Preparation, Characterization, and Localization of Antisera Against Bovine MP26, an Integral Protein from Lens Fiber Plasma Membrane

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ABSTRACT Polyclonal antisera were prepared in rabbits using both native and chymotrypsin-digested bovine lens fiber plasma membranes. MP26, the principal protein of lens fiber plasma membranes, and CT20, a chymotryptic fragment of MP26, were isolated electrophoretically and used to purify anti-MP26 and anti-CT20 activity from the respective antisera by affinity chromatography. These affinity-purified antisera were characterized by immunoreplica. Immunofluorescence microscopy localized MP26 on sections of methacrylate-embedded lenses in the lens fiber plasma membranes, but not the lens epithelium. Immunocytochemistry of isolated native or chymotrypsin-digested lens fiber plasma membranes localized both the MP26 and the CT20 only in the nonjunctional plasma membranes, with no detectable activity in the lens fiber junctions themselves. Electron microscopy revealed a second set of pentalaminar profiles, thinner by 4 nm than the lens fiber junctions, which contained demonstrable anti-MP26 and anti-CT20 activity following immunocytochemistry. These results indicate either that MP26 is not a component of the lens fiber junctions, or that significant conformational changes accompany assembly of MP26 into lens fiber junctions, resulting in the masking of MP26 antigenic determinants.

Lens fiber plasma membranes are rich in an intrinsic protein, called MP26 (4), with an apparent molecular weight on SDS polyacrylamide gels of 26,000 daltons (1, 5, 7). Evidence has been presented that indicates that the MP26 is a structural component of the unusual intercellular junctions joining adjacent lens fibers (2, 3, 12, 20). These lens junctions occupy a large percentage of the lens fiber surface area (17), and the MP26 is quantitatively the most abundant lens fiber membrane protein. Dunia et al. (9) have reported the enrichment of a 34,000 Mr polypeptide (MP34) in a detergent-enriched subfraction of lens junctions, perhaps reflecting the two morphological classes of junctions in the lens that have been described (2, 13, 24).

Antisera to the MP26 have been generated in several laboratories. Immunofluorescence studies have localized the MP26 in the plasma membrane of the lens fibers, but not in the lens epithelium (8, 29). Electron microscopic immunocytochemical studies of urea-washed, isolated lens plasma membranes reveal staining of the cytoplasmic aspect of both the junctional and nonjunctional membranes using an anti-MP26 polyclonal antiserum (6).

Numerous studies indicate that the MP26 protein, although highly conserved in lenses from different organisms (27), may be much less abundant or absent in other tissues, notably liver (15). Peptide mapping and sequencing studies have demonstrated that the MP26 and the principal polypeptide from liver gap junctions (27,000 Mr) are clearly different gene products (15, 23). In addition, immunological cross-reactivity could not be demonstrated (15, 33). Due to the loss of protein synthetic machinery during lens fiber differentiation (3), the MP26 must be a long-lived protein, with little or no turnover, in contrast to the liver junctional polypeptide, which has a measured half-life of 5 h (10, 31).

Taken together, these data characterize the MP26 as a membrane protein detectable thus far only in differentiated lens tissue, appearing to be localized in both the junctional and extrajunctional lens fiber plasma membranes. The MP26 appears quite distinct biochemically and immunologically from the principal polypeptide of liver gap junctions.

It has been questioned whether the lens fiber junctions mediate physiological communication (15, 32). The lens behaves functionally as a spherical syncitium with respect to ions (22), fluorescent dyes (25, 26), and small metabolites (13). These physiological data suggest that the lens cells are joined
by low-resistance pathways (11). The lens fiber junctions share some structural features in common with gap junctions in other tissues, but differ structurally in several respects. The junctions usually do not show a "gap" in thin-sectioned material and there is little tendency for the component subunits (connexons) to crystallize in response to routine tissue preparation for electron microscopy (12, 14). In addition, the MP26 in isolated lens membranes has been described in a crystalline form not seen in isolated gap junctions from liver and myocardium (32). Thus any direct role of the lens fiber junctions in cell-cell communication must await further experimentation.

In this manuscript, we describe the preparation of antisera against bovine lens MP26 and a chymotryptic fragment of MP26, called CT20. These antisera are affinity purified and characterized by immunoreplica. Immunofluorescence microscopy localizes the αMP26 to the lens fiber plasma membranes, and not the epithelium, as described by others previously. Electron microscopy localizes the αMP26 and the αCT20 only on the cytoplasmic surface of the nonjunctional lens fiber plasma membrane, not on the lens fiber junctions.

MATERIALS AND METHODS

Isolation of Antigens: Lens membranes enriched in gap junctions were prepared according to Goodenough (12) except that DOC washing was omitted. Membranes were solubilized in Laemmli (18) gel dissolving buffer at 2°C at concentrations of 1-2 mg/ml. One mg total protein was electrophoresed on 120 x 120 x 1.5 mm slab gels according to Laemmli (18) with 5% and 12.5% stacking and running gels. The protein bands were visualized by incubating gel in 1 M KCl for 1-5 min. Bands were excised with grease-free razor blades and eluted with an ISKO electrophoretic concentrator (ISCO, Instrumentation Specialities Co., Lincoln, NE) in 10 mM PO₄ buffer, pH 6.8 with 0.1% SDS. 200 μg highly purified MP26 was obtained from 1 mg total membrane protein. A 20,000 M₄ fragment of the MP26 was prepared by suspending urea-washed membranes in 50 mM Tris, pH 7.6 at 4 mg/ml with T/L-CH-Chymotrypsin (Sigma Chemical Co., St. Louis, MO) at 200 μg/ml. Membranes were incubated at 4°C for 90 min, made 1 mM in fresh PMSF (Sigma Chemical Co.), washed twice by centrifugation, and pre pared for electrophoresis as described.

Preparation and Characterization of Antisera: New Zealand white rabbits (Charles River Breeding Laboratories, Inc., Wilmington, MA) were injected subcutaneously at multiple sites with urea-washed or chymotryptically treated membranes emulsified in complete Freund's adjuvant. Usually, 1 ml of the emulsion containing 2 mg/ml membranes was injected. At monthly intervals thereafter, rabbits were boosted with membranes emulsified in incomplete Freund's and bled at 6, 10, and 13 d after each booster shot.

Antisera were affinity purified using isolated MP26 or CT20 covalently attached to Sepharose CL-4B by the procedure of March et al. (21). 1% SDS was present in coupling and initial washing buffers. Affinity purification was done according to Hudson and Hay (16), except that 0.1% Tween 80 (Atlas Chemical, Wilmington, DE) was present in washing buffers.

Antisera and affinity-purified antibodies were characterized by immunoreplica as described by Towbin et al. (28) using HRP-conjugated goat α rabbit (Cappel Laboratories Inc., Cochranville, PA).

Immunocytochemistry: For fluorescence microscopy, tissue samples were fixed in 1% formaldehyde, made from paraformaldehyde, in PBS at 4°C overnight, dehydrated in ethanol solutions, and embedded in OCT (Sakura Finetek USA Inc., Torrance, CA). 5-μm sections were cut on a cryostat (Leica SM 5000, Wetzlar, Germany) and air dried at room temperature. Sections were then blocked in 3% normal sheep serum (NSS) for 30 min at RT and incubated with the primary antibody. The primary antibodies were rabbit anti-MP26 (Cappel Laboratories Inc.) at 1/100 dilution or rabbit anti-CT20 (Cappel Laboratories Inc.) at 1/20 dilution. Secondary antibodies were fluorescein-conjugated goat anti-rabbit (Cappel Laboratories Inc.) at 1/100 dilution. Sections were washed three times with PBS (10 min each) and mounted with Vectashield medium for observation under a Nikon E800 fluorescence microscope.

RESULTS

Antigen and Antibody Characterization

Rabbit antibodies against MP26 were prepared by injecting urea-washed membranes (Fig. 1, lane B), then affinity purifying the resultant serum with electrophoretically isolated MP26 (Fig. 1, lane D) bound to Sepharose. Immunoreplica staining with the crude antisera (Fig. 2, lanes C and D) indicated activity against a variety of membrane and several soluble proteins. The affinity-purified antiMP26 (αMP26) showed activity against only MP26, one larger protein, and a few smaller proteins.

![Figure 1](image-url)
FIGURE 2 Characterization of specificity of αMP26 using immunoreplica. Lanes A and B: Coomassie-stained SDS PAGE containing urea-washed lens fiber membranes (lane A) and soluble lens proteins (lane B). Lanes C and D: Immunoreplica of lanes A and B stained with rabbit antiserum produced by injecting membranes. Lane E and F: Immunoreplica of lanes A and B stained with same antiserum after affinity purification using SDS-purified MP26. High molecular weight band corresponds to first order aggregation seen in Fig. 1, lane D.

ones (Fig. 2, lanes E and F). The higher molecular weight band has been shown to be an aggregation of MP26 that occurs in SDS solution (30). The lower molecular weight peptides are proteolytic fragments of the MP26 because preadsorption of the αMP26 with isolated MP26 abolished all immunoreplica staining. αMP26 monoclonal antibodies stained the aggregate and the lower molecular weight bands in addition to the MP26 (data not shown).

As reported previously (27), exhaustive proteolytic digestion of lens membranes quantitatively reduced the molecular weight of MP26 to ~20,000 Mr (CT20 fragment) (Fig. 1, lane C; Fig. 3, lanes C and G). Immunoreplica staining of digested membranes with αMP26 showed no reaction with the CT20 fragment (Fig. 3, lane E). In order to generate a probe for this portion of the molecule, chymotryptically digested membranes were injected into rabbits. The resultant antiserum was affinity purified using isolated CT20 (Fig. 1, lane F). Immunoreplica staining with affinity-purified αCT20 demonstrated activity against only the CT20 and not the MP26 (Fig. 3, lanes H and I). This appears to conflict with the notion that CT20 is derived from MP26. However, this could be explained if CT20 provoked a very limited antigenic response, producing antibodies directed against only those sites that are exposed after chymotryptic cleavage. We believe that this must be the case for the following reasons. First, the CT20 is clearly related to MP26 because two dimensional peptide maps of the two peptides show considerable homology (27). Second, it is clear that a very limited type of antigenic response has occurred. αCT20 does not react with any proteins in urea-washed, unproteolyzed membranes (Fig. 3, lane H). Thus, regardless of the derivation of the CT20, chymotryptic cleavage of its parent molecule must occur before reactive sites are exposed. Third, it can be seen that the shape and size of the protein band stained in immunoreplica (Fig. 3, lane I) mirror the shape and size of the CT20 band in the Coomassie-stained gels (Fig. 3, lane G). This suggests that the immunoreactive peptide is not a minor contaminant of the CT20 band.

Immunocytochemical Localization

Indirect immunofluorescence microscopy was performed on methacrylate-embedded sections of adult lens (Fig. 4A and B). Using αMP26, only lens fibers were observed to specifically stain. No staining of the epithelial cells was detectable with this method. The fibers appeared to stain over their entire surface, including that portion in contact with the epithelial cells. Since this area exhibits a much lower density of junctions than fiber-
FIGURE 4 Immunofluorescence localization of MP26 in methacrylate-embedded sections of lens. Panel a: Section at anterior surface of lens included capsule (cap), epithelium (epith), and cross-sectioned cortical fibers. Specific staining is observed only on fiber membranes. Staining fiber membranes is uniform with occasional “hot-spots”, including areas of epithelial-fiber contact (arrows) where gap junctions are rare. Panel b: Subcortical fibers, again cross-sectioned, from posterior of lens. Circular profiles (single arrows) represent ball-and-socket invaginations of one cell into another. Membrane staining is uniform again, with occasional “hot-spots”, even on ball-and-socket invaginations which are extremely rich in gap junctions. A portion of one of the posterior lens sutures is visible (double arrow). Bar, 10 μm. × 2,000.
fibrin contact areas (13), a strong component of nonjunctional staining was indicated. On the other hand, the ball-and-socket invaginations of one fiber cell membrane into another were strongly stained (Fig. 4a, arrows). These have been shown to be junction-rich areas (12, 19).

Antibody staining at the EM level was performed on isolated lens fiber membranes. Due to the continuity of the junctional with the nonjunctional membranes, the cytoplasmic/extracellular topology of the nonjunctional membranes can be determined by inspection. In Fig. 5 B, αMP26 is detected with a ferritin-labeled goat antiserum. Specific staining was observed on the cytoplasmic surfaces of nonjunctional membranes (closed arrows). Patches of nonjunctional membrane without staining were detectable but no staining was ever observed on junctional regions (closed long arrows). In some cases, areas of close membrane apposition were seen with ferritin on the cytoplasmic surface (open arrows). These areas are distinctly more narrow by ~4 nm than the usual lens fiber junction thickness of ~16 nm. These areas of close membrane apposition may be an artefact of the isolation procedure, although their true nature is not known (see Discussion). Fig. 5 A illustrates a typical control stained with preimmune serum instead of αMP26. Fig. 5 C shows staining with αMP26 as in Fig. 5 B, except that no ferritin-labeled 2° antibody was applied. The 1° antibody is directly visualized by tannic acid staining as fuzzy electron-dense masses (short arrows). As in Fig. 5 B, staining is confined to the cytoplasmic aspects of nonjunctional membranes. Due to the closure of membrane sheets into large vesicles, immune staining is frequently not observed on the enclosed nonjunctional membrane (Figs. 5 C and 6).

Electron microscopic antibody localization was also performed using the affinity-purified antibody to the CT20 fragment of MP26 (αCT20). Fig. 6 shows the antibody staining pattern on urea-washed membrane that had been digested with chymotrypsin, visualized directly with tannic acid staining. Proteolytic digestion produced no detectable structural alterations in thin sections of lens membranes. As with αMP26 staining on undigested membranes, specific staining with αCT20 was observed only on the cytoplasmic surfaces of nonjunctional membranes (closed arrows), and on the areas of close membrane apposition (open arrows) described above. As was expected from the immunoreplica characterization of these antibodies, αMP26 did not stain digested membranes and αCT20 did not stain undigested membranes (data not shown).

These localization results have been consistent with a variety of different antisera. Polyclonal antisera have been generated with both whole lens fiber membranes and electrophoretically purified MP26 as antigen. Monoclonal antisera have been generated also with both sets of antigen. In all cases, the resultant antisera stain the MP26 on immunoreplicas and exclusively stain the nonjunctional membranes (data not shown). No lens fiber junction staining has ever been observed with an antiserum which recognizes only the MP26. These results were observed whether the lens membranes were urea-washed or examined without urea treatment.

DISCUSSION

In this paper we have shown that antisera prepared by using lens fiber membranes as antigen show activity against many of the polypeptides in the preparation, including the MP26. Affinity purification of the αMP26 activity using electrophoretically purified MP26 bound to Sepharose yields antibodies that react with the MP26, its aggregates, and some of its degradation products, as assayed by immunoreplica. Immunofluorescence photographs of methacrylate-embedded lenses show strong staining of the lens fiber membranes, but not the epithelial membranes. Immunoelectron microscopy localizes this staining exclusively to the cytoplasmic surface of the nonjunctional membranes, with no detectable activity on the surfaces of the lens fiber junctions themselves. These findings contrast with those published by Bok et al. (6), who localized MP26 on the cytoplasmic surfaces of lens fiber junctions by similar techniques.

Interpretations of the Data

These results are discussed by considering two different interpretations: either the MP26 is located in both the junctional and nonjunctional membranes, and the MP26 antigenic sites recognized by our antiserum become inaccessible to antibody binding within the junction structure, or the MP26 is not contained in the lens fiber junctions.

Considering the first possibility, the large surface area of lens fiber junctions that has been reported, taken together with the quantitatively prominent MP26 on SDS gels of isolated lens fiber membranes, has provided the initial conclusion that the MP26 is located within the junctional structure. A more rigorous quantitative comparison is needed to provide more critical data for this conclusion. Detergent subfractionation of the morphologically identifiable lens fiber junctions yields an apparent enrichment of the MP26 (12), but it should again be emphasized that these latter results were not quantitative, and the small percentage of lens fiber junctions enriched by detergent subfractionation may not be representative. Our immunofluorescence photographs (Fig. 4a and b) reveal staining of the MP26 over the entire fiber cell surface, both at the epithelium/fiber interface, where junctions are relatively rare, and on the ball-and-socket fiber/fiber interactions, where junctions are plentiful. However, this same antiserum will not stain lens fiber junctions in the electron microscope. In the immunofluorescence technique used, the antiserum is applied to the surfaces of the methacrylate-embedded sections of the cells, where intramembrane and extracellular portions of the MP26 are exposed to the antibody by the sectioning process. It is possible, then, that the MP26 antigenic determinants, normally exposed on the nonjunctional membranes, are masked as a result of junction assembly and are exposed by the sectioning process. Taken together, these data suggest that the MP26 may be localized within both the junctional and nonjunctional plasma membranes, and that either the junctional assembly masks some of the MP26 antigenic sites, or our isolation procedure selectively alters the MP26 in only the lens fiber junctions. If that is true, the antiserum of Bok et al. (6) may recognize additional antigenic determinants not recognized by our antiserum.

A second possible interpretation would hold that the MP26 is not located in the lens fiber junctions and is usually found only in the nonjunctional membranes. Our antisera show no antigenic activity on the junctional surfaces. Chymotrypsin digestion of isolated lens fiber membranes quantitatively converts the MP26 to the CT20 fragment. There are no detectable changes in the thin-sectioned appearance of the membranes. An affinity-purified antiserum prepared against the CT20 still localizes only on the nonjunctional membranes, indicating no detectable antigen redistribution. Thus the removal of an apparent 6,000 daltons of mass from the MP26 does not expose common buried antigenic determinants found in MP26 mole-
FIGURE 5  Electron microscopic localization of MP26 in urea-washed lens plasma membrane. Panel A: Preimmune control. Panel B: Membranes stained with αMP26 followed by ferritin-conjugated goat arabbit. Staining is observed only on cytoplasmic surfaces of non-junctional membrane (short closed arrows). No staining is observed on lens fiber junctions (long closed arrows). Areas of close membrane apposition are frequently observed (open arrows). These areas are more narrow than normal fiber junctions, and αMP26 localizes in these regions. Panel C: αMP26 is directly visualized on the cytoplasmic surface of non-junctional membrane. No ferritin-conjugated 2º antiserum was used. Bar, 100 nm. × 275,000.

We have now generated several different polyclonal and monoclonal antisera, directed against MP26; all of these localize the MP26 in the non-junctional membranes and not in the lens fiber junctions.

We have used the antiserum prepared by Bok et al. (6) to immunolabel membranes prepared in our laboratory and have obtained electron microscopic staining patterns identical to
FIGURE 6. Electron microscopic localization of CT20 on chymotryptically-digested lens membranes. Antibody is visualized directly without ferritin as in Fig. 5, panel C. Staining is visible on cytoplasmic surfaces of nonjunctional membrane (closed arrows). Areas of close membrane apposition also exhibit aCT20 staining (open arrows). Staining is never observed on junctions. Staining is absent from other half of membrane pair because isolated membranes tend to form sealed vesicles. Bar, 100 nm. × 275,000.

those produced by our own antisera, reported here. We therefore feel that MP26 cannot be located in the lens fiber junctions isolated by our protocols. This does not rule out the possibility that, due to differences in the procedures used to prepare the lens plasma membrane fractions, we are actually studying a class of "junctional" membranes separate from that studied by Bok et al. (6). In this regard, it is important to emphasize that our micrographs of thin sections of isolated lens plasma membranes actually reveal two classes of membrane interactions. Both these interactions appear as double membranes, or pentalaminar structures, viewed in profile. They are often continuous with each other in the membrane plane (Figs. 5B and 6). One class of these pentalaminar profiles (long arrows, Fig. 5B) measures ~16 nm in thickness and is indistinguishable from lens fiber junctions observed in whole lens and in detergent-enriched lens fiber junctions (12). The average density of the three dark laminae in the 16-nm pentalaminar profile appears approximately equal. The other pentalaminar profile (open arrows, Fig. 5B) measures ~12 nm in thickness and the central lamina appears more electron dense than the two adjacent dense laminae. This narrower type of pentalaminar profile has not been reported in intact lenses and thus may be an artifactual association of nonjunctional membranes following cell disruption. The aMP26 antisera label the 12-nm profiles, as does the aCT20 if membranes are proteolytically digested.

Zampighi et al. (32) have also reported two classes of pentalaminar profiles, shown in their Fig. 4. They also report a 3–4-nm difference in width between the two classes of pentalaminar profiles, and the narrower of the two profiles contains a more electron-dense central lamina, as reported for our data above. Zampighi et al. (32) have demonstrated that the narrower class of pentalaminar profile contains the MP26 protein crystallized in a square lattice. Our electron microscopic localization studies support the conclusion that these narrow pentalaminar profiles contain, at least in part, the MP26. Zampighi et al. (32) conclude that the narrow pentalaminar profiles, with the crystalline arrays, are a second class of intercellular junction formed between lens fibers.

The use of tannic acid in the fixation protocol for isolated lens fiber plasma membranes has resulted in a dramatic im-
process in the preservation of nonjunctional membranes as seen in thin-section electron microscopy. Zampighi et al. (32) use tannic acid in their fixatives, while Bok et al. (6) do not. The degree of increased preservation is dramatic, such that the junction-enriched preparations of Goodenough (12) are now seen to be substantially contaminated with nonjunctional membranes (data not shown). It is possible, then, that the differences in localization observed between our antisera and those of Bok et al. (6) may be resolved by the use of different fixation protocols.

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