Galectin-3 Is Associated with the Plasma Membrane of Lens Fiber Cells

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Purpose. To discover proteins that have the potential to contribute to the tight packing of fiber cells in the lens.

Methods. Crude fiber cell membranes were isolated from ovine lens cortex. Proteins were separated by two-dimensional gel electrophoresis, and selected protein spots identified by microsequencing. The identification of galectin-3 was confirmed by immunoblotting with a specific antibody. The association of galectin-3 with the fiber cell plasma membrane was investigated using immunofluorescence microscopy, solubilization trials with selected reagents, and immunoprecipitation to identify candidate ligands.

Results. A cluster of three protein spots with an apparent molecular weight of 31,000 and isoelectric points ranging between 7 and 8.5 were resolved and identified as galectin-3. This protein was associated peripherally with the fiber cell plasma membrane and interacted with MP20, an abundant intrinsic membrane protein that had been identified previously as a component of membrane junctions between fiber cells.

Conclusions. The detection of galectin-3 in the lens is a novel result and adds to the growing list of lens proteins with adhesive properties. Its location at the fiber cell membrane and its association with the junction-forming MP20 is consistent with a potential role in the development or maintenance of the tightly packed lens tissue architecture. (*Invest Ophthalmol Vis Sci.* 2000;41:199–203)

Lens transparency critically depends on the crystalline packing of fiber cells to reduce light scattering by minimizing extracellular space. Several types of membrane junctions and specialized "ball and socket" interdigitations underpin this close apposition of fiber cells, and it is evident that proteins with adhesive properties play an important role.

Indeed, several proteins with adhesive properties are known to be expressed in the lens. Laminin appears to be limited to the capsule whereas fibronectin also is present between fiber cells.[1] Integrins with specificities for these matrix proteins also have been identified in the lens.[2] Fiber cells express N-cadherin[3] consistent with the abundant presence of adherens junctions.[4] The 26-kDa major intrinsic polypeptide MIP, which functions as an aquaporin in the lens, also possesses adhesive properties,[5] and is a constituent of the 11-nm "thin" junctions between fiber cells.[6] Another abundant intrinsic membrane protein, MP20, formerly referred to as MP18, also has been identified as a component of membrane junctions between fiber cells.[7,8] Connexins, which form gap junction channels, constitute yet another type of cell–cell contact between fiber cells.[9]

Interactions are less well understood for two recently discovered proteins that might play a role in lens cell adhesion. One is the SPARC (secreted protein acidic and rich in cysteine) protein, which is a matricellular protein that in other tissues regulates cellular adhesion and proliferation. In the lens, SPARC is expressed predominantly in the epithelium, and when absent, lens development is severely disrupted resulting in cataract.[10,11] The other is GRIFIN (galectin-related inter-fiber protein), which is a novel lens-specific protein related to the galectin family of adhesion molecules.[12] However, GRIFIN lacks the β-galactoside binding ability that the galectins normally have, and its role in the lens remains unclear.

We now report the identification of galectin-3, formerly also known as Mac-2,[13–15] as yet another member to be added to this list of lens proteins with adhesive properties. This identification has been achieved using a proteomic approach based on the separation of membrane associated proteins by two-dimensional gel electrophoresis and subsequent microsequencing of individual protein spots. Our data show that galectin-3 is associated peripherally with the fiber cell membranes and that the junction-forming MP20 is a candidate ligand.

Materials and Methods

Preparation of Crude Lens Fiber Cell Membranes

Lenses were obtained from sheep at the local abattoir. They were harvested immediately after death and stored at −80°C until used. Typically 20 lenses were processed at a time. Lenses were thawed, and cortical tissue was collected by scraping it from the tougher nucleus with a surgical blade. The tissue was homogenized in 5 ml ice-cold homogenization buffer (5 mM

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Supported by the Health Research Council of New Zealand, the New Zealand Lottery Grants Board, the University of Auckland Research Committee, and a University of Auckland Doctoral Scholarship.

Submitted for publication March 5, 1999; revised July 15, and August 6, 1999; accepted August 17, 1999.

Commercial relationships policy: N.

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Tris, pH 8.0, 5 mM EDTA, and 5 mM EGTA). Crude membranes were pelleted in an SS34 rotor (RC 5C; Sorvall, Newton, CT) at 12,000 rpm for 20 minutes and subsequently were washed twice in the same buffer. Pellets were resuspended in storage buffer (5 mM Tris, pH 8.0, 2 mM EDTA, 2 mM EGTA, and 100 mM NaCl) at a protein concentration of approximately 4 mg/ml, and kept at −80°C until further use.

Membrane Proteomics

Two-dimensional (2D) gel electrophoresis was carried out with crude cortical membrane proteins to which 2D gel markers were added (Bio-Rad, Cambridge, MA). For the first-dimension isoelectric focusing, Immobiline Dry Strips (pH 3–10 nonlinear, Pharmacia LKB Biotechnology, Piscataway, NJ) were used. Strips were rehydrated in 8 M urea, 1% octyl-β-D-glucoside, 0.52% Pharmalyte 3–10 (Pharmacia), 13 mM dithiothreitol (DTT), and 0.01% bromophenol blue. Crude cortex membranes were pelleted at 12,000 rpm for 20 minutes (model 5402; Eppendorf, Westbury, NY), and washed once with 10 mM HEPES, pH 7.2. The pellet was solubilized in 9 M urea, 4% octyl-β-D-glucoside, 2% Pharmalyte 3–10, 65 mM DTT, and 0.01% bromophenol blue. Isoelectric focusing was carried out according to the manufacturer's instructions using a Pharmacia LKB Multiphor II apparatus.

Once focused, isoelectric strips were equilibrated with 100 mM Tris, pH 6.8, 6 M urea, 30% glycerol, 3.5 mM sodium dodecyl sulfate (SDS), and 52 mM DTT, and subsequently with 100 mM Tris, pH 6.8, 6 M urea, 30% glycerol, 3.5 mM SDS, 0.01% bromophenol blue, and 20 mM iodoacetamide. A second-dimension gel was run using ExcelGel SDS 12% to 14% acrylamide (Pharmacia LKB Biotechnology) according to the manufacturer's instructions. Protein spots were visualized either by Coomassie blue or silver staining.

Coomassie blue–stained proteins were given numbers and cut from the gel using a surgical blade. Pooled proteins were digested in the gel pieces according to Rosenfeld et al.[16] using 0.2 mg trypsin per sample for 16 hours at 37°C. Peptides were extracted from the gel pieces and separated using reversed-phase high-performance liquid chromatography (HPLC) using a 10% to 70% linear gradient of buffer A (0.08% TFA) and buffer B (80% acetonitrile, 0.016% TFA) and a 5-μm C_{18} 250 × 2-mm column (Phenomenex, Torrance, CA). Sequencing was done using a Procise 492 protein sequencer (Applied Biosystems, Foster City, CA).

For single dimension SDS-polyacrylamide gel electrophoresis (PAGE), 15% or 17.5% acrylamide gels were run in a Mini-PROTEAN II cell (Bio-Rad). Proteins were solubilized without boiling in sample buffer containing 15 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.01% bromophenol blue. Proteins in the gels were visualized by silver staining.

For immunoblotting, proteins were transferred electrophoretically in a Mini-PROTEAN Trans-Blot cell (Bio-Rad) onto Hybond-C pure nitrocellulose membranes (Amersham Life Science, Arlington Heights, IL). Blots were stained with 0.1% Ponceau S/1% acetic acid to visualize and record the positions of lens proteins and molecular weight markers. After washing in milliQ water, the blots were blocked overnight at 4°C in 5% nonfat milk powder in TBS. Galectin-3 was detected with a commercially available monoclonal anti–galectin-3 antibody (Affinity Bioreagents, Deerfield, IL)[17] at a dilution of 1:200 in TBS containing 1% BSA. MP20 was detected with a 1:1000 dilution of a carboxyl tail peptide–specific antibody that was kindly donated by Charles Louis, University of Minnesota, St. Paul.[18] Bound antibodies were detected with alkaline phosphatase–conjugated secondary antibodies at 1:1000 dilution according to the manufacturer's instructions (Immun Star kit; Bio-Rad), or with biotinylated secondary antibodies at 1:1000 and streptavidin–horseradish peroxidase at 1:1000 according to the manufacturer's instructuctions (ECL; Amersham Life Science).

Probing the Membrane Association of Galectin-3

Immunofluorescence microscopy was used to determine the cellular distribution of galectin-3 in lens fiber cell tissue. Equatorial cryosections (18-μm thick) were cut from an ovine lens and fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS). They were labeled with anti–galectin-3 antibody at 1:20 dilution in PBS and detected using fluoroscein isothiocyanate–conjugated secondary antibody.

The nature of the association of galectin-3 with the fiber cell membrane was probed in two ways. First, crude cortical membranes were pelleted at 12,000 rpm for 20 minutes (model 5402; Eppendorf) and resuspended in 4 M urea, 5 mM Tris, pH 9.5, 5 mM EDTA, and 5 mM EGTA. After incubation for 10 minutes at room temperature, soluble proteins and membranes were separated by centrifugation at 40,000 rpm in a SW65 rotor (Discovery 100S; Sorvall) for 40 minutes. For a more complete extraction of peripheral membrane proteins, the pellet was further treated three times with 20 mM NaOH in water. Second, the carbohydrate specificity of galectin-3 binding was assessed by extracting crude fiber cell membranes with 100 mM glucose or 100 mM lactose in 5 mM Tris, pH 8, 2 mM EDTA, and 2 mM EGTA. Membranes and soluble proteins were separated as above and analyzed by SDS-PAGE and immunoblotting.

Candidate ligands of galectin-3 were identified by immunoprecipitation of detergent solubilized membrane proteins with anti–galectin-3 antibody and protein A Sepharose. Proteins were solubilized with 0.5% *n*-decyl-β-D-maltopyranoside (Sigma Chemical Co., St. Louis, MO), 10 mM HEPES, pH 7.2, for 5 minutes at room temperature and separated from insoluble material by centrifugation at 40,000 rpm in the SW65 rotor for 1 hour. The supernatant was incubated with anti–galectin-3 antibody at 1:100 dilution overnight at 4°C. Protein complexes recognized by the antibody were precipitated with protein A Sepharose CL-4B (Pharmacia LKB Biotechnology) for 1 hour at room temperature. The beads were washed four times with 10 mM HEPES, pH 7.2, and 200 mM NaCl. Proteins were released from the beads by incubating them in sample buffer for 10 minutes at room temperature and analyzed by SDS-PAGE and immunoblotting. Controls were treated the same way except that the anti–galectin-3 antibody was omitted.

Results

Our rationale was to apply proteomics to lens cortical membranes because some proteins undergo age-related cleavage in the lens nucleus. Furthermore, we used "crude" buffer washed membrane preparations so that intrinsic as well as peripheral membrane proteins could be discovered. This was particularly important for the detection of adhesion molecules but had the disadvantage that many spots sequenced turned out to be

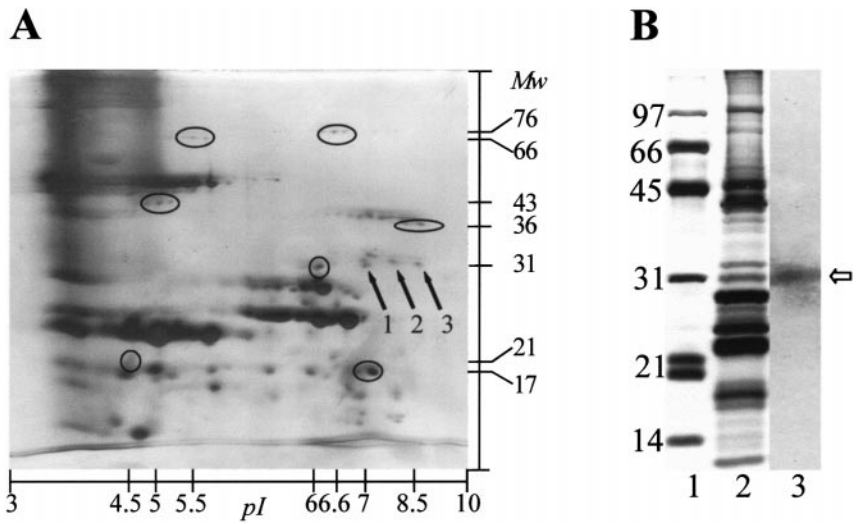

FIGURE 1. Identification of galectin-3 in crude fiber cell membranes. (**A**) Crude membrane proteins separated by isoelectric focusing horizontally and according to molecular weight vertically. *Arrows* point to three spots identified as galectin-3. *Circled spots* represent marker proteins with known isoelectric point (pI) and molecular weight (Mw) that were added to the sample. (**B**) Single-dimension SDS-PAGE (15% acrylamide) of molecular weight markers (*lane 1*) and crude fiber cell membranes (*lane 2*). Proteins were visualized by silver staining. *Lane 3*: immunoblot of a replica of *lane 2* identifying the 31-kDa band as galectin-3 (*arrow*).

crystallins and cytoskeletal proteins, which also have a tendency to adhere to the fiber membranes. However, a cluster of three spots with an apparent molecular weight of 31,000 and isoelectric points (pIs) between 7 and 8.5 were identified as galectin-3 (Fig. 1A). This identification was achieved by cutting out each of the three protein spots from the gel and digesting them with trypsin to generate a series of peptides, which were separated by reversed-phase HPLC. For each digest, the fraction that produced the largest and sharpest peak in the elution profile was used for sequencing. All three sequences matched 100% with portions of galectin-3 from rabbit (Swiss-Prot accession no. P47845) despite the species difference (Table 1). The octapeptides derived from spot 1 and 2 were identical with residues 202 to 209 and 191 to 198, respectively. The tetrapeptide derived from spot 3 matched residues 202 to 205. To further verify the identification of galectin-3 as a component of lens fiber cells, crude fiber cell membrane proteins also were analyzed by single-dimension SDS-PAGE and immunoblotting with a specific galectin-3 antibody.[17] Accordingly, a significant band was detected immediately adjacent to the 31-kDa marker (Fig. 1B, lanes 1, 2), and this band was identified as galectin-3 on the immunoblot of a gel replica (Fig. 1B, lane 3). After galectin-3 was identified in crude fiber cell membranes, its association with the membrane was probed in several ways. First, cryo-sectioned ovine lens cortical tissue was labeled with galectin-3 antibodies. This procedure localized galectin-3 clearly to the fiber cell plasma membrane (Fig. 2).

Second, two different kinds of protein extraction protocols were used to test the peripheral nature of galectin-3 and its β-galactoside specificity, respectively. Treatment of crude fiber cell membranes with 4 M urea enriched the 26-kDa major intrinsic polypeptide MIP (Fig. 3, lane 3), and extracted a significant portion of adherent proteins including the 31-kDa galectin-3 (Fig. 3, lane 4). Further alkaline extractions with 20 mM NaOH removed nearly all galectin-3 from the membranes (Fig. 4A, lane 2). This confirmed that galectin-3 was associated

TABLE 1. Sequence of Rabbit Galectin-3 and Matching Peptides from Ovine Lens Protein

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ADGFSLNDAL      SGSGHPPNQG      WPGPWGNQPA      GPGGYPGAAY     40
PGAYPGHAPG      AYPGQAPPGP      YPGPGAHGAY      PGQPGGPGAY     80
PSPGQPSGAG      AYPGASPYSA      SAGPLPVPYD      LPLPGGVMPR    120
MLITIVGTVK      PNANRLALDF      KRGNDVAFHF      NPRFNENNRR    160
VIVCNTKVDN      NWGREERQTT      FPFEIGKPFK      IQVLVEPDHF    200
                                                :::::::::
                                                IQVLVEPD*
KVAVNDAHLL      QYNHRMRNLK      EINKLGISGD      IQLTSASHAMI   241
 :::::::::
 VAVNDAHL†
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 VAVN‡
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Swiss-Prot accession number P47845.
* Internal peptide sequence of spot number 2.
† Internal peptide sequence of spot number 1.
‡ Internal peptide sequence of spot number 3.

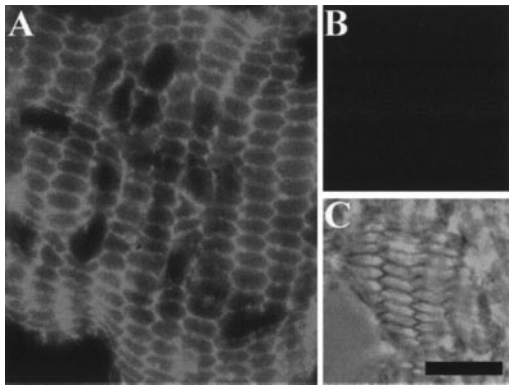

FIGURE 2. Immunolocalization of galectin-3 in lens cortical tissue. (**A**) The fluorescence labeling pattern indicates that galectin-3 is predominantly membrane associated. (**B**, **C**) Fluorescence and phase contrast micrograph, respectively, of a control specimen without the primary antibody. Identical exposure times were used to record the fluorescence images. Scale bar, 20 μm.

with the lens fiber cell membranes as a peripheral protein similar to the situation in other tissues.[13–15] Further, we examined whether the β-galactoside binding specificity, which is characteristic for galectin-3, also played a role in the case of the lens fiber cells. This was confirmed at least partially as treatment of crude fiber cell membranes with 100 mM lactose (Fig. 3, lanes 8, 10) but not with 100 mM glucose (Fig. 3, lanes 6, 9) solubilized small but detectable amounts of galectin-3. Repeated treatments with lactose removed more but not all galectin-3 consistent with another report that showed that this type of "elution" with lactose was also unable to remove all galectin-3 from the surface of mast cells.[19] Hence, an additional lactose-independent attachment mechanism might be involved.

The third way to probe the association of galectin-3 with lens fiber cell membranes was to identify candidate ligands from among the fiber cell intrinsic membrane proteins that form the insoluble fraction after urea/alkali extraction (Fig. 4A, lane 2). Integral and peripheral proteins were solubilized from crude membranes with 0.5% decylmaltoside (Fig. 4A, lane 4), and the solubilizate incubated with the galectin-3 antibody. Precipitation with protein A Sepharose enriched several proteins (Fig. 4B, lane 2) that were present in lesser amounts or not at all in the control (antibody omitted, Fig. 4B, lane 1). One of these enriched proteins comigrated with MP20, and its identity as MP20 was confirmed by immunoblotting using an antibody specific for this membrane protein [18] (Fig. 4A, lane 5; Fig. 4B, lanes 3, 4). This evidence identifies MP20 as one of the candidate ligands of galectin-3 in the lens fiber cell plasma membrane.

DISCUSSION

Galectin-3 is a β-galactoside binding protein with reported involvement in development, oncogenesis, and inflammation.[13–15] It occurs early during embryonal development, and in the adult is expressed in several epithelial cell types, in activated macrophages, and some sensory neurons. Although there is evidence that galectin-3 also might perform intracellular functions, it appears to be mainly secreted and to have a role in cell adhesion.

The detection of galectin-3 in the lens is novel. Our data demonstrate that it is associated with the fiber cell membrane as a peripheral protein, and there is evidence pointing to some involvement of its lectin domain in this association. Most interestingly, the junction-forming intrinsic fiber cell membrane protein MP20 [8] has been identified as a candidate ligand of galectin-3.

Lens fiber cells are unusual in that they pack tightly into a crystalline array. One would predict that this arrangement critically depends on the presence of appropriate adhesion molecules. The discovery in the lens of yet another adhesion protein in addition to those that have already been identified in this tissue does therefore not come as a surprise. A role for galectin-3 in cell–cell adhesion in the lens is supported by its association with MP20, which was previously identified as a component of membrane junctions between fiber cells.[8] It is possible that galectin-3 facilitates the interactions between MP20 molecules in the apposing membranes. A recent report shows several hypothetical models how galectin-3 might promote cell–cell adhesion via a mechanism that involves dimerization/oligomerization, and uses its lectin site either for the interaction between galectin-3 molecules or for the binding to integral membrane proteins.[20] MP20 has a consensus site for glycosylation,[7] but it is not known whether this site is used in vivo and whether any interaction between this protein and galectin-3 would involve this site.

In conclusion, this initial report documents that galectin-3, a protein with known adhesive properties, is associated with the plasma membrane of lens fiber cells. Its association with MP20, which is a component of membrane junctions, supports a role for galectin-3 in cell–cell adhesion in the lens. Future research will have to consolidate such a role with a more comprehensive identification of other ligands and will investigate the temporal and spatial expression of galectin-3 in the lens to decide whether its primary function is in the develop-

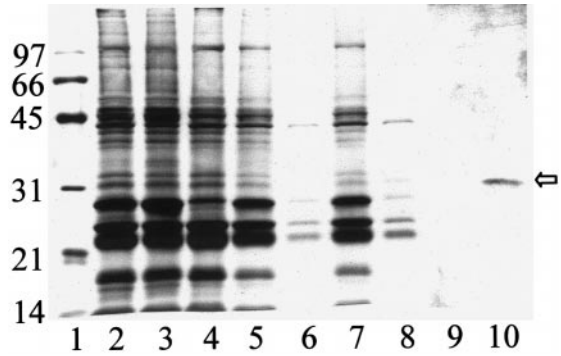

FIGURE 3. Probing the association of galectin-3 with the fiber cell plasma membrane. SDS-PAGE (15% acrylamide) of crude fiber cell membranes after extraction with various reagents. Proteins were visualized by silver staining. *Lane 1*: molecular weight markers. *Lane 2*: total crude membrane proteins. *Lane 3*: membranes treated with 4 M urea. *Lane 4*: membrane adherent proteins solubilized in 4 M urea. *Lanes 5* and *6*: membranes and solubilized proteins, respectively, after treatment with 100 mM glucose. *Lanes 7* and *8*: membranes and solubilized proteins, respectively, after treatment with 100 mM lactose. *Lanes 9* and *10*: immunoblots of replica gels with 25-fold sample loading of *lanes 6* and *8*, respectively, confirming the identity of the 31-kDa band (*arrow*) in the 100 mM lactose extract as galectin-3.

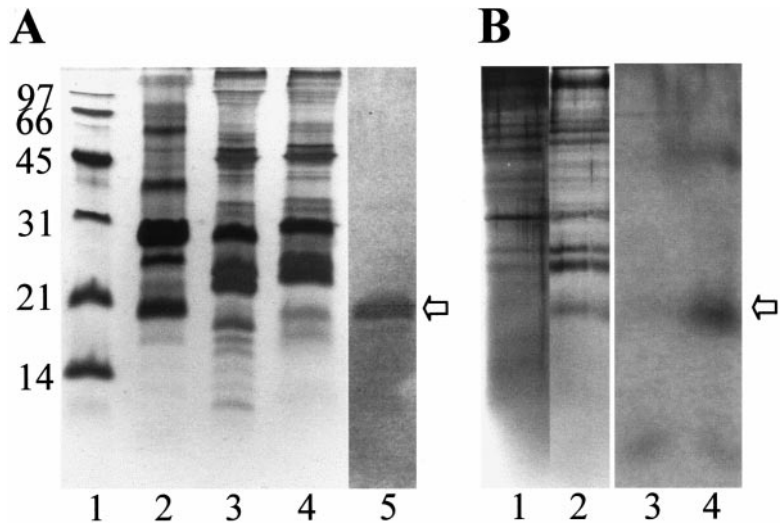

FIGURE 4. Identification of MP20 as a candidate ligand of galectin-3. Proteins were separated by SDS-PAGE (17.5% acrylamide) and visualized by silver staining. (**A**) Identification of MP20 in membrane protein fractions. *Lane 1*: molecular weight markers. *Lane 2*: urea/alkali stripped fiber cell membranes showing the major intrinsic membrane proteins. *Lane 3*: crude fiber cell membranes. *Lane 4*: proteins solubilized from crude fiber cell membranes with detergent. *Lane 5*: immunoblot of a replica of *lane 4* identifying MP20 (*arrow*). (**B**) Immunoprecipitation of proteins associated with galectin-3. *Lanes 1* and *2*: proteins precipitated with protein A Sepharose without and with anti–galectin-3 antibody, respectively. *Lanes 3* and *4*: immunoblots of replica gels of *lanes 1* and *2*, respectively, identifying MP20 (*arrow*).

ment or in the maintenance of the remarkable crystalline tissue architecture.

Acknowledgments

The authors thank Charles Louis (University of Minnesota, St. Paul) for the generous gift of anti MP20 antibody, David Christie for his support with advice and reagents, Catriona Knight for protein microsequencing, and Paul Edgar and Judy Douglas for help with 2D gel electrophoresis.

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