Versican and Fibrillin-1 Form a Major Hyaluronan-Binding Complex in the Ciliary Body

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PURPOSE. In this study, biochemistry, molecular biology, immunohistochemistry, and electron microscopy techniques were used to examine whether versican, which is known to bind fibrillin-1, interacts with fibrillin-1 in the ciliary body and vitreous, and whether the versican in this complex binds to hyaluronan.

METHODS. The new polyclonal antibodies against the amino and carboxyl termini of versican were raised and characterized. The mRNA expression levels of versican and fibrillin-1 were analyzed by RT-PCR and real-time PCR, and their protein levels were evaluated by Western blot analysis and immunohistochemistry. Isolation of versican bound to fibrillin-1-containing microfibrils from ciliary bodies was performed by extraction studies. Slot-blot analyses and rotary shadowing electron microscopy were applied to identify versican associated with fibrillin-1-containing microfibrils after gel filtration chromatography and density gradient centrifugation.

RESULTS. The newly prepared polyclonal antibodies recognized amino and carboxyl termini of chicken versican. Versican, principally V0 and V1, was found to be securely bound to fibrillin-1-containing microfibrils, forming a major hyaluronan-binding structure in the ciliary nonpigmented epithelium. In addition, Western blot analysis revealed two cleaved complexes, the carboxyl-terminal end of versican bound to fibrillin microfibrils and the amino terminal end of versican bound to hyaluronan in the vitreous body.

CONCLUSIONS. Fibrillin-1, versican, and hyaluronan form a unique complex in the ciliary nonpigmented epithelium, and two cleavage products of this complex were shown to exist in the vitreous body. This newly clarified fibrillin-versican-hyaluronan (FiVerHy) complex and its cleavage products may be indispensable for the physiological properties important to the ciliary body and vitreous. (Invest Ophthalmol Vis Sci. 2008;49:2870–2877) DOI:10.1167/iovs.07-1488

One of the most important functions of the ciliary body is accommodation.1–3 The ciliary muscle consists of longitudinal, circular, and radial fibers and contributes to accommodation. The distributions of some extracellular matrix components in the ciliary muscle have already been delineated, via immunohistochemical studies of collagen-I, -II, -IV, and -VI; elastin; fibrillin; fibronectin; and laminin.4–9 Ciliary zonules arise from the vicinity of the apex of the nonpigmented epithelium of the ciliary processes of the ciliary body and insert into the lens capsule. The zonules anchor the lens to the wall of the eye and transmit accommodation forces from the ciliary muscle to the lens. To perform these physiological functions, the structural properties of these tissues must include both elasticity and strength.

The glycoprotein fibrillin is a major component of recognizable structural elements, designated microfibrils, in various connective tissues, and was reported to be the principal component of the ciliary zonules.10 Microfibrils play important roles in the strength and elasticity of ocular connective tissues.11 In the eye, microfibrils are also present in the vitreous body,12 although their origin is unknown. In humans and chickens, there are three fibrillins (fibrillin-1, -2 and -3), each encoded by a distinct gene.13 Fibrillin-1 and -2 perform compensatory functions in elastic fiber formation during development. Postnatal tissues require fibrillin-1, since fibrillin-2 is largely restricted to fetal development and to early postnatal life. The microfibrils of the ciliary zonules are almost exclusively composed of fibrillin.14 Fibrillin-1 is involved in certain systemic connective tissue diseases with ocular manifestations, such as Marfan syndrome.15 One of the major ocular complications in Marfan syndrome is dislocation of the lens induced by defective fibrillin-1-containing microfibrils in the ciliary zonules.

Versican, also known as PG-M, is a large hyaluronan-binding chondroitin sulfate proteoglycan that belongs to the lecithin family.16 Several different isoforms of versican (V0, V1, V2, and V3) containing different sets of chondroitin sulfate-attachment domains are generated by alternative splicing.17 Versican, especially the V0 isoform, which has the most chondroitin sulfate attachment sites, plays important roles in retinal differentiation, particularly in the regulation of ganglion cells during retinal development.17 Versican is widely expressed in many tissues, including the ciliary muscle and trabecular meshwork.18,19 Although a previous study revealed that versican interacts with fibrillin-1 in some tissues,20 it is unknown whether versican interacts with fibrillin-1 in the ciliary body and zonules. Chondroitin sulfate proteoglycans and hyaluronan were colocalized in the ciliary zonules and ciliary nonpig-

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clonal antibody 6084 against the amino terminus of versican is characterized elsewhere. The polyclonal antibody 9543 against fibrillin-1 and the monoclonal antibody 2B1 against the carboxyl terminus of versican are also characterized elsewhere. Peroxidase-conjugated goat IgG fractions against mouse immunoglobulins (IgG, IgA, and IgM) were purchased from Organon Teknika Corp. (Durham, NC). Anti-GST antibody was purchased from GE Healthcare (Little Chalfont, UK). Chondroitinase ABC (protease-free) was purchased from Seikagaku Corp. (Tokyo, Japan).

Peptide Fragments of Chicken Versican

PCR amplifications were performed with a chicken retina cDNA library as a template and oligonucleotide primer pairs. We used the sense primer 5′-GTGATGTATGGAGTTGAGGACACAC-3′ (533-559) and the antisense primer 5′-AGTAGCCATCAAACCTGATCTGACG-3′ (1175-1190) to amplify a cDNA encoding the amino terminal of chicken versican. We used the sense primer 5′-CAGAGTTCCATG-CAAAAGTAATCCCTGC-3′ (9907-9933) and the antisense primer 5′-GGCCCTGAGTGTGTCGGACGTT-3′ (10830-10810) to amplify a cDNA encoding the carboxy-terminal of chicken versican. The numbers indicate the nucleotide positions (GenBank accession no. D15542).

Each purified cDNA was ligated into the pGEX6p-1 vector (GE Healthcare). Each ligated construct was then transformed into Escherichia coli BL21 cells (Novagen, Madison, WI) for expression of glutathione S-transferase (GST) fusion proteins. Expression was induced by the addition of isopropyl-β-D-thiogalactoside. After lysis of the BL21 cells in PBS (pH 7.5), 1 mM PMSE, 10 mM DTT, 100 mM MgCl₂, 1.0 mg/mL lysozyme, and 20 U/mL DNAse I, the released GST fusion proteins were purified (GSTrap FF; GE Healthcare). Positive colonies were checked by sequencing.

Western Blot Analysis

To test the binding affinities of the above-mentioned antibodies and biotinylated hyaluronan (b-HA) for versican, conditioned medium from human fibroblasts was concentrated by using O-diethylaminoethyl (DEAE)-Sepharcel. The preparation of b-HA was described previously. The bound fractions were washed with 0.3 M NaCl, 50 mM Tris-HCl (pH 7.5) and eluted with 4 M guanidine HCl, 50 mM Tris-HCl (pH 7.5). We used the DEAE partially purified sample for Western blot analyses. The eluted fractions were further separated by density gradient centrifugation. The bottom fraction was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 3% to 8% polyacrylamide gels in nonreducing conditions. The separated proteins were electrotransferred to nitrocellulose membranes, blocked with 10% nonfat milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-Tween) for 1 hour and incubated with the antibodies 7080 (diluted 1:5000) and 7390 (1:2000) in PBS-Tween. The antibodies were incubated with horseradish peroxidase (HRP)-conjugated protein A (Zymed, San Francisco, CA) for 7080 and 7390, anti-mouse IgG fractions against mouse immunoglobulins (IgG, IgA, and IgM) were purchased from Organon Teknika Corp. (Durham, NC). Anti-GST antibody was purchased from GE Healthcare (Little Chalfont, UK). Chondroitinase ABC (protease-free) was purchased from Seikagaku Corp. (Tokyo, Japan).

Materials and Methods

To investigate the amino terminus and carboxyl terminus of versican separately, polyclonal antibodies 7080 and 7390, recognizing the amino and carboxyl termini of versican, respectively, were produced by Operon Biotechnology (Tokyo, Japan). The antibodies were raised by immunizing rabbits with the synthetic peptides CYVDHLDGD-VFHTVTSPS259 and STLQYENWRPNGPS257 located within the hyaluronan-binding region and the lectin-like domain of human versican (NCBI accession no. NP 004376), respectively (Fig. 1). The poly-
a positive control, since alternatively spliced isoforms of versican had been detected by RT-PCR, performed as described previously. The primers for the RT-PCR amplifications of fibrillin-1 (Table 1) were chosen from the published sequence of chicken fibrillin-1 (NCBI accession no. U88872), and PCR amplification was performed in the same thermal cycler conditions.

The levels of the mRNAs for six versican isoforms in the ciliary body were measured by real-time PCR with the appropriate primers (Table 2). Primers were designed to yield products from each isoform as similar in size as possible. Product sizes were: V0(+) (Table 2). Primers were designed to yield products from each isoform chosen from the published sequence of chicken fibrillin-1 (NCBI accession no. U88872), and PCR amplification was performed in the same thermal cycler conditions.

### Immunohistochemical Analysis

Newly hatched chicken eyes were fixed in 10% formalin neutral-buffered solution (pH 7.4), for 4 hours at room temperature and embedded in paraffin. Antibodies (6084, 7080, 7590, and 9545) were used at 1:100 dilution in PBS containing 1% normal goat serum. Antibody binding was detected by fluorophore-labeled goat anti-rabbit IgG antibodies (Alexa Fluor 488; Invitrogen, Carlsbad, CA). For the detection of hyaluronan, biotinylated hyaluronic acid-binding protein (b-HABP; Seikagaku Corp.) was incubated instead of the primary antibody. Hyaluronidase (Seikagaku Corp.) was used for negative control of stainings by b-HABP. Nonimmune serum was used instead of the primary antibody as a negative control. For the detection of chondroitin sulfate, biotin-conjugated anti-proteoglycan ΔDI-0S, ΔDI-4S, and ΔDI-6S (Seikagaku Corp.) were used in the incubation instead of the primary antibody. Antibody binding was detected by streptavidin-FITC (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Immunolabeled tissue sections were observed by fluorescence microscope (Axioplan2, and AxioCam; Carl Zeiss Meditec, Oberkochen, Germany) with bovine serum albumin as the standard.

Slot-blot analyses were performed with a nitrocellulose filter spotted with 100-μL aliquots from odd fractions. The blots were blocked with 10% nonfat milk in PBS-Tween for 1 hour and then incubated with 6084 and 7080 (each diluted 1:500), 7590 (1:500), 9545 (1:2000), b-HA (1:500), or biotinylated hyaluronan binding protein (b-HABP) (1:500) purchased from Seikagaku Corp. in PBS-Tween. After they were washed with PBS-Tween, the blots were incubated with HRP-conjugated protein A for the detection of antibodies and streptavidin for the detection of b-HA and b-HABP. The development procedure was performed as just described.

### Density Gradient Centrifugation

Aliquots of the void volume fractions 13, 14, and 15 from gel filtration were brought to a density of 1.37 g/mL by the addition of CsCl. A direct gradient was established by centrifugation at 40,000 rpm at 10°C for 45 hours (P90AT Hitachi, Tokyo, Japan). The gradients were partitioned into 12 fractions. Slot blots spotted with 20-μL aliquots of each fraction were created as has been described. To analyze the top fractions, we pooled fractions 1, 2, 3, and 4 and brought them to a density of 1.30 g/mL. A similar centrifugation procedure was performed, and the gradients were partitioned into 10 fractions. Slot blots spotted with 100-μL aliquots of each fraction were created. Samples on the membranes were stained with b-HABP before and after alkaline treatment with 0.2 M NaOH for 2 hours at room temperature.

### Rotary Shadowing Electron Microscopy

Samples around the density of 1.29 g/mL, after density gradient centrifugation, were dialyzed against H2O and rotary shadowed, as described previously.

### Vitreous Bodies

Vitreous bodies from six newly hatched chickens were extracted and subjected to further investigation, as described for the ciliary bodies.

### Binding Assays

ELISAs were performed on samples after CsCl density gradient centrifugation. Samples were incubated in microtiter wells overnight at 4°C. The wells were then washed three times with PBS-Tween and samples were incubated for 1 hour at room temperature in 9543, 6084, 7590, or b-HA, which were threefold serially diluted, beginning with an initial dilution of 1:50. After the samples were washed three times with PBS-Tween, the samples were incubated for 1 hour at room temperature in HRP-conjugated protein A to detect the antibodies and streptavidin to detect b-HA. After they were washed again, color reaction was

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**Table 1. Primers Used for PCR Amplifications**

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<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence Chicken Fibrillin-1</th>
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<tr>
<td>FBN-f (S)</td>
<td>2089-2108</td>
<td>CTTAACATCTGGTCTATGG</td>
</tr>
<tr>
<td>FBN-r (A)</td>
<td>2538-2519</td>
<td>AACTGATAATAGGTTTGTC</td>
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* Nucleotide positions. NCBI accession no. U88872.

**Table 2. Primers Used for Real-time RT-PCR Amplifications of Chicken Versican**

<table>
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<tr>
<th>Primer</th>
<th>Position*</th>
<th>Sequence</th>
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<tr>
<td>V0(+) (S)</td>
<td>4291-4313</td>
<td>CAACCCACAGAGGTGTCTCTCTCA</td>
</tr>
<tr>
<td>V0(+) (S)</td>
<td>4463-4441</td>
<td>GATTCTGCATGATGGTTGTC</td>
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<tr>
<td>V1(+) (S)</td>
<td>1552-1575</td>
<td>GAAGTGGACACACTTCTCTGAA</td>
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<td>V1(+) (S)</td>
<td>4505-4482</td>
<td>CAACTCGTCAGGACTGCTGAGTA</td>
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<td>V1(+) (S)</td>
<td>1135-1157</td>
<td>ACAGGGTTCTCTCATCAGGATAG</td>
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<td>V1(+) (S)</td>
<td>1463-1457</td>
<td>GAATCTGCATGATGGTTGTC</td>
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<td>V2(+) (S)</td>
<td>4291-4313</td>
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<td>V2(+) (A)</td>
<td>9994-9975</td>
<td>ACCCTGGCAAACATCGTACAG</td>
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<td>V3(+) (S)</td>
<td>1552-1575</td>
<td>GAAGTGGACACACTTCTCTGAA</td>
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<tr>
<td>V3(+) (S)</td>
<td>9994-9975</td>
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<td>V3(+) (S)</td>
<td>1135-1157</td>
<td>ACAGGGTTCTCTCATCAGGATAG</td>
</tr>
<tr>
<td>V3(+) (A)</td>
<td>9994-9975</td>
<td>ACCCTGGCAAACATCGTACAG</td>
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* Nucleotide positions (GenBank accession no. D13542).
were also specifically stained with 7080 and 7390 (Fig. 2B), indicating that both reacted with chicken versican.

Identification of Versican and Fibrillin-1 mRNAs in Chicken Ciliary Bodies

RT-PCR amplifications were performed to detect expression of versican and fibrillin-1 in ciliary bodies from newly hatched chickens. Amplified cDNAs for all four versican isoforms (V0, V1, V2, and V3; data not shown), as well as fibrillin-1 (Fig. 3A), were detected. Although it is hard to rigorously compare relative expression levels among different mRNAs, we measured the levels of the mRNAs for six versican isoforms in the ciliary body by real-time PCR. This analysis showed that V0 and V1 are the major isoforms in the ciliary body (Fig. 3B).

Colocalization of Versican, Fibrillin-1, and Hyaluronan in Ciliary Body

Marked staining in ciliary zonules and diffuse staining in nonpigmented epithelium were observed by using 9543 polyclonal antibodies (Fig. 4, 9543). The 6084 polyclonal antibodies strongly stained the nonpigmented epithelium on ciliary processes (Fig. 4, 6084). Identical stainings as 6084 were observed by 7080 polyclonal antibodies (Fig. 4, 7080). The 7390 polyclonal antibodies similarly stained the nonpigmented epithelium especially and also stained ciliary zonules (Fig. 4, 7390). Similar staining was intensely found in the nonpigmented epithelium by biotin conjugated anti-proteoglycan ΔDi-6S and ΔDi-4S antibodies, but faintly by ΔDi-0S antibody (Fig. 4, ΔDi-6S, ΔDi-4S, and ΔDi-0S). Similar staining was also shown by b-HABP (Fig. 4, b-HABP), and the stained area in the tissues was achieved in the samples with 100 μL of soluble blue peroxidase substrate (Roche Diagnostics, Indianapolis, IN). Color absorbance was determined at 450 nm with a microplate reader (Model 680; Bio-Rad, Hercules, CA).

Reduction Procedures

Western blot analyses were also performed on extracts from chicken vitreous bodies under reducing conditions. Samples were incubated with or without 10 mM dithiothreitol (DTT) for 30 minutes at 37°C. Then, samples were filtered (Microcon YM-100 filter units; Millipore, Billerica, MA) at 12,000 rpm for 10 minutes in a microcentrifuge (5415R: Eppendorf) and subjected to SDS-PAGE in 10% polyacrylamide gels. The separated proteins were electrotransferred to nitrocellulose membranes, blocked with 10% nonfat milk in PBS-Tween for 1 hour, and incubated with 6084 (diluted 1:5000) or 7080 (1:5000). All experiments were repeated three times, and the results are presented as the mean ± SE.

RESULTS

Characterization of Antibodies against Versican

The new polyclonal antibodies 7080 and 7390 raised against synthetic peptides of human versican were characterized by Western blot analysis of partially purified samples of human versican after chondroitinase ABC treatment. Human versican was specifically detected by 7080 and 7390, which recognize the amino and carboxyl terminus of versican, respectively (Fig. 2A, arrowhead). These antibodies, which potentially react with all forms of versican, detected only the V0 isoform, the alternatively spliced form with the most abundant sites for chondroitin sulfate attachment, according to molecular weight, suggesting that the human fibroblasts used for this experiment dominantly yield the V0 isoform under the present conditions. The previously characterized antibodies 6084 and 2B1, as well as b-HA, were used in positive control experiments for versican detection. To verify that 7080 and 7390 react with chicken versican, we checked the reactivity of both antibodies with GST fusion proteins matching the amino and carboxyl termini of chicken versican expressed by E. coli BL21 cells, respectively, by Western blot analysis. Proteins that reacted with anti-GST antibody

FIGURE 2. Characterization of the newly prepared polyclonal antibodies against versican. (A) Western blot analyses with the anti-versican antibodies 6084, 7080, 2B1, and 7390 on partially purified samples of conditioned medium from human fibroblasts. Versican was detected by all the antibodies examined (arrowhead). Versican was also stained by biotin-conjugated hyaluronan (b-HA). (B) Western blot analysis with 7080 and 7390 antibodies on GST fusion proteins matching the amino and carboxyl termini of chicken versican. Both fusion proteins were specifically stained with 7080 and 7390 antibodies.

FIGURE 3. PCR analysis for fibrillin-1 and versican in chicken ciliary bodies. (A) RT-PCR analysis for fibrillin-1 expression. The band demonstrates the existence of fibrillin-1. As positive controls, RT-PCR amplifications of α-tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed. (B) Relative expression levels of versican isoforms. The levels of mRNAs for six isoforms of versican in the ciliary body were measured by real-time PCR. The analysis showed that V0 and V1 are the major isoforms in the ciliary body.

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Visualization and Characterization of Versican-Bound Microfibrils
The fractions around 1.29 g/mL after density gradient centrifugation were visualized by rotary shadowing electron microscopy. Vitreous body samples appeared as a multiple beads and strings structure (Fig. 7, vitreous body), characteristic of microfibrils. By contrast, the morphology of the ciliary body microfibrils was different from that of the vitreous body microfibrils (Fig. 7, ciliary body). The ciliary body microfibrils showed additional projections (Fig. 7, ciliary body, arrows) from the interbead strands compared with the vitreous body microfibrils.

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When extracts were sieved on Sepharose CL-2B, the protein concentration profile of the eluted fractions revealed two peaks (Fig. 5A). By slot-blot analysis, the fractions around the void volume (Fig. 5A, bar) reacted with antibodies 6084, 7080, and 7390 against versican, antibody 9543 against fibrillin-1, b-HA, and b-HABP. These results suggest that versican and fibrillin-1 form a complex and that this complex retains the ability to bind hyaluronan (Fig. 5B). Intense reactivity to b-HA was found in the low-molecular-weight fractions from 29 to 33, but these fractions showed only faint reactivity with b-HABP.

Next, the versican- and fibrillin-1-positive fractions around the void volume were separated by CsCl density gradient centrifugation. First, we analyzed a sample with an initial density of 1.37 g/mL (Fig. 6A). Slot-blot analysis revealed that the top fraction reacted with antibodies 6084, 7080, 7390, and 9543. This fraction also reacted with b-HA and b-HABP. To further separate the top fraction, a pooled sample of fractions 1, 2, 3, and 4 was again separated by CsCl density gradient centrifugation with an initial density of 1.30 g/mL. Subsequent slot-blot analyses revealed that the fractions around 1.29 g/mL reacted with antibodies 6084, 7080, 7390, 9543, and b-HA (Fig. 6B). Although there was faint staining of b-HABP in fractions 5 to 8 before alkaline treatment, strong reactivity was observed in fractions 3 to 8 after alkaline treatment.

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To isolate versican-bound microfibrils from ciliary bodies, chicken ciliary bodies were extracted with 6 M guanidine HCl, a nondegradative procedure for isolating microfibrils. Western blot analysis revealed that fibrillin-1 monomers and the versican core protein, previously reported to be 350 kDa and 550 kDa, respectively, were not present in the extracts, even after chondroitinase ABC digestion, because they were not detected in the materials entering the running gels. Instead, both fibrillin-1 and versican remained in the stacking gel (data not shown), indicating that most of the fibrillin-1 and versican present in the extract was in the form of macroaggregates.

When extracts were sieved on Sepharose CL-2B, the protein concentration profile of the eluted fractions revealed two peaks (Fig. 5A). By slot-blot analysis, the fractions around the void volume (Fig. 5A, bar) reacted with antibodies 6084, 7080, and 7390 against versican, antibody 9543 against fibrillin-1, b-HA, and b-HABP. These results suggest that versican and fibrillin-1 form a complex and that this complex retains the ability to bind hyaluronan (Fig. 5B). Intense reactivity to b-HA was found in the low-molecular-weight fractions from 29 to 33, but these fractions showed only faint reactivity with b-HABP.

Next, the versican- and fibrillin-1-positive fractions around the void volume were separated by CsCl density gradient centrifugation. First, we analyzed a sample with an initial density of 1.37 g/mL (Fig. 6A). Slot-blot analysis revealed that the top fraction reacted with antibodies 6084, 7080, 7390, and 9543. This fraction also reacted with b-HA and b-HABP. To further separate the top fraction, a pooled sample of fractions 1, 2, 3, and 4 was again separated by CsCl density gradient centrifugation with an initial density of 1.30 g/mL. Subsequent slot-blot analyses revealed that the fractions around 1.29 g/mL reacted with antibodies 6084, 7080, 7390, 9543, and b-HA (Fig. 6B). Although there was faint staining of b-HABP in fractions 5 to 8 before alkaline treatment, strong reactivity was observed in fractions 3 to 8 after alkaline treatment.

Visualization and Characterization of Versican-Bound Microfibrils
The fractions around 1.29 g/mL after density gradient centrifugation were visualized by rotary shadowing electron microscopy. Vitreous body samples appeared as a multiple beads and strings structure (Fig. 7, vitreous body), characteristic of microfibrils. By contrast, the morphology of the ciliary body microfibrils was different from that of the vitreous body microfibrils (Fig. 7, ciliary body). The ciliary body microfibrils showed additional projections (Fig. 7, ciliary body, arrows) from the interbead strands compared with the vitreous body microfibrils.
When the samples after CsCl density gradient centrifugation (fractions under 4 M guanidine HCl) were subjected to slot blot analysis, vitreous body microfibrils did not react with antibodies against the hyaluronan-binding region of versican or with b-HA (Fig. 8A). By contrast, ciliary body microfibrils showed reactivity to 6084 and 7080 antibodies and a binding affinity for hyaluronan. All samples examined reacted with the antibodies against fibrillin-1 and the carboxyl-terminus of versican. The same fractions were studied by ELISA (Fig. 8B). The vitreous body sample reacted negatively with 6084 in all dilutions. The ratio of 6084 to 7390 in dilution 1:50 was significantly small in vitreous body comparison with ciliary body (Fig. 8C; \( P < 0.0001 \)). We detected a distinct 80-kDa band reactive to 6084 and 7080 antibodies but not reactive to 7390 antibodies in the vitreous samples under reducing conditions with DTT by Western blot study (Fig. 8D, and data not shown). This 80-kDa fragment was not detected in samples derived from ciliary bodies, regardless of the presence or absence of DTT, whereas some degraded bands were seen in each lane (Fig. 8D). This finding suggests that ciliary bodies are mainly equipped with...
the entire FiVerHy complex, which remains in the stacking gel. Amino acid sequence analysis of this 80-kDa band revealed an N-terminal peptide KKTLVKG, which is identical with the amino terminus of chicken versican.

Reactivity of 7080 with ciliary and vitreous bodies was absent in ELISA analysis (data not shown). We think that this is due to masking of the epitope by the loop structure of versican, which is not present in the blot, but is present in the fluid, as the antibodies were specifically raised against the hyaluronan-binding region near the disulfide bonds.

**Discussion**

Comparisons of the amino acid sequences used for preparing antibodies 7080 and 7390 with the respective chick regions by BLAST searches revealed identities of 50% and 93%, respectively. These moderate and high similarities of the amino acid sequences allowed for cross-reactivity of the antibodies with the corresponding chicken sequences, thereby enabling us to perform these studies on chick samples. In addition, these antibodies showed staining patterns for versican in chick retinal samples similar to those shown previously by immunohistochemical and Western blot analyses (data not shown). There is a possibility that these antibodies also react with chicken aggrecan. However, an anti-aggrecan antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) showed no staining of human, mouse, and rat ciliary bodies (data not shown). Therefore, our newly prepared polyclonal antibodies appeared to react well with chicken versican in the tissues examined.

We found intense reactivity to b-HA in the low-molecular-weight fractions from 29 to 33 after gel filtration chromatography, but the fractions showed only faint reactivity with b-HABP. These fractions showed relatively stronger reactions with antibodies 6084 and 7080 than with antibody 7390, suggesting that they may contain a fragment corresponding to the hyaluronan-binding region of versican that is not bound to hyaluronan.

Versican of the FiVerHy complex in vitreous samples releases gigantic hyaluronan because of the split of disulfide bond in hyaluronan-binding region of versican under the reducing conditions, and it may enable the 80 kDa of amino terminus versican fragment to enter the running gels as shown in Figure 8D. The 80-kDa fragment we observed in the chicken vitreous body may correspond to the 55 kDa of amino terminal versican fragment detected in the bovine vitreous body. In extracts from brain, a fragment corresponding to the hyaluronan-binding region of versican has been also reported. Some proteolytic reactions may be involved in these processes. Taken together, the present data allow us to propose the hypothesis that the hyaluronan-binding region of versican in the vitreous body is cleaved from the FiVerHy complex in the ciliary body. We estimate that this may be reflected in the ultrastructural evidence suggests that the regions involved in the attachment of chondroitin sulfate to theaminoterminal end of versican are shed into the vitreous body and that only the carboxyl-terminal end of versican bound to microfibrils and forms huge complexes with hyaluronan. Association of the complex in the presence of 4 M guanidine HCl further suggests the presence of strong linkages, such as covalent bonds, among versican, fibrillin-1, and hyaluronan.

Our previous report showed that monomeric versican eluted in the middle fractions of sieve chromatography with Sepharose CL-2B. In this study, versican from the ciliary body sample eluted in the void volume, rather than in the middle of the sieved materials. This result suggests that most of the versican is bound to microfibrils and forms huge complexes with hyaluronan. Association of the complex in the presence of 4 M guanidine HCl further suggests the presence of strong linkages, such as covalent bonds, among versican, fibrillin-1, and hyaluronan.

Our slot-blotting results, shown in Figure 6B, suggest that the FiVerHy complex is mainly composed of proteins, because it had a density of ~1.29 g/mL. Although the fractions around 1.29 g/mL strongly reacted with the antibodies, they hardly reacted with b-HABP before alkaline treatment. However, the samples on the membrane reacted strongly with b-HABP after alkaline treatment (Fig. 6B, fractions 3 and 4), suggesting that some protein constituents of the FiVerHy complex may prohibit the exposure of hyaluronan.

The anti-proteoglycan ΔDi6S and ΔDi4S antibodies showed no staining in ciliary zonules but detected immunoreactivity in the nonpigmented epithelium. This finding may suggest that the regions involved in the attachment of chondroitin sulfate to theaminoterminal end of versican are shed into the vitreous body and that only the carboxyl-terminal end of versican bound to fibrillin microfibrils accumulates in ciliary zonules. The flexibility and water-content property of integrant FiVerHy complex in the ciliary body may be essential for accommodation and aqueous humor secretion, respectively. The mobility of hyaluronan accompanied with chondroitin sulfate shed into the vitreous body, being free from behavioral restriction by fibrillin-1 attached to the carboxyl terminus of versican, may be also significant for it to accomplish its physiological function there.

Exfoliation syndrome (XFS) is an age-related disorder that constitutes the most common identifiable cause of glaucoma. The hallmark of the disease is the pathologic production and accumulation of an abnormal fibrillar exfoliation material (XFM) in many ocular tissues. Ultrastructural evidence suggests that XFM is mainly produced by epithelial cells of the iris, ciliary body, and lens. Of interest, Ovodonko et al. identified both fibrillin-1 and versican in XFM, and genome-wide scan of XFS suggested the involvement of fibrillin-1, MMP-2, and TIMP-1. Thus, FiVerHy complex as well as the proteolytic enzymes and the inhibitors may be components of the molecular background of XFS.

Chondroitin sulfates are required in this tissue. An appropriate balance of each splice isofrom may be important for regulation of chondroitin sulfates. Hence, an imbalance of splice variants may result in ocular disorders such as Wagner disease and erosive vitreoretinopathy. We previously tried immunoprecipitation to purify the FiVerHy complex by using 7080, 6084, and 7390 antibodies, but all failed. We speculate that the cause of these failures is because the complex is too gigantic. Indeed, the FiVerHy complex eluted in the void fractions as shown in Figure 5. In addition, maybe for the same reason, hyaluronan affinity columns are not suitable for purification of this complex. Further, hyaluronan affinity columns are not suitable as the purifying sample also includes hyaluronan in the complex, which may competitively affect their affinity. We also failed to separate the sample by electrophoresis for Western blot analysis, as the complex had such a high molecular weight that it remained trapped at the top of the gel. Thus, we came to the present approach for purification.
References