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MP20, the second most abundant lens membrane protein and member of the tetraspanin superfamily, joins the list of ligands of galectin-3

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Abstract

Background: Although MP20 is the second most highly expressed membrane protein in the lens its function remains an enigma. Putative functions for MP20 have recently been inferred from its assignment to the tetraspanin superfamily of integral membrane proteins. Members of this family have been shown to be involved in cellular proliferation, differentiation, migration, and adhesion. In this study, we show that MP20 associates with galectin-3, a known adhesion modulator.

Results: MP20 and galectin-3 co-localized in selected areas of the lens fiber cell plasma membrane. Individually, these proteins purified with apparent molecular masses of 60 kDa and 22 kDa, respectively. A 104 kDa complex was formed *in vitro* upon mixing the purified proteins. A 102 kDa complex of MP20 and galectin-3 could also be isolated from detergent-solubilized native fiber cell membranes. Binding between MP20 and galectin-3 was disrupted by lactose suggesting the lectin site was involved in the interaction.

Conclusions: MP20 adds to a growing list of ligands of galectin-3 and appears to be the first representative of the tetraspanin superfamily identified to possess this specificity.

Background

Tetraspanins constitute a superfamily of integral membrane proteins, which share a common membrane topology characterized by four membrane-spanning segments and the location of amino- and carboxy-termini at the cytoplasmic surface [1]. Most members further have a consensus N-linked glycosylation site in one of the extracellular loops [2]. Tetraspanins have been implicated in a variety of cellular processes including activation, proliferation, differentiation, migration, adhesion, and apoptosis [3]. Tetraspanins form contacts with other cells or the extracellular matrix by binding to other tetraspanins, to adhesion receptors such as integrins, and to extracellular proteins [4–6].

The PMP22/EMP/MP20 gene family is a subfamily of the tetraspanins [7]. PMP22, also known as CD25, has been associated with fibroblast apoptosis [8]. It is also

expressed in myelinating Schwann cells where it plays a role in differentiation [9–11]. When absent or dysfunctional, it causes peripheral neuropathies that result in progressive distal muscle weakness [12]. This report is concerned with MP20 [13,14]. Previously also referred to as MP17 and MP18, it is the second most abundant integral membrane protein of lens fiber cells, which appears to be distributed uniformly in the plasma membrane but also occurs in distinct membrane junctional domains [15–17]. Mutations in MP20 severely disrupt the normally crystalline fiber cell arrangement in the lens and cause cataractogenesis [18,19]. This indicates an important role for MP20 in the lens cell membranes, but its functional relationship with other proteins remains an enigma. Recently, galectin-3 was identified as a membrane-associated protein in the lens [20]. In other tissues galectin-3 functions as an adhesion modulator [21–23]. It appears therefore that MP20 and galectin-3 share a common involvement in adhesive processes, which raises the possibility that they might be binding partners in the lens. The present report shows that MP20 and galectin-3 co-localize in selected areas of the cell plasma membrane. Biochemical analysis confirmed that MP20 and galectin-3 interact with each other. Thus MP20 should be added to a growing list of ligands of galectin-3. MP20 is the first member of the tetraspanin superfamily identified to have this binding specificity.

Results

Co-localization of MP20 and galectin-3 in lens fiber cells

Both MP20 and galectin-3 have been shown to be expressed in lens fiber cells [15–17,20]. The alleged involvement of both proteins in adhesion processes raises the possibility that they interact with each other in the lens fiber cell membranes. As a first step to examine this possibility, the spatial distributions of these two proteins were determined using immunocytochemistry (Figure 1). MP20 was expressed widely in the lens, both in elongating fiber cells near the lens periphery, as well as in mature cells deeper in the lens, which had already lost the cell nuclei (Figure 1A). In the peripheral elongating fiber cells, a significant portion of MP20 appeared to be concentrated in vesicles, possibly representing a precursor state to insertion into the plasma membrane (Figure 1B). Similarly, galectin-3 appeared to be present predominantly in vesicles, occasionally in the same ones as for MP20 (Figure 1B). A strikingly different pattern was observed in the mature fiber cells deeper in the lens: both MP20 and galectin-3 were entirely membrane associated (Figure 1C). MP20 was more uniformly distributed in the plasma membrane than galectin-3, which had a more punctate appearance. In many areas, the two proteins appeared to co-localize, supporting the notion that they might interact with each other. Some galectin-3 also localized in membrane regions where MP20 appeared to be missing, suggesting that galectin-3 might also have other binding partners.

Following on from the observation that MP20 and galectin-3 co-localized in many areas of the fiber cell plasma membrane, we now investigate their ability to bind to each other biochemically using two protocols. In the first protocol, we purified MP20 and galectin-3 individually and characterized their molecular mass. The purified proteins were then combined and the mixture was analyzed for complex formation. In the second protocol, conditions were optimized for the solubilization of native complexes of MP20 and galectin-3 directly from the fiber cell membranes.

Biochemical purification of MP20 and galectin-3

MP20 was purified using a previous procedure [26] with some modifications. Outcomes of the individual steps in the procedure are documented in Figure 2. Ovine fiber cell membranes were "fully stripped" of peripheral proteins leaving predominantly the major integral membrane proteins including the 26 kDa major intrinsic polypeptide (MIP) and its 22 kDa cleaved form, MP20 (20 kDa), and the 38 kDa cleaved form of connexin46 and 50 (Figure 2A, lane 2). N-decyl-beta-D-maltopyranoside (DM) solubilized more than 50% of MP20 together with variable amounts of the other integral membrane proteins (Figure 2A, lane 4). All of these proteins including about half of MP20 bound to a MonoQ column. The other half of MP20 appeared in the flow-through fraction and could thus be easily separated from the other proteins (Figure 2B, arrow; Figure 2A, lane 5). Immunoblotting analysis confirmed the identity of MP20. Note that the antibody also recognizes a dimer of MP20 that is not normally detected in stained gels (Figure 2A, lane 8). Gel filtration also indicated that MP20 existed as an oligomer: the protein eluted from the column as a single peak at 15 ml (v_0 = 8 ml) giving it an apparent molecular mass of 60 kDa (Figure 2A lane 7, C & D). This is consistent with a dimer of MP20 associated with a substantial amount of detergent, or less likely, with a trimer with no detergent present.

The purification of galectin-3 started from crude fiber cell membrane preparations, which included the membrane-integral proteins mentioned above and in addition a large number of membrane-adherent proteins (Figure 3A, lane 2). A single alkaline-extraction step removed a sufficient amount of galectin-3 for purification together with variable amounts of other membrane adherent proteins (Figure 3A, lane 4). The presence of galectin-3 in this fraction was confirmed by immuno blotting (Figure 3A lane 7). Galectin-3 from was purified by affinity chromatography on a α-lactose-agarose column [27] taking advantage of the lectin binding site of the protein. A sin

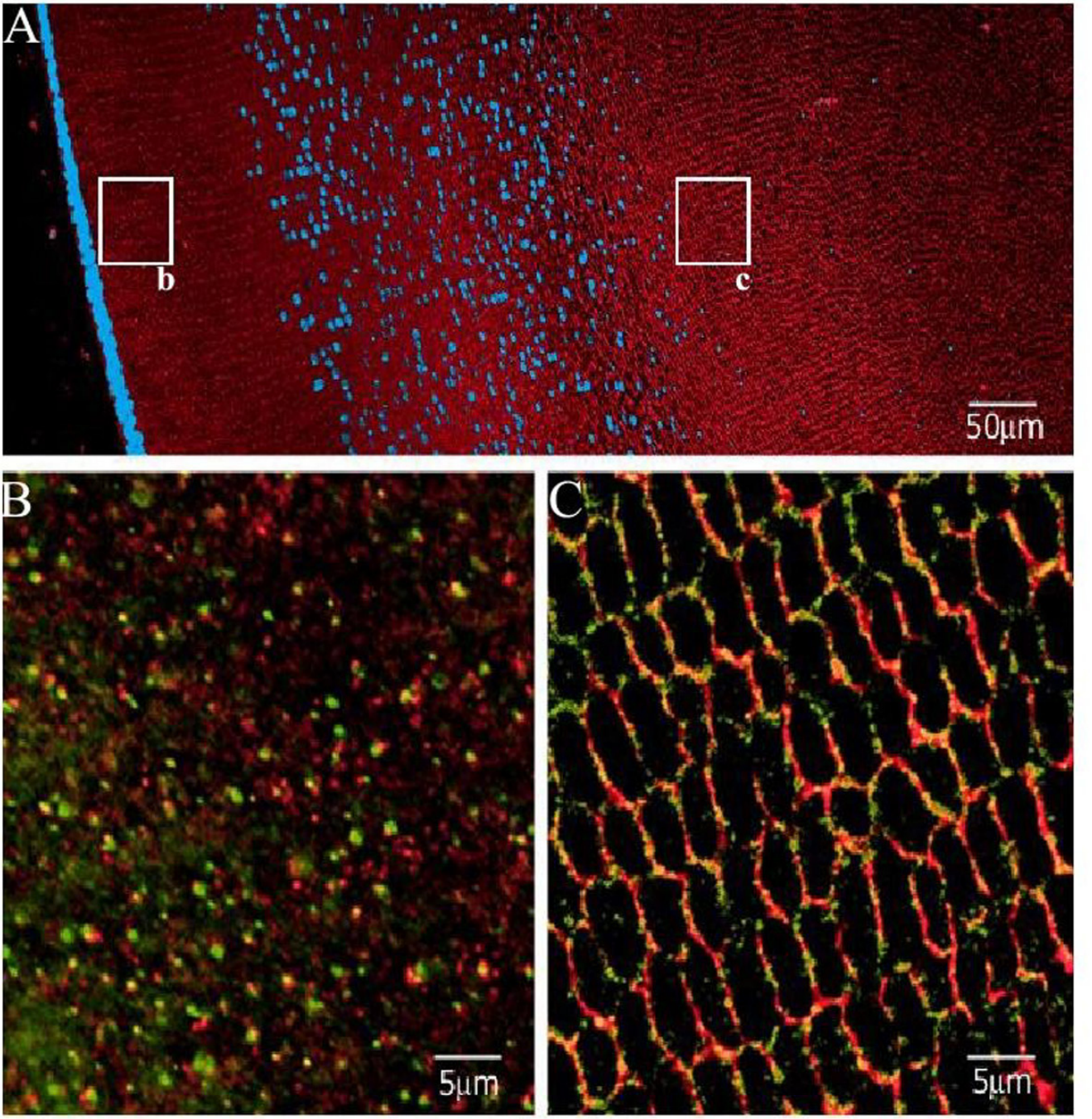

Figure 1
Spatial distribution of MP20 and galectin-3 in the lens. (A) Overview of the distribution of MP20 (red) in relation to cell nuclei (blue) as a marker for fiber cell differentiation in equatorial sections. (B) Co-localisation of MP20 (red) and galectin-3 (green) in peripheral cortex as indicated by box b in panel A. (C) Co-localisation of MP20 and galectin-3 in an area approximately 500 m into the cortex as indicated by box c in panel A. Yellow indicates regions of overlap of both proteins.

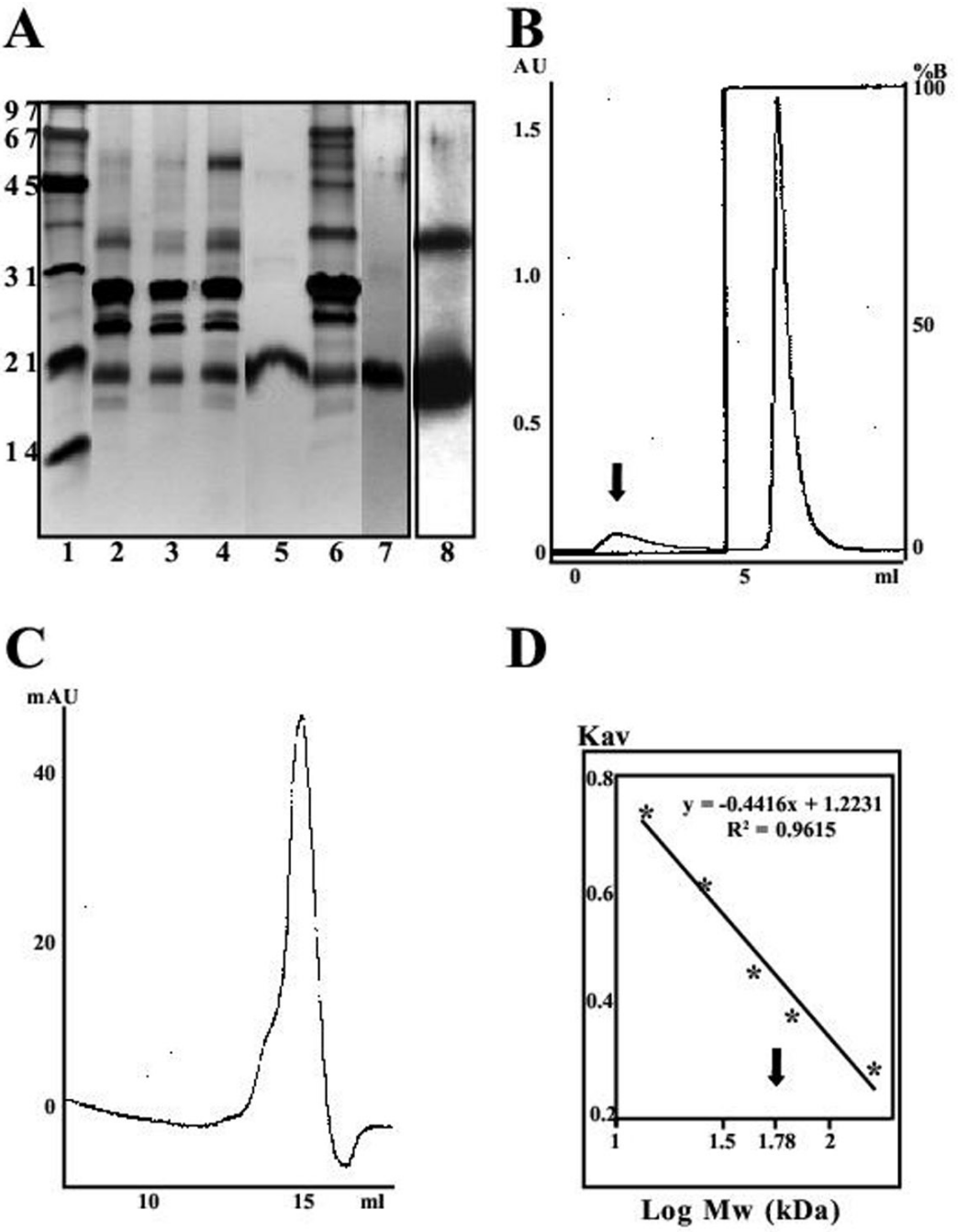

Figure 2

Purification and characterization of lens MP20. (A) SDS-PAGE and Western blot documentation of the purification process. *Lane 1:* Molecular weight markers. *Lane 2:* Fully stripped membranes showing significant enrichment in MP20 (20 kDa), and other integral membrane proteins including MIP (26 kDa) and its 22 kDa cleavage product, and MP38 which is the cleavage form of connexins 46 and 50. *Lane 3 and 4:* Insoluble and soluble fractions, respectively, following treatment of membranes with DM. *Lane 5 and 6:* Soluble proteins that did not bind (mostly MP20) and proteins that did bind to the MonoQ, respectively. *Lane 7:* Pure MP20 eluted from size exclusion chromatography column S-200. *Lane 8:* Immunoblot of lane 5 identifying MP20 and its dimer that is not detected in the stained gel. (B) Single step purification of MP20 on a MonoQ column. MP20 is the only major integral membrane protein that does not bind to the column at pH 8 (arrow). (C) Size exclusion chromatography of pure MP20. The protein was eluted at 15 ml. AU and mAU are absorbance units at 280 nm. (D) Calibration of the S-200 column. Vo = 8 ml. MP20 has an apparent molecular mass of 60 kDa (arrow).

gle peak was eluted with 100 mM lactose (Figure 3B) and contained exclusively galectin-3 with an apparent molecular mass of 31 kDa by SDS-PAGE (Figure 3A, lane 5). Its identity was confirmed by immuno blotting analysis (Figure 3A lane 8). On a gel filtration column (Figure 3C) galectin-3 eluted at 12.5 ml (Vo = 8.3 ml) (Figure 3A lane 6) corresponding to an apparent molecular mass of 22 kDa (Figure 3D), which is smaller then the apparent molecular mass on SDS PAGE. This suggests that the purified galectin-3 is in a (hydrodynamically) compact conformation and is monomeric.

In vitro and in vivo complex formation between MP20 and galectin-3

Having obtained pure preparations of MP20 and galectin-3, their ability to interact with each other was investigated by mixing them and monitoring for the formation of a complex composed of both proteins. This was achieved by gel filtration of the mixture, which should either show a peak for each protein individually with the predetermined molecular weights thus indicating a lack of interaction, or a major peak at a higher molecular weight containing both proteins thus indicating a positive interaction. When mixing pure MP20 (Figure 4A lane 2) and pure galectin-3 (Figure 4A lane 3) with a molar ratio of 1:6, the predominant peak eluted at 13 ml (v_o = 7.8 ml) corresponding to an apparent molecular mass of 104 kDa (Figure 4B and 4C). SDS-PAGE revealed the presence of MP20 and galectin-3 (Fig 4A, lane 4), the latter appearing as a doublet due to minor proteolysis. Such proteolysis had previously been observed and apparently did not abolish binding [28]. The peak at the right edge of Figure 4B contained unbound galectin-3 but no free MP20 was observed. When a similar experiment was carried out using a molar ratio of 1:2, the elution profile was similar to the above except that an additional peak was obtained that contained exclusively MP20 (data not shown). Hence an excess of galectin-3 had to be used to saturate all MP20 indicating that not all galectin-3 had retained the ability to bind to MP20, or that purified MP20 and galectin-3 have a moderate affinity to each other. Nevertheless the data confirm that galectin-3 is a ligand to MP20 and that the two proteins can form a complex *in vitro*.

This ability of MP20 and galectin-3 to bind to each other was further supported by the second protocol aimed at purifying native MP20/galectin-3 complexes directly from fiber cell membranes. Starting from "partially stripped" membranes, which still contained some galectin-3 (Figure 5A, lane 2), MP20 consistently co-purified with galectin-3 on the MonoQ column (Figure 5B arrow; 5A, lane 3). Gel filtration of this fraction (indicated by the arrow on Figure 5B) produced a single peak eluting at 13.8 ml (vo = 8 ml) corresponding to an apparent molecular mass of 102 kDa (Figure 5C & 5D) and containing both proteins (Figure 5A lanes 5–7). This strongly indicates that galectin-3 and MP20 interact with each other also *in vivo*.

The lectin site of galectin-3 appears to be involved in binding to MP20

Since galectin-3 had previously been shown to bind other protein ligands via its lectin domain or other segments of the molecule [21], the interaction between MP20 and galectin-3 was further analyzed to distinguish between these two possibilities. Native MP20/galectin-3 complexes were first solubilized from crude membranes, and then the involvement of the lectin site was investigated by immunoprecipitation with anti MP20 and protein A sepharose beads in the absence or presence of 100 mM lactose, which competes for the lectin site. In the absence of lactose, galectin-3 co-precipitated with MP20 as expected (Figure 5E lanes 1 and 3). However, in the presence of 100 mM lactose most galectin-3 was removed from the native complexes leaving only MP20 (Figure 5E lanes 2 and 4). Lactose did not affect MP20 binding to the protein A sepharose beads as equal amounts of MP20 bound to the beads irrespective of whether lactose was present or not (Figure 5E lanes 3 and 4). This result suggests that galectin-3 binds to MP20 via its lectin site.

Discussion

The interaction between galectin-3 and a member of the tetraspanin superfamily appears to be novel. A recent list of ligands for galectin-3 includes a variety of membrane and extracellular matrix proteins but none of these proteins adopts the tetraspanin topology [29]. However, many of the ligands for galectin-3 have a known involvement in adhesion. This feature is shared by MP20, which has been reported to form membrane junctions between the fiber cells in the lens [15–17]. Hence, while MP20 may be the first of a new class of galectin-3 ligands, it easily fits into an emerging pattern of ligand function.

Evidence presented here that supports a specific binding of galectin-3 and MP20 is based on the following observations: (1) MP20 and galectin-3 co-localize in selected areas of the lens membranes; (2) pure MP20 and galectin-3 spontaneously reconstitute into a 104 kDa complex upon mixing; (3) MP20 consistently co-purifies from crude membranes as a 102 kDa complex with galectin-3; and (4) the co-purification after anion exchange chromatography of MP20 with a 31 kDa protein was previously observed although the latter was not commented on and remained unidentified [26]. All four observations are consistent with each other and unambiguously identify MP20 as a new member among ligands of galectin-3.

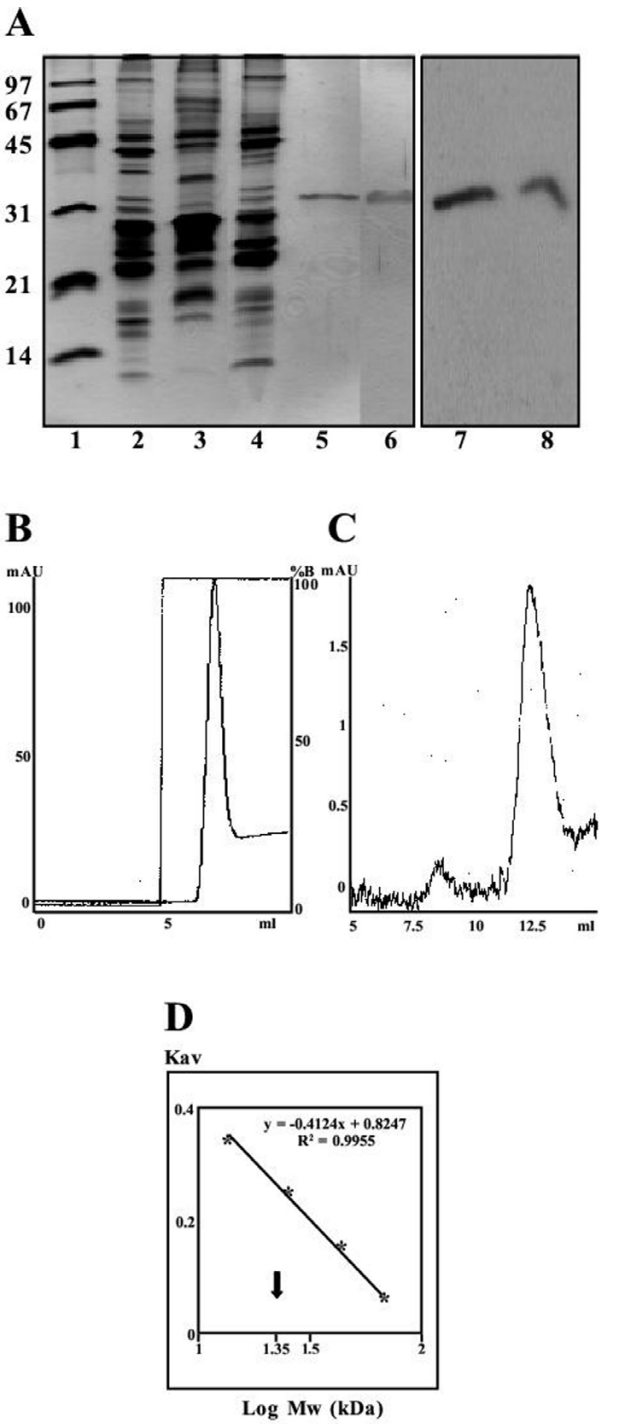

Figure 3

Purification and characterization of lens galectin-3. (A) SDS-PAGE and Western blot documentation of the purification process. *Lane 1:* Molecular weight markers. *Lane 2:* Crude lens fiber cell membranes. *Lane3 and 4:* Insoluble and soluble fractions, respectively, after treatment with 20 mM NaOH. *Lane 5:* Pure galectin-3 eluted from the α-lactose-agarose column with 100 mM lactose. *Lane 6:* Galectin-3 eluted from a size exclusion chromatography column S-75. *Lanes 7 and 8:* Immunoblot of the preparations in lanes 4 and 5 identifying galectin-3. (B) Galectin-3 bound to an α-lactose-agarose column, and was eluted with 100 mM lactose. (C) Size exclusion chromatography of the pure galectin-3. The protein was eluted at 12.5 ml. mAU refers to absorbance units at 280 nm (D) Calibration of the S-75 column. Vo = 8.3 ml. Galectin-3 has an apparent molecular mass of 22 kDa (arrow).

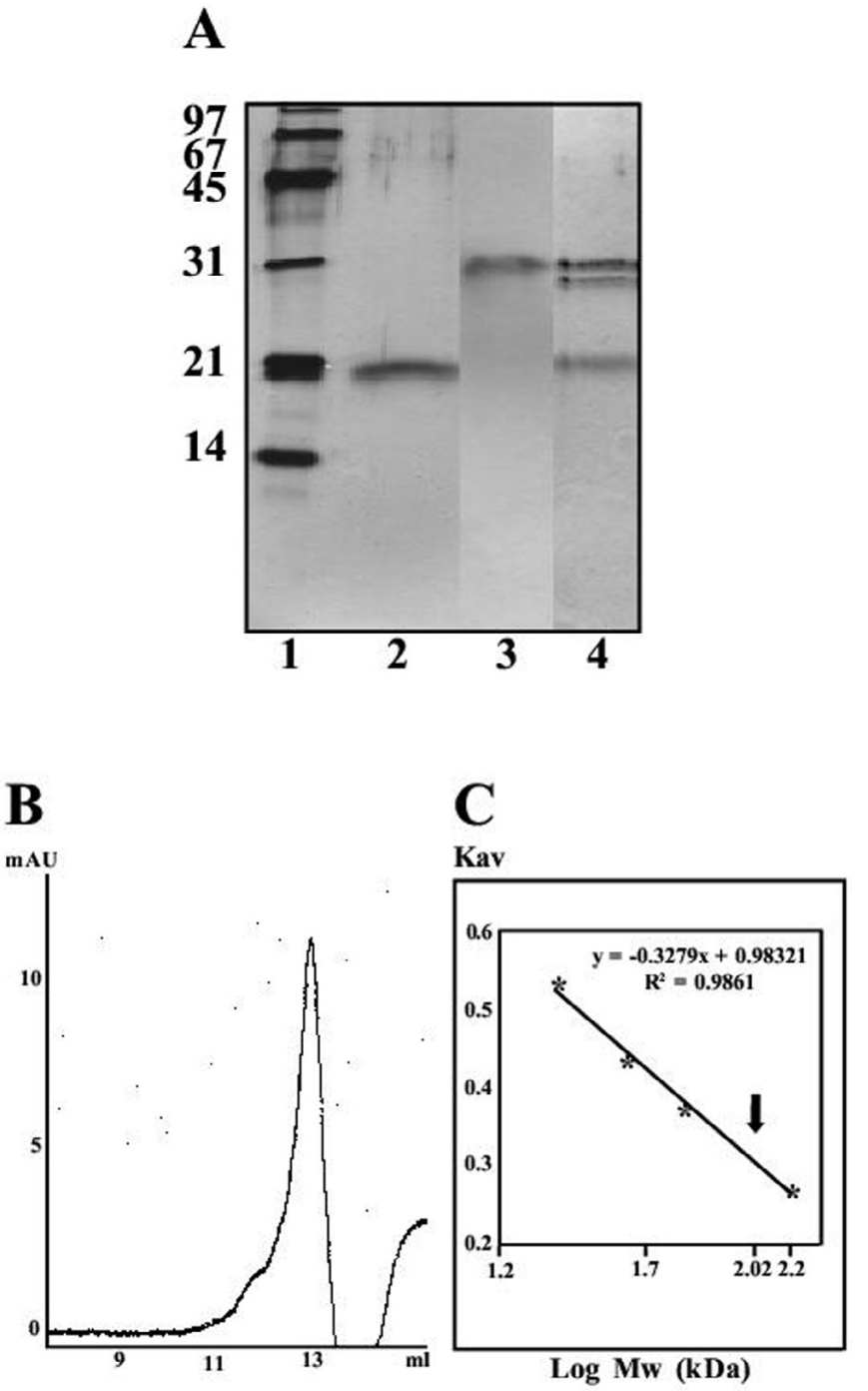

Figure 4
MP20/galectin-3 complex formation *in vitro*. (A) SDS-PAGE analysis of pure MP20 and pure galectin-3 and of the complex formed. *Lane 1*. Molecular weight markers. *Lanes 2 and 3*. Pure MP20 and galectin-3 used for the binding experiment, respectively. *Lane 4*. The complex eluted from the gel filtration column. Galectin-3 appears as a doublet due to minor proteolysis [28]. (B) Size exclusion chromatography of MP20/galectin-3 complexes formed following the mixing of purified lens MP20 with purified galectin-3. The complex eluted at 13 ml. mAU refers to absorbance units at 280 nm. (C) Calibration of the S-200 column. V_o = 7.8 ml. MP20/galectin-3 complex has an apparent molecular mass of 104 kDa (arrow).

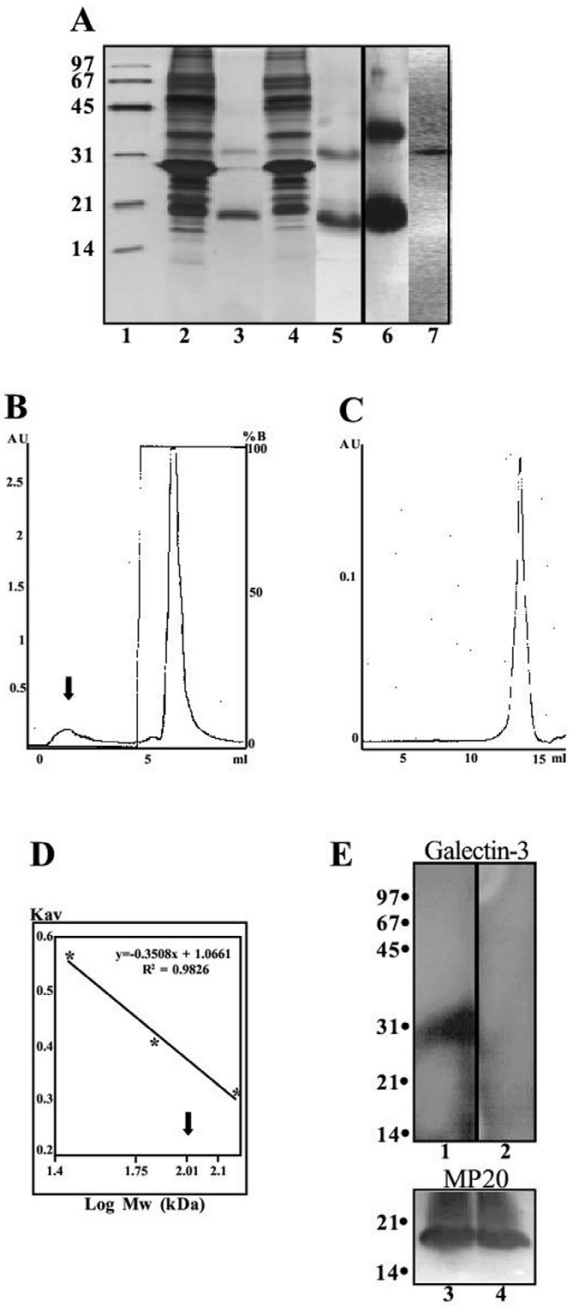

Figure 5

Purification of a native MP20/galectin-3 complex from lens membranes and role of lectin site. (A) SDS-PAGE and Western blot documentation of the purification process. *Lane 1:* Molecular weight markers. *Lane 2*: Partially stripped lens fiber cell membranes showing both galectin-3 and enriched MP20. *Lanes 3 and 4:* Proteins that did not bind (including MP20 and galectin-3) and proteins that did bind to the MonoQ column, respectively. *Lane 5:* MP20 and galectin-3 eluted from the S-200 column. *Lanes 6 and 7:* Immunoblot of lane 5 identifying MP20 and galectin-3, respectively. Note that anti MP20 also recognizes dimeric MP20 that is not normally detected on stained gels. (B) Galectin-3 and MP20 did not bind to the column at pH8 (arrow). (C) Size exclusion chromatography of the complex. A single peak eluted from the S-200 column at 13.8 ml. AU refers to absorbance units at 280 nm. (D) Calibration of the S-200 m column. Vo = 8 ml. MP20/galectin-3 complex has an apparent molecular mass of 102 kDa (arrow). (E) Immunoprecipitation of MP20 and galectin-3 and protein detection by Western blotting. *Lanes 1 and 2:* galectin-3 detected without and with lactose present, respectively. Note the removal of galectin-3 from the complex in the presence of lactose. *Lanes 3 and 4:* Equal amounts of MP20 are detected without and with lactose present, respectively.

The stochiometry of the MP20/galectin-3 complex remains somewhat uncertain mainly because it is unknown how much detergent is bound to purified MP20. The apparent molecular mass of 60 kDa allows for a trimer with no detergent bound, or for a dimer with a substantial amount of bound detergent. By comparison, detergent-solubilized 26 kDa major intrinsic membrane protein of lens fiber cells, MIP, has substantial amounts of detergent bound. The molecular mass for detergent-solubilized MIP has been reported to be in a range between 147 kDa and 229 kDa [30–32]. We know from electron microscopy that detergent solubilized MIP is a tetramer, hence the protein alone accounts for a molecular mass of only 104 kDa, the difference being detergent [31]. Using this analogy, a dimeric conformation for MP20 clearly appears as the more likely option. Given that there is only one potential glycosylation site per MP20 monomer [14] we would expect that two galectin-3 molecules can bind per MP20 dimer. The increase of molecular mass from 60 kDa to 104 kDa upon addition of purified galectin-3 is consistent with this stochiometry.

The observation that lactose effectively releases galectin-3 from isolated native complexes MP20, strongly points to an involvement of the lectin site in the binding interaction. It also suggests that MP20 is a glycoprotein. MP20 has one consensus glycosylation site, however, we have so far failed to demonstrate the presence of carbohydrate despite using different kinds of biochemical detection methods (data not shown). It is possible that the kits used were not sensitive enough, or that the amount of carbohydrate on MP20 is very small. A more rigorous investigation using mass spectrometry will be required to detect such smaller carbohydrate moieties.

What role could the MP20/galectin-3 complex play in the lens? A single point mutation in the MP20 gene of the *To3* mouse results in complete lens opacification and microphthalmia [18]. The lens is vacuolated and the fiber cells are grossly disorganized. This suggests that MP20 plays a role in lens development. This view is supported by our immunolabeling results, which show that MP20 is inserted in the membrane at a distinct point of fiber cell maturation. It is conceivable that galectin-3 plays a critical role in modulating the ability of MP20 to form adhesive junctions at this critical stage of development. Such a modulating function for galectin-3 has previously been proposed from evidence that its presence can increase or decrease cell adhesion in other systems, and that its secretion is developmentally regulated [21,29]. It remains to be established for lens MP20 whether the interaction with galectin-3 is required for junction formation or whether it counteracts this process.

In summary, the major lens membrane protein MP20 is a new member on the list of ligands for galectin-3. MP20 belongs to the superfamily of tetraspanins, which have diverse cell surface functions associated with proliferation, differentiation, migration, and adhesion. It would appear that these functions are complementary with that of galectin-3 as a modulator of adhesion. Hence, it might be worthwhile to test other tetraspanins for their ability to bind galectin-3.

Materials and methods

Immunocytochemistry

Rat lenses were fixed in 0.75% paraformaldehyde in phosphate buffered saline (PBS) for 24 h at room temperature. They were washed 3 times for 10 min in PBS, then cryoprotected by incubation in 10% sucrose in PBS for 1 hr at room temperature, followed by 20% sucrose in PBS, and then 30% sucrose in PBS at 4°C overnight. Lenses were mounted in Tissue-Tek O.C.T. compound at 4°C on pre-chilled chucks. The chucks were immersed in liquid nitrogen for 25 s to freeze the lenses then stored on dry ice. 10 μm thick cryosections were cut at -18°C on a cryostat (Leica CM3050), and placed onto slides coated with poly-L-lysine. All following procedures were carried out either at room temperature for the times indicated, or at 4°C overnight, in a humid box. Slides were washed with PBS and treated for 1 hr with blocking solution (3% bovine serum albumin, 3% fetal calf serum in PBS), washed 3 times for 5 min in PBS, and treated for 2 hrs with a mouse monoclonal anti galectin-3 (Affinity Bioreagents Inc [24]) and rabbit anti MP20 [17] diluted 1:25 and 1:100, respectively, in blocking solution. Slides were washed in PBS to remove unbound antibodies and incubated for 1.5 hr in the dark with 1:200 diluted anti rabbit and anti mouse immunoglobulins conjugated with Alexa Fluor 488 and 568, respectively (Molecular Probes). After washing in PBS, cell nuclei were stained with propidium iodide (0.2% in PBS) for 5 min, and slides were washed and mounted in 10 l Citifluor AF1. Images of each chromophore-staining pattern were recorded digitally using a laser scanning confocal microscope (Leica TCS 4D), then pseudo-colored and combined using Adobe Photoshop software.

Preparation of lens fiber cell membranes

For binding studies of MP20 and galectin-3, proteins were purified from ovine lens membranes. Three different membrane preparations were made. All procedures were carried out at 4°C except where mentioned otherwise. Following removal of capsule and epithelium, batches of 100 lenses were homogenized in 100 ml 5 mM Tris-HCl pH 8.0, 5 mM EDTA and 5 mM EGTA. Membranes were pelleted at 18,000 g for 20 min, and were subsequently washed twice in the same buffer. This preparation was called "crude membranes". Membrane

adherent crystallins and cytoskeletal proteins were removed from crude membranes with a urea/alkali stripping procedure [25]. In one protocol, membrane adherent proteins were only partially removed; the crude membrane preparation was centrifuged and the pellet resuspended in 15 ml 4 M urea, 5 mM Tris-HCl pH 9.5, 5 mM EDTA, 5 mM EGTA. Following centrifugation at 110,000 g for 40 min the pellet was washed twice in 15 ml 20 mM NaOH, and finally resuspended in storage buffer containing 5 mM Tris-HCl pH 8.0, 2 mM EDTA, 2 mM EGTA. The resulting membranes were labeled "partially stripped membranes" as they contained membrane-integral proteins as well as some of the more strongly bound peripheral membrane proteins including galectin-3. They were stored at -20°C at a protein concentration of approximately 4 mg/ml. Protein concentrations were determined using the BCA protein assay method (Pierce).

"Fully stripped membranes" were also prepared from crude membrane preparations following a similar procedure to that outlined above but using larger quantities of the urea and alkaline solutions, and adding a lactose extraction step to remove any remaining galectin-3. Fully stripped membrane preparations contained only the membrane-integral proteins. The crude membrane preparation was centrifuged and the pellet resuspended in 100 ml 4 M urea, 5 mM Tris-HCl pH 9.5, 5 mM EDTA, 5 mM EGTA. Membranes were pelleted at 140,000 g for 1 hr and subsequently washed three times in 100 ml 20 mM NaOH. Next, membranes were incubated in 100 mM lactose in storage buffer for 10 min at room temperature, and pelleted as above. The lactose step was repeated once, and fully stripped membranes were resuspended in storage buffer and stored at -20°C at a protein concentration of approximately 1.5 mg/ml until further use.

Purification of MP20

MP20 was purified with a single ion exchange chromatography step. Aliquots of fully stripped membranes were solubilized in 1% n-decyl-beta-D-maltopyranoside (DM) (Sigma Chemical Co) in storage buffer at room temperature. Insoluble material was pelleted at 110,000 g for 1 hr. Soluble proteins were separated on a MonoQ column (Pharmacia Biotech) equilibrated with 0.3% DM in storage buffer on an AKTA FPLC (Pharmacia Biotech). All proteins except MP20 bound to the column. MP20 in the flow-through fraction was concentrated with Centricon-10 cartridges as required. MP20 was analysed by size exclusion chromatography using an S-200 10/30 column (Pharmacia Biotech) equilibrated with 0.3% DM, 0.5 M NaCl in storage buffer. The column was calibrated using ovalbumin, bovine serum albumin (BSA), aldolase, chymotrypsinogen A, and ribonuclease A as marker proteins, and blue dextran for determination of the void column volume. For the calculation of molecular weight a calibration plot of log Mw *vs*. Kav was constructed. Kav = (Vc-Vo/Vt-Vo) where Vc = protein elution volume, Vt = column volume (24 ml) and Vo = void column volume.

Purification of galectin-3

A single treatment step of crude membranes with 20 mM NaOH was used to solubilize sufficient galectin-3 for further purification. Insoluble material was pelleted at 140,000 g for 1 hr, and the supernatant dialyzed against 2 L 50 mM HEPES pH 6.8, 2 mM EDTA, 2 mM EGTA, at room temperature for 24 hours. The supernatant was then loaded onto an α-lactose-agarose column (Sigma) pre-equilibrated with 10 mM HEPES pH 6.8, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, and eluted with 100 mM lactose in the same buffer. Pure galectin-3 was obtained by this single purification step and was further analyzed by size exclusion chromatography using a S-75 10/30 column (Pharmacia Biotech) equilibrated with the same buffer. The column was calibrated using ribonuclease A, chymotrypsinogen A, ovalbumin, and BSA as marker proteins, and blue dextran to determine the void volume.

Isolation of complexes containing MP20 and galectin-3

The ability of MP20 and galectin-3 to interact with each other was investigated in two ways: first, by analyzing the complex formed when combining the purified proteins, and second, by determining whether a similar complex could be isolated from lens fiber cell membranes. The protocol for the first approach was as follows. Lactose was removed from purified galectin-3 by dialysis against 2 L 10 mM Tris-HCl pH 8.0, 2 mM EDTA, 2 mM EGTA at room temperature for 24 hours. Galectin-3 was then incubated with purified MP20 for 24 hrs at 4°C at a molar ratio 6:1. The mixture was analyzed by size exclusion chromatography using an S-200 10/30 column (Pharmacia Biotech) equilibrated with 0.3% DM, 0.5 M NaCl in storage buffer. The column was calibrated using aldolase, BSA, ovalbumin, and chymotrypsinogen A as marker proteins, and blue dextran for the determination of the void column volume.

Alternatively, a native complex of MP20 and galectin-3 was isolated from lens fiber cell membranes. For this, the same protocol was employed as for the purification of MP20 except that "partially stripped" membranes were used as starting material. Isolated MP20/galectin-3 complexes were analyzed by gel filtration as above using carbonic anhydrase, BSA, alcohol dehydrogenase, and blue dextran for calibration.

Investigating the interaction between galectin-3 and MP20

Complexes of MP20 and galectin-3 were first solubilized from crude membranes, and the soluble complexes treated with lactose to determine whether this separated the two proteins. Crude membranes were treated with 1% DM in 10 mM HEPES pH 6.8, 2 mM EDTA, 2 mM EGTA for 1 hr at room temperature, and the soluble material was collected following centrifugation at 140,000 g for 1 hr. Soluble proteins were then incubated for 1 hr with anti MP20 at a dilution of 1:50 in 10 mM HEPES pH 6.8, 2 mM EDTA, 2 mM EGTA, 0.3% DM. Three volumes of a solution containing 50% protein A sepharose beads, 10 mM HEPES pH 6.8, 2 mM EDTA, 2 mM EGTA, 100 mM NaCl, 0.3% DM, and either none or 100 mM lactose, were added to the immunoprecipitated MP20/galectin-3 complexes and incubated overnight at room temperature. The beads were washed several times with the respective buffers (with or without lactose), collected by centrifugation, and analyzed by immunoblotting. For this purpose, 17.5% acrylamide gels were run in a Mini-PROTEAN II cell (BIO-RAD). Proteins were solubilized in sample buffer without boiling as heat tended to aggregate some of the membrane integral proteins. Proteins 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 SCIENCES). Blots were stained with 0.1% Ponceau S/1% acetic acid to visualize and record the positions of lens proteins and molecular weight markers. Following washing in milliQ water, the blots were blocked overnight at 4°C in 5% non-fat milk powder in Tris-buffered saline (TBS). The blots were incubated with either anti galectin-3 (1:200 in TBS containing 1% BSA) or anti MP20 (1:1000), and bound antibodies detected with biotinylated secondary antibodies at 1:1000 and streptavidin-horseradish peroxidase at 1:1000 according to the manufacturer's instructions (ECL, Amersham LIFE SCIENCES).

Abbreviations

DM, n-decyl-beta-D-maltopyranoside; TBS, Tris buffered saline; PBS, phosphate buffered saline; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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