Identification in Vitreous and Molecular Cloning of Opticin, a Novel Member of the Family of Leucine-rich Repeat Proteins of the Extracellular Matrix*

Anthony J. Reardon‡‡, Magali Le Goff‡‡, Michael D. Briggs‡‡, David McLeod‡, John K. Sheehan‡, David J. Thornton‡, and Paul N. Bishop‡‡‡

From the ¤Wellcome Trust Centre for Cell-Matrix Research, School of Biological Sciences, Room 2.14 Stopford Building, University of Manchester, Oxford Road, Manchester M13 9PT, the ¶Research Group in Eye and Vision Science (The Medical School), Royal Eye Hospital, Oxford Road, Manchester M13 9WH, United Kingdom

A prominent 45-kDa component was identified by protein staining following SDS-polyacrylamide gel electrophoresis of a 4 M guanidine hydrochloride extract from bovine vitreous collagen fibrils. Peptide sequences obtained from this component were used as a basis for the cloning (from human retinal cDNA) and sequencing of a novel member of the leucine-rich repeat extracellular matrix protein family that we have named opticin. Opticin mRNA was found by reverse transcription polymerase chain reaction (RT-PCR) in ligament and skin as well as in retina. An open reading frame containing 332 amino acids was identified, the first 19 amino acids representing a signal peptide. The deduced amino acid sequence of the mature protein encodes a 35-kDa protein with a calculated isoelectric point of 5.4. The central domain of this protein consists of six B-type leucine-rich repeats. This domain is flanked by cysteine clusters including a C-terminal two-cysteine cluster containing an additional leucine-rich repeat. The N-terminal region contains a cluster of potential O-glycosylation sites, and analysis of bovine vitreous opticin demonstrated the presence of sialylated O-linked oligosaccharides substituting the core protein. Opticin shows highest protein sequence identity to epiphycan (42%) and osteoglycin (55%) and belongs to Class III of the leucine-rich repeat extracellular matrix protein family.

The vitreous gel is a highly hydrated, virtually acellular, transparent extracellular matrix (ECM) containing a dilute dispersion of structural macromolecules. It contains thin heterotypic collagen fibrils composed of collagen types II, IX, and V/IX that are essential to its gel structure (1). Hyaluronan is the predominant glycosaminoglycan in mammalian vitreous (2). Smaller quantities of sulfated glycosaminoglycans are also present, and we have previously demonstrated the presence of two CS proteoglycans, versican and type IX collagen (2, 3).

Human vitreous is a gel at birth, but it undergoes an inevitable process of liquefaction with age. Vitreous liquefaction is implicated in the pathogenesis of a number of blinding ocular conditions including rhegmatogenous retinal detachment (4). During age-related vitreous liquefaction, the collagen fibrils aggregate (5). The process of fibril aggregation appears to be the primary event in age-related vitreous liquefaction; therefore, molecules that modulate collagen fibril diameter, fusion, or aggregation could play a key role in this process.

Fibrillar collagen systems in tissues other than vitreous generally contain at least one component that is a member of the leucine-rich repeat (LRR) family of ECM proteins (6). There are currently 10 members of this family: biglycan (7), decorin, fibromodulin (8), PRELP (9), keratan (10), osteoatherin/osteomodulin (11), limican (12), epiphycan/PG-Lb/DSPG3 (13), osteoglycin/mimecan (14, 15), and chondroadherin (16). All of these proteins have a central region containing 6 or 10 copies of a LRR motif that is characterized by the sequence LX(L)XX-, where X can be any amino acid. The leucine residues can be replaced by other amino acids with a hydrophobic aliphatic side chain (Ile, Val, or Met). The central LRR domain is flanked by N- and C-terminal domains. The N-terminal region contains a four-cysteine cluster, and the C-terminal domain has two cysteine residues with an additional LRR consensus sequence between them. With the exception of chondroadherin and PRELP, the LRR family of ECM proteins usually exist as proteoglycans substituted with one or more KS or CS/DS glycosaminoglycan chains. Therefore, the members of this family are sometimes referred to as small leucine-rich proteoglycans. Some members of the family of LRR proteins of the ECM bind noncovalently to collagen fibrils, and their presence is essential for normal fibril (and hence tissue) morphology. Their importance is highlighted by the phenotype of decorin-null (17), limican-null (18), and fibromodulin-null (19) mice, which all have abnormal collagen fibrils with uneven diameter and irregular structure.

We have identified a 45-kDa macromolecule in bovine vitreous that we have shown to be a novel member of the family of LRR proteins of the ECM, and we have determined the primary structure of the human form. We have named this molecule opticin based on the tissue in which it was initially discovered, although its mRNA was subsequently identified in ligament and skin. The 45-kDa bovine vitreous form was not substituted with KS or CS/DS glycosaminoglycan chains but was substituted with sialylated O-linked oligosaccharides.
Identification and Cloning of Opticin

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine eyes (from 2-year-old steers) were obtained from a local abattoir, and the vitreous gels were isolated within 5 h post mortem. Retina, ciliary body, and vitreous were dissected from a pair of human (age 52) eyes 22 h postmortem and pooled. Samples of skin, articular cartilage, and (cruciate) ligament were obtained from diabetic patients (ages 30 and 55 years) following amputations. Hyaluronan lyase from Streptomyces (EC 3.2.1.97) and O-glycosidase (endo-α-N-acetylgalactosaminidase from Streptococcus pneumoniae, EC 3.2.1.97) were obtained from Oxford Glycosystems. The monoclonal antibodies 1B5, 2B6, and 3B3 (1B5, 2B6, 3B3, and 5D4) were obtained from ICN. Sequencing grade modified trypsin was obtained from Promega. Clones containing ESTs (1B5, 2B6, 3B3, and 5D4) were obtained from CLONTECH.

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Identification and Cloning of Opticin

Peptides identified by protein sequence analysis and comparison with the translation of the human cDNA

The 45-kDa opticin components on SDS-PAGE were subjected to N-terminal sequencing (as indicated), or internal tryptic peptides were purified and sequenced. The mass (m/z) of the peptides is shown where applicable. The bovine peptide sequences were compared with the translation of the human cDNA, and equivalent sequences were identified.

| Peptide | Sequence | m/z | Position |
|---------|----------|-----|----------|
| ASLPEE | ND<sup>a</sup> | 20–25 | ASLPRK |
| LQPAIQLQPAFAR | 1336 | LQSSQIGQAFFR | 233–244 |
| XQFLYA | 1715 | LQFLYLQ | 249–254 |
| RPLEDIR | 900 | RQLEDIR | 299–305 |
| LDGBPENLDFPXAY | 1840 | LDGNPNFPLFSAY | 306–320 |

<sup>a</sup> Subjected to N-terminal sequencing.  
<sup>b</sup> ND, not determined.  
<sup>c</sup> Peptide sequence incomplete.

TABLE I

RESULTS

Isolation of Collagen-associated Macromolecules from Bovine Vitreous—Hyaluronan lyase digestion and subsequent washing of vitreous gels resulted in the isolation of collagen fibrils and associated macromolecules, which were then concentrated by centrifugation and extracted with 4 M guanidine HCl. The collagen fibril extract was analyzed by SDS-PAGE on a 4–12% gradient gel, and protein bands were visualized with Brilliant Blue G Coomassie stain (Fig. 1A, lane 2). A number of components were observed that were minor components of an extract containing total soluble vitreous proteins (Fig. 1A, lane 1), thus demonstrating that a very specific subpopulation of vitreous macromolecules was isolated using this procedure. A prominent 45-kDa component that frequently migrated as a doublet was identified in the collagen fibril extract, and this component was subjected to further analysis.

Glycosylation of the 45-kDa Component from Bovine Vitreous—The collagen fibril extract was analyzed using the GlycoTrack<sup>®</sup> carbohydrate detection system, which, by labeling carboxylic groups present in the glycoproteins, detects the presence of glycans. The 45-kDa component was clearly labeled using this system (Fig. 1B, lane 1).  

Pretreatment of the reduced fibril extract with endoglycosidase F peptide-N-glycosidase F had no effect on the mobility of the 45-kDa component as compared with untreated control. The migration on SDS-PAGE of the reduced 45-kDa component after digestion with endoglycosidase F peptide-N-glycosidase F was minimally retarded as compared with the un-reduced form, and this probably reflects the cleavage of intrachain disulfide bonds (Fig. 1A, lane 3).
Following chondroitin ABC lyase digestion of the collagen fibril extract, the electrophoretic migration of the 45-kDa component was unaltered (Fig. 1A, lane 4). The collagen fibril extract was analyzed for the presence of CS/DS proteoglycans by Western blotting using monoclonal antibodies that recognize the terminal stubs of CS/DS chains following digestion with chondroitin ABC lyase (21). The 1B5 antibody recognizes the unsulfated stub, the 2B6 antibody recognizes the 4-sulfated stub, and the 3B3 antibody recognizes the 6-sulfated stub. These antibodies were used in combination to probe the collagen fibril extract with (Fig. 1B, lane 3) and without (lane 2) prior chondroitin ABC lyase digestion. Two components of ~200 kDa were labeled in the chondroitin ABC lyase-digested sample, but the antibodies did not detect any lower molecular weight components. These data demonstrate that the 45-kDa component was not substituted with CS or DS chains and that the collagen fibril extract does not contain known members of the family of ECM LRR proteins substituted with CS or DS chains.

A monoclonal antibody that recognizes highly sulfated KS chains, 5D4 (22), was used to probe the collagen fibril extract by Western blotting; this labeled a diffuse smear of material ~120 kDa but did not detect the 45-kDa component (Fig. 1B, lane 4). The collagen fibril extract was analyzed by SDS-PAGE and Brilliant Blue G colloidal staining after digestion with keratanase II (Fig. 1A, lane 5) and endo-β-galactosidase (lane 6). Neither of these enzymes altered the electrophoretic migration of the 45-kDa component, providing further evidence that it is not substituted with KS chain(s). The fibril extract, after digestion with these two enzymes, was also analyzed by Western blotting with the 5D4 antibody (data not shown). The 5D4 reactivity of the component(s) of ~120 kDa was removed by the enzymes, thus acting as a positive control for these digests.

Partial Purification of the 45-kDa Component—The collagen fibril extract was subjected to two rounds of Superose 12 gel filtration chromatography and then MonoQ anion-exchange chromatography (Fig. 2). SDS-PAGE analysis of fractions from these columns allowed the identification of the 45-kDa component for subsequent purification (data not shown). The 45-kDa component was contained within included fractions following Superose 12 gel filtration chromatography and eluted at 0.2–0.35 M lithium perchlorate at pH 5. The partially purified 45-kDa component was analyzed by SDS-PAGE on a 4–12% gradient gel before and after digestion with sialidase or sialidase and then O-glycosidase (Fig. 2). Digestion with sialidase produced a single band of approximately 40 kDa. Subsequent digestion with O-glycosidase decreased the molecular weight of most but not all of this 40-kDa component to 35 kDa. Digestion with O-glycosidase without prior sialidase digestion produced virtually no effect upon the electrophoretic migration of the 45-kDa component (data not shown).

Protein Sequence Analysis of Opticin—The 45-kDa component was resolved as a doublet by SDS-PAGE, and the N-terminal sequence was obtained from each band following Western blotting with the 5D4 antibody (data not shown). The N-terminal sequence that represents the human equivalents of the bovine sequences obtained by Edman degradation are underlined (solid lines); see Table I. Residues corresponding to potential tyrosine sulfation (*) and N-glycosylation (N) sites are indicated, and the putative site of action of signal peptidase is shown (†). The Ser/Thr-rich region that contains potential O-glycosylation sites is underlined (broken line). The positions of the forward and reverse primer pairs (1 and 2) were used to generate a cDNA clone for screening the MTE™ poly(A)+ RNA array and for PCR-based screening of multiple tissue cDNAs are indicated by dotted lines.
transfer to polyvinylidene difluoride. The N-terminal sequence obtained was ASLPEE in both cases. Each band of the 45-kDa doublet was subjected to in-gel trypsin digestion following SDS-PAGE, and the extracted peptides were analyzed by MALDI-TOF MS. The peptide mass spectrum from each band was identical over the range m/z 600–5000 Da (data not shown), further demonstrating that the two bands were derived from the same protein. Individual peptides were purified and subjected to N-terminal amino acid sequencing (Table I).

**cDNA Cloning and Sequencing**—The derived internal peptide sequences were used to search EST databases (using BLAST). An internal peptide sequence (m/z 1840 Da) almost exactly matched the translation of part of a human EST (GenBankTM accession number AA318186) derived from a retinal cDNA library. This EST sequence was used to search for other ESTs, and two more were identified that contained parts of the same cDNA sequence (GenBank accession numbers AA317820 and AA457366); these were also derived from retinal cDNA libraries. The clones containing these ESTs were obtained, and the inserts were fully sequenced. Analysis of the sequences revealed that these ESTs encoded part of a novel member of the LRR family of ECM proteins and when the sequences were combined they provided approximately 750 bp of cDNA sequence from the 3’ end of the gene (Fig. 3).

The 5’ end of the cDNA sequence was obtained by 5’ rapid amplification of cDNA ends using Marathon-ReadyTM human retinal cDNA. Two primers that were complementary to the EST cDNA sequence (Fig. 3) were used in conjunction with the primers supplied for the 5’ adaptor region for PCR amplification. After two rounds of PCR amplification using nested primers, a single product of approximately 800 bp was obtained, which was cloned and sequenced. The sequencing was performed twice in both directions, and after combining this sequence with the sequence from the EST clone AA318186, a full-length cDNA sequence was obtained (Fig. 3). This sequence and its translation were scanned against the nonredundant data base, and no closely related sequences were identified, showing that it represented a novel full-length cDNA sequence.

**cDNA and Deduced Amino Acid Sequence Analysis**—The full-length cDNA consisted of 1403 base pairs, and an open reading frame was found to be flanked by base pairs 2 and 1100 that encoded a protein of 332 amino acids (Fig. 3). The N-terminal amino acid sequence (ASLPEE) allowed a 19-amino acid signal peptide to be defined. The calculated mature protein has a molecular mass of 35,189 Da and a pI of 5.4. Equivalent human sequences were found for all the bovine peptide sequences obtained by Edman degradation sequencing (Table I). The putative core protein contained an N-terminal domain with a four-cysteine cluster between positions 128 and 140. The central LRR domain contained six B-type LRRs. Two cysteine residues were identified at positions 289 and 322 that define a C-terminal disulfide bonded loop containing a seventh LRR consensus sequence. One potential tyrosine sulfation site was located at Tyr71 (23), and one potential N-glycosylation site was located at position 312. A Ser/Thr-rich domain in the N-terminal region between residues 81 and 112 containing 16 potential O-glycosylation sites was identified using the NetOGlyc 2.0 program (Fig. 3). Furthermore, this program did not predict

![FIG. 4. A dendrogram showing the predicted relationship between the members of the family of LRR proteins of the ECM.](image)

Horizontal distances of bars are proportional to the evolutionary distance and based upon human protein sequences. Where alternative names have been given to the same family members, these names are shown. Three classes have been identified within this family, i.e. Classes I, II, and III, and these are identified with Roman numerals on the dendrogram. The dendrogram was generated using the program CLUSTAL W (Version 1.74), and the output was generated using Njplot.

![FIG. 5. Alignment of the sequences of human opticin, epiphycan and osteoglycin/mimecan.](image)

Residues identical to opticin are shown. Conservative substitutions are indicated (+), and blank spaces represent amino acid differences. Gaps (−) have been inserted to optimize alignment. The LRRs are underlined, and the conserved cysteine residues are shaded. The alignment was performed on human protein sequences using the CLUSTAL W (Version 1.74) software.
that any of the other Ser or Thr residues in the cDNA sequence would be O-glycosylated.

**Similarity to Other Members of the LRR Proteins of the ECM—**A dendrogram comparing the human sequences of the known members of the family of LRR proteins of the ECM (Fig. 4) shows that opticin is a distinct member of this family but is most closely related to epiphycan and osteoglycin/mimecan. These three proteins form one of three clearly definable branches on the dendrogram. This branching pattern has allowed the subclassification of the LRR proteins of the ECM into Classes I, II, and III (24). As well as having the greatest similarities in protein sequence, members of each class have a similar genomic structure and conserved spacing of the four-Cys cluster that is N-terminal to the LRRs. In the Class III members, i.e. opticin, epiphycan, and osteoglycin/mimecan, the spacing of the Cys residues is C\_X\_3CXCX\_2C, whereas in Class II it is C\_X\_3CXCX\_2C and in Class I it is C\_X\_2CXC\_3C. Alignment of the human sequences of opticin, epiphycan, and osteoglycin/mimecan (Fig. 5) revealed that opticin has a 42% sequence identity with epiphycan and a 35% sequence identity with osteoglycin/mimecan. Most similarity was observed in the central LRR-containing and C-terminal region, with the N-terminal regions being more divergent. All three members of this group have six LRRs in the central domain and conserved spacing of the Cys residues in the C-terminal region.

**Northern Blot and Tissue Distribution of Opticin—**Northern blot analysis of the combined total RNA from ocular tissues showed a single 1.4-kilobase mRNA (Fig. 6). This would be the predicted size of the mRNA from the cDNA sequence. Hybridization of the MTE\textsuperscript{TM} poly(A)\textsuperscript{+} RNA array with opticin cDNA did not produce a signal with any tissue-specific poly(A)\textsuperscript{+} RNAs; only the DNA controls were weakly labeled (data not shown). However, the MTE\textsuperscript{TM} array did not contain poly(A)\textsuperscript{+} RNA from the eye or specifically from connective tissues. Therefore, in addition, a PCR-based screening strategy was used. RT-PCR products from human connective tissues and the CLONTECH multiple tissue cDNA panels were screened. Two primer pairs were used, which we have previously shown produce different sized products from genomic DNA. Both primer pairs produced single bands of the correct size with ligament and skin as well as retinal cDNA, demonstrating expression of opticin in these tissues (Fig. 7). None of the other tissue-specific cDNAs produced bands with either opticin primer pair.

**DISCUSSION**

We have shown for the first time that a member of the LRR family of ECM proteins is present in vitreous and is associated with the heterotypic collagen fibrils. Furthermore, we have shown that it is a novel member of this family, and we propose for it the name "opticin," based upon the word optic (from Greek optikos), meaning relating to the eye. Although our data cannot exclude the presence of other LRR proteins of the ECM associated with vitreous collagen fibrils, there do not appear to be any known members of this family that are substituted with CS or DS chains.

Opticin has six LRRs in the central region and an additional LRR between the two Cys residues in the C-terminal domain. The seven LRRs in opticin are heterogeneous in length, containing 24, 24, 20, 26, 21, 31, and 32 residues. The number of residues per repeat matches exactly that found in epiphycan (13) and osteoglycin/mimecan (14). The central five LRRs of these three proteins form a pattern (long-short-long-short-long) that differs from that of decorin, biglycan, PRELP, keratocan, osteoadherin, lumican, and fibromodulin, which all have a triplet repeat pattern (long-long-short). Chondroadherin has repeats that are regularly spaced at intervals of 24 amino acids. The significance of the different numbers and lengths of the LRRs in the family of LRR proteins of the ECM is unclear.

The three-dimensional structure of the LRR protein ribonuclease inhibitor has been determined. Ribonuclease inhibitor possesses 15 LRRs containing 28 or 29 amino acids with each LRR containing a roughly parallel \( \beta \)-strand and \( \alpha \)-helix. These LRRs form a horseshoe-shaped structure in which the \( \beta \)-strands form a parallel \( \beta \)-sheet on the inner concave surface and the \( \alpha \)-helices align on the outer circumference (25). Similar structures have been suggested for other LRR-containing molecules despite the presence of shorter repeats (26), and a model structure of the LRR region of decorin was shown to fit well to the ribonuclease inhibitor structure (27). Therefore, other members of the family of LRR proteins of the ECM, including opticin, may have similar structures.

The 45-kDa form of bovine opticin was not substituted with KS, CS, or DS glycosaminoglycan chain(s). The human opticin...
sequence contained one potential N-glycosylation site at Asn\(^{312}\) but, at least in the 45-kDa bovine vitreous opticin, this was probably not glycosylated. This conclusion was reached because a peptide containing this residue (LDGP\(\text{NXLFPXAY}\)) was sequenced by Edman degradation and a good yield of Asn was obtained. Furthermore, digestion of bovine vitreous opticin with endoglycosidase F-peptide-N-glycosidase F did not alter its electrophoretic migration.

Sequential digestion of the bovine 45-kDa opticin component with sialidase and then O-glycosidase produced a product with an electrophoretic migration at 35 kDa, which is in agreement with the predicted molecular mass of the core protein of opticin. O-Glycosidase specifically cleaves the core structure Galβ1-3GalNAcα1- from Ser/Thr; therefore, some of the oligosaccharides on opticin must be composed of this structure capped with Ser or Thr residues, all of which are potential alternative oligosaccharides with a different core structure. Analysis of the translated human cDNA sequence revealed a 31-amino acid sequence in the N-terminal region containing 16 Ser or Thr residues, all of which are potential O-glycosylation sites; no similar region is found in any of the other known LRR ECM proteins. If this region of the core protein is substituted with endoglycosidase F/peptide-N-glycosidase, it may be important to the function of opticin. Most members of family of LRR proteins of the ECM possess GAG chain(s), but opticin instead has sialylated O-linked oligosaccharides that may provide an alternative form of anionic carbohydrate for certain, as yet undefined, functions. Three members of the family of LRR proteins of the ECM, decorin, lumican, and fibromodulin (28, 29), are known to bind fibrillar collagens; in the case of decorin, the binding sites for type I collagen have been located to the central LRRs (30, 31).

As opticin was extracted specifically from a pool of macromolecules that were associated with vitreous collagen fibrils, this molecule is also likely to bind to fibrillar collagen. The interaction of LRR proteins of the ECM with fibrillar collagens is essential for the regulation of fibril diameter as demonstrated by decorin-, lumican-, and fibromodulin-null mice (17–19). In these mice, the collagen fibrils had irregular cross-sections and variable diameter. In addition, the decorin- and lumican-null mice showed evidence of uncontrolled lateral fusion of collagen fibrils.

During normal aging, human vitreous collagen fibrils aggregate or fuse into thick fibers. This process is associated with vitreous liquefaction (5) and posterior vitreous detachment (4), a splitting of the attachment of the residual vitreous gel away from the inner surface of the retina that eventually occurs in approximately one-quarter of the population (32). Posterior vitreous detachment may in turn predispose to various ocular pathologies, including rhegmatogenous retinal detachment (4). In view of the role of some LRR proteins of the ECM in regulating the shape and lateral fusion of collagen fibrils, it is possible that opticin plays a role in preventing these age-related processes.

The range of tissues analyzed, opticin was found to be expressed in retina, ligament, and skin. Abnormalities in ligaments can cause joint laxity, and there are a number of hereditary conditions in which vitreoretinal disorders and joint laxity co-exist, including some forms of Ehlers-Danlos syndrome (which also have skin abnormalities) and Stickler syndrome. A majority of patients with Stickler syndrome have mutations in the genes encoding collagen types II and V/XI, but a proportion are not linked to collagen genes (33, 34). Therefore, the opticin gene represents a candidate gene for these hereditary conditions.

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