immune-precipitated proteins were digested with N-glycanase to remove all glycosylation before separation by SDS-PAGE and autoradiographic detection. C, proteins from unfractionated guanidine-HCl extracts of a variety of tissues were separated by SDS-PAGE on 7–15% gels and KSPG25-related proteins detected on blots using the osteoglycin monoclonal antibody. +, pretreatment with endo-β-galactosidase; −, not treated with endo-β-galactosidase.
most abundant in cornea, sclera, cartilage, aorta, and skin. Antigenic proteins in extracts of bone were much less abundant than in other tissues. The intact antigenic molecules in non-corneal tissues were smaller and more homogeneous than those in cornea, and the molecular size was not reduced as dramatically by treatment with endo-β-galactosidase. These results indicate that only in cornea is the KSPG25 protein linked to long, sulfated keratan sulfate chains. The proteins released by endo-β-galactosidase of non-corneal tissue extracts exhibited a molecular mass of about 44–46 kDa, clearly differing from the two forms of corneal KSPG25.

**KSPG25 mRNA—** Northern blotting using total RNA from a range of bovine tissues (Fig. 7) showed the presence of KSPG25 transcripts in many tissues. Binding of the probe to corneal and scleral RNA was strongest, suggesting higher steady state concentrations of KSPG mRNA in these tissues. KSPG25 mRNA was distributed more widely than lumican transcripts, and both were more generally present than keratocan mRNA. Unlike lumican and keratocan, several sizes of KSPG25 transcripts were present in different tissues. The major form in cornea and cultured keratocytes of 2.4 kb was clearly distinguishable from the 2.5-kb form present in most tissues. A minor 2.6-kb band was present in some tissues as well.

RNase protection was carried out on samples of total RNA using mixed probes for KSPG25, lumican, and keratocan. Using this technique (shown in Fig. 8), the abundance of the protected fragments reflects the quantitative relationships among these transcripts within a single tissue as well as among tissues. Of several antisense probes examined, all KSPG25 probes produced multiple protected fragments, suggesting the possibility of splice variants of KSPG25 mRNA. The 660-base probe used in Fig. 8 generated two protected fragments ~600 bases. The intensity of the these protected mRNA fragments varied among the different tissues. The smaller fragment was particularly strong in cornea and sclera, whereas the larger fragment was weak in these tissues but represented the strongest band in several other tissues. The single protected lumican mRNA fragment was found with similar intensity to the KSPG25 mRNA but in fewer overall tissues. Keratocan was the least highly expressed mRNA and of the most limited distribution. RNase protection products of keratocan were present in detectable levels only in cornea, sclera, and cultured keratocytes.

**DISCUSSION**

The work presented here ties together independent lines of research to show that two proteins having no apparent *a priori* relationship to one another are actually products of the same gene, and are likely to be proteolytic products of the same propeptide. The relationship between KSPG25 and osteoglycin was established initially in the identity of 10 of 13 amino acids in a GenBank search, a coincidence that is unlikely in unrelated proteins. We went on to identify mRNA with the osteoglycin sequence in cornea and to show that the KSPG25 amino acid sequence was part of the open reading frame present in this RNA. Northern blotting showed that this mRNA was present in many tissues but was most abundant in sclera, cornea, and cultured keratocytes. A monoclonal antibody made to bovine osteoglycin and a polyclonal antibody affinity-purified against the KSPG25 N-terminal peptide (a sequence of amino acids not present in the osteoglycin protein) both bound the same protein. This protein was identified as KSPG25 by its chromatographic properties, its attachment to keratan sulfate, and its molecular size on SDS-PAGE. All of these observations led to the conclusion that the gene originally identified as that producing osteoglycin is the gene for the corneal KSPG 25-kDa protein.

The protein known as osteoglycin, isolated from demineralized bone, was originally named osteoinductive factor (OIF). Later, it was determined that co-purifying bone morphogenic proteins were the source of its growth stimulatory activity in this preparation, and the protein and its gene were renamed osteoglycin. Our current results add a new level of understanding about this gene and its products. Western and Northern blotting show that both mRNA and protein products of this gene are common in connective tissues but most abundant in eye tissue. Immunoreactive material was not abundant in extracts of bone, demonstrating that expression of this gene is not bone-related. The major protein bands detected by the anti-osteoglycin monoclonal antibody were at least as large as the corneal KSPG25; thus, the 12-kDa “osteoglycin” form of this protein is not common. These observations suggest that the name osteoglycin may be inappropriate for this gene and its major translation products. The small and elusive nature of

**FIG. 7. Tissue distribution of mRNA transcripts of KSPG proteins.** Total RNA (5 μg) from a variety of tissues was separated by electrophoresis and transferred to nylon membranes, and mRNA for KSPG proteins detected by hybridization with 32P-labeled cDNA probes as described under “Experimental Procedures.” A, RNA reacting with a probe for KSPG25. B, RNA reacting with a probe for keratocan. Each probe had similar specific activity, and each autoradiogram was exposed a similar length of time; thus, intensity of the bands should reflect the abundance of the three RNA species.

**FIG. 8. RNase protection analysis of mRNA for the three KSPG proteins.** Excess 32P-labeled antisense RNA probes for mimecan (KSPG25), lumican, and keratocan were hybridized with 1 μg of total RNA from the same panel of bovine tissues shown in Fig. 7. RNase digestion and separation of protected probes was carried out as described under “Experimental Procedures.” Identification of the fragments (shown on the left) was based on experiments with individual probes. Migration of end-labeled DNA molecular size markers is shown on the right.
this protein suggested the name “mimecan” for this gene and its products, based on the deceitful dwarf, Mime, from Wagnerian opera and Norse legend whose head continued to survive and was consulted as a source of wisdom even after being beheaded.

The probes we used to detect the mimecan products identified several forms of this protein and its mRNA. Western blotting identified a minor corneal KSPG core protein antigenically related to KSPG25 but of a size similar to lumican. This material exhibited different chromatographic properties from lumican and keratocan and thus did not appear to be a result of antigenic cross-reactivity with these proteins, suggesting the possibility that it was a full-length translation product of the mimecan cDNA. Immune precipitation of mimecan-related proteins from pulse-labeled keratocyte cell cultures identified only a larger not the 25-kDa form. The estimate of 36 kDa for this protein after enzymatic deglycosylation makes it consistent with a full-length translation product having calculated molecular mass of 34 kDa, based on the cDNA sequence. Our interpretation of these results is that the mimecan gene product is secreted as a full-length protein and in situ is cleaved to form the 25-kDa form that predominates in the adult bovine cornea. A Lys residue just next to the N terminus of the 25-kDa protein could serve as a cleavage site for serine proteases. Proteolytic cleavage might also be the explanation for the apparently larger size of the antigenically related protein present in nonocular tissues. However, the data are not conclusive regarding the origin of the non-corneal form of the mimecan protein.

Northern blotting revealed three distinct size classes of mimecan mRNA that varied in abundance depending on the tissue source (Fig. 7). The smallest (2.4 kb) form was predominant in cornea and sclera. Such heterogeneity can arise as a result of alternate splicing of RNA or by alternate usage of tissue source (Fig. 7). The smallest (2.4 kb) form was predominant in cornea and sclera. These results are consistent with the presence in cornea and sclera of a 5′ splice variant that is about 100 bases shorter than the predominant mimecan RNAs of non-ocular tissues. Should the splice site occur in the translated region of the mRNA, it could be responsible for the difference in protein sizes observed between ocular and non-ocular tissues (shown in Fig. 6C). Investigations are currently under way in our laboratory to clarify the nature of these heterogeneities in mimecan mRNA and protein size.

Since its initial cloning, the mimecan gene has been recognized as a member of the gene family known as SLRPs. Alignment of the amino acid sequence of the mimecan protein with that of lumican and keratocan does not show the high sequence identity present between lumican and keratocan. However, comparison of structural domains among all three proteins, as shown in Fig. 9, indicates good conservation of certain molecular features. Each of the three proteins has one or more tyrosines adjacent to acidic amino acids (Glu or Asp) in the N-terminal region. These represent consensus sites for tyrosine sulfation.

Each of the three KSPG proteins has 4 cysteines immediately preceding the leucine-rich domains. Each has five evenly spaced leucine-rich domains following the N-terminal cysteines and a C-terminal leucine-rich domain centered between two Cys moieties spaced 33/34 residues apart. One consensus site for N-linked glycosylation is common among all three sequences, just N-terminal to the fifth leucine-rich repeat. This site has recently been shown to be modified with keratan sulfate in chicken limican, and thus serves as the leading candidate for the location of the single keratan sulfate chain attached to mimecan (23). Closer comparison of these features may help us understand exactly what sequence elements are required for a SLRP protein to become a KSPG.

As with lumican and keratocan, the mimecan protein is present in many tissues as a non-sulfated glycoprotein. Treatment with endo-β-galactosidase shifted the molecular size of this protein up to 10 kDa in several tissues indicating that, like arterial lumican, these proteins are modified with unsulfated oligolactosamine (24). In cornea, mimecan as well as the other two KSPG proteins is transcribed and translated several fold greater than the other tissues in which they are found. This phenomenon appears to be a new example of the phenomenon known as gene sharing. In the lens, genes for common intracellular proteins are massively overexpressed to produce the crystalline proteins that provide transparency to this tissue (25). Recent studies of transketolase in the corneal epithelium suggest a similar mechanism may also be important to corneal transparency (26). In the kerocytes, it would appear that an entirely analogous process is taking place with a family of common extracellular proteins. In the stroma, however, a tissue-specific glycosylation of these proteins converts them from common extracellular matrix glycoproteins to a unique class of proteoglycans. Elucidation of mechanisms by which keratocytes upregulate expression of these genes and then provide unique glycosylation to selected sites on the proteins will greatly enhance our understanding of how this tissue obtains transparency and how it might be restored in pathological situations.

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REFERENCES
1. Suzuki, M. (1939) J. Biochem. (Tokyo) 30, 185–191
2. Hassell, V. C. (1982) Prog. Clin. Biol. Res. 110B, 3–15
3. Hassell, J. R., Newsome, D. A., Kucharner, J., and Rodrigues, M. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3705–3709
4. Funderburgh, J. L., Funderburgh, M. L., Mann, M. M., and Conrad, G. W. (1991) Biochim. Biophys. Acta 1071, 871–876
5. Funderburgh, J. L., and Conrad, G. W. (1990) J. Biol. Chem. 265, 8287–8303
6. Funderburgh, J. L., Funderburgh, M. L., Mann, M. M., and Conrad, G. W. (1991) J. Biol. Chem. 266, 14226–14231
7. Funderburgh, J. L., Funderburgh, M. L., Brown, S. J., Vergnes, J. P., Hassell, J. R., Mann, M. M., and Conrad, G. W. (1993) J. Biol. Chem. 268, 11874–11880
8. Corpas, I. M., Funderburgh, J. L., Bottomley, G. W., Prakash, S., and Conrad, G. W. (1996) J. Biol. Chem. 271, 9759–9753
9. Iozzo, R. V. (1997) Crit. Rev. Biochem. Mol. Biol. 32, 141–174
10. Bonta, H., Nathin, R. M., Rosen, D. M., Armstrong, R. M., Thompson, A. Y., Segarini, P. R., Mathews, M. C., Dasch, J. R., Piez, K. A., and Seyedin, S. M. (1989) J. Biol. Chem. 264, 20805–20810
11. Bonta, H., Thompson, A. Y., Armstrong, R., Chang, R. J., Piez, K. A., and Rosen, D. M. (1991) Matrix 11, 269–275

FIG. 9. Structural features of the three KSPG proteins. Structural domains present in the amino acid sequences of mimecan, lumican, and keratocan are compared graphically. Circles show consensus sites for tyrosine sulfation. Broken lines connect adjacent Cys pairs. Open rectangles mark leucine-rich repeat regions (LXXLLXXX, where L is a hydrophobic amino acid, N is Asn, and X is any amino acid). Incomplete leucine-rich repeats in which the first or second consensus site does not contain a hydrophobic moiety are shown as shaded rectangles. Arrows show consensus sites for N-glycosylation, and the asterisk marks the proposed site for keratan sulfate attachment to mimecan. The short vertical line on mimecan marks the experimentally determined N terminus.
KSPG25 and Osteoglycin Are Products of the Mimecan Gene

12. Madisen, L., Neubauer, M., Plowman, G., Rosen, D., Segarini, P., Dasch, J., Thompson, A., Ziman, J., Bentz, H., and Purchio, A. F. (1990) DNA Cell Biol. 9, 303–309
13. Bentz, H., Chang, R. J., Thompson, A. Y., Glaser, C. B., and Rosen, D. M. (1990) J. Biol. Chem. 265, 5024–5029
14. Fukuda, M. N. (1981) J. Biol. Chem. 256, 3900–3905
15. Funderburgh, J. L., Caterson, B., and Conrad, G. W. (1987) J. Biol. Chem. 262, 11634–11640
16. Jost, C. J., Funderburgh, J. L., Mann, M., Hassell, J. R., and Conrad, G. W. (1991) J. Biol. Chem. 266, 13336–13341
17. Conrad, G. W., Ager-Johnson, P., and Woo, M. L. (1982) J. Biol. Chem. 257, 464–471
18. Dasch, J. R., Pace, D. R., Avis, P. D., Bentz, H., and Chu, S. (1993) Connect. Tissue Res. 30, 11–21
19. Funderburgh, J. L., Funderburgh, M. L., Mann, M. M., Prakash, S., and Conrad, G. W. (1996) J. Biol. Chem. 271, 31431–31436
20. Tasheva, E., and Roufa, D. (1995) Genes Dev. 9, 304–316
21. Funderburgh, J. L., Stenzel-Johnson, P. R., and Chandler, J. W. (1983) Curr. Eye Res. 2, 769–776
22. Antonsson, P., Heinegard, D., and Oldberg, A. (1991) J. Biol. Chem. 266, 16859–16861
23. Hassell, J., Dunlevy, J., J.-P. V., and Neame, P. (1997) Invest. Ophtalmol. Vis. Sci. 38, S681
24. Funderburgh, J. L., Funderburgh, M. L., Mann, M. M., and Conrad, G. W. (1991) J. Biol. Chem. 266, 24773–24777
25. Piatigorsky, J., Gopal-Srivastava, R., and Tomarev, S. I. (1994) EXS 71, 241–250
26. Sax, C. M., Salamon, C., Kays, W. T., Guo, J., Yu, F. X., Cuthbertson, R. A., and Piatigorsky, J. (1996) J. Biol. Chem. 271, 33568–33574
27. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254