Junctions between Lens Fiber Cells Are Labeled with a Monoclonal Antibody Shown to be Specific for MP26

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ABSTRACT   A monoclonal antibody (mcAb) that recognizes an intracellular domain of the major lens membrane protein in both chicken and bovine lenses is described. Mice were immunized with chicken lens fiber cell membranes that had been washed with 7 M urea. Hybridomas were screened by means of enzyme-linked immunosorbent assays and the molecular specificities of the mcAbs were determined using electrophoretic transfer procedures, "Westerns." One of these mcAbs, an IgG designated B2, reacted with a single band of 28,000 M_r from the chicken embryo lens (MP28) and the analogous 26,000 M_r protein in the bovine lens (MP26). Monoclonal B2 was shown to be specific for these proteins, since (a) heating in SDS caused MP26 to aggregate and reduced B2 binding to the protein band at an M_r of 26,000 in Western transfer analysis; (b) apparent dimers were bound by B2 in Western transfers; (c) soluble protein fractions from the lens contained no detectable B2 antigens; and (d) a cyanogen bromide fragment of MP26 was bound by B2.

Studies with several proteases indicated that the antigenic site for B2 resides on a 2-kd, protease-sensitive region at the C-terminal end of MP26 and MP28. Evidence for B2 binding on the cytoplasmic side of the membrane comes from labeling studies done at the ultrastructural level. These studies, utilizing indirect methods with peroxidase and colloidal gold markers, clearly demonstrated that B2 labels two types of junctional profiles. In our calf lens membrane preparations after tannic acid staining, the predominant type (80%) measured 16–18 nm thick, with the second type measuring only 12–14 nm.

Chick embryo lens cells that had differentiated in vitro and formed groups of lens fiber-like cells (termed lentoids), fluoresced brightly only when they had been permeabilized before labeling with B2 and a fluorochrome-conjugated antibody. This binding was concentrated at the plasma membranes of cells within the lentoids, even outside areas of cell-cell contact. Surrounding epithelioid cells were not stained. Solubilized lens cultures, examined by Westerns, displayed a single immunoreactive band, which co-migrated with MP28.

It has been recognized for several years that the fiber cells in the vertebrate lens form extensive intercellular junctions, resembling gap junctions seen in a variety of other tissues. Consequently, investigators have sought to better understand the detailed ultrastructure (1, 2) and protein composition (3, 4) of these junctions. The dominant hypothesis has been that the bovine lens junction is composed of a protein of 26,000 M_r (5).

We have developed monoclonal antibodies (mcAbs)[1] to

lens junctional proteins, since these proteins are difficult to purify and presumably contain multiple antigenic determinants. Antibodies specific for distinct domains on these proteins are desirable in order to investigate the presumed transmembrane nature of junctional components. Different domains would likely be involved in different functions, e.g., recognition and adhesion, possible channel formation and intracellular regulation. Monoclonal antibodies (6) are uniquely applicable to such a study.

This communication describes the production and proper-

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[1] *Abbreviations used in this paper:* CEL, chick embryo lens; DME, Dulbecco's modified Eagle's medium; DPT, diazophenylthioether; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum;

GAM, goat-anti-mouse; HRP, horseradish peroxidase; mcAbs, monoclonal antibodies; PBS, phosphate-buffered saline.

ties of a monoclonal antibody specific for the most abundant protein of lens fiber cell plasma membranes in the calf (M_r 26,000) and in the chicken (28,000). This protein has been referred to as either main intrinsic protein, MIP (7), or membrane protein MP26 (8). Some controversy has arisen recently over whether or not this protein is junctional, based on electron microscope immunocytochemistry. One study has reported that labeling with a rabbit serum directed against MP26 is distributed over junctional as well as nonjunctional regions of isolated lens membranes (9). The same group reports nonjunctional and junctional staining in frozen lens sections as well (10). However, another laboratory has stated that MP26-specific labeling is found on nonjunctional regions and on only one subset of the junctions visualized (11). A previous report had indicated that two junctional classes exist in the lens, one somewhat thicker in cross-section than the other (12). The monoclonal antibody described here is shown to bind to junctions of both thicknesses. The implications of these results are discussed below.

We also report that the antibody recognizes the chicken lens membrane protein, MP28 (13), in cultures of chick embryo lens cells (14). The cultured lens cells provide a variety of experimental possibilities for studying the properties of this junctional protein. A preliminary report describing this mcAb has already appeared (15).

MATERIALS AND METHODS

Cell and Tissue Culture: BALB/c female mice were purchased from the mouse colony in the Department of Laboratory Medicine at the University of Minnesota. NS-1 myeloma cells (a kind gift of Dennis Smith in the lab of Dr. Leo Furcht) were maintained in Dulbecco's modified Eagle's medium (DME) plus 10% fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY, and KC Biologicals, Inc., Lenexa, KS). Calf lenses were purchased from Forster's Meat Market in New Hope, MN, chicken lenses were from Butterfield Foods Co., in Butterfield, MN, and fertilized white leghorn chicken eggs were from the Department of Animal Science at the University of Minnesota. Lens cultures were obtained from trypsin-dispersed lenses of 10-d chick embryos, as detailed in another publication (14).

Hybridoma Production: Bovine and chicken lens membranes, purified according to the method used by Goodenough (5) through the first sucrose gradient following extraction with 7 M urea, have been used for immunizations. For the production of the monoclonal antibody, B2, described in this report, 10–12 wk-old BALB/c female mice were given a single intraperitoneal injection of urea-washed chicken lens plasma membranes (100 μg, see Fig. 1, lane 3) in Freund's complete adjuvant. 3–4 wk later the mice were boosted intravenously with 100 μg of the same membrane preparation. Their spleens were removed 3–4 d later and used for hybridization. A standard hybridization procedure was used (16).

Screening Assay: Antibody production was screened by the enzyme-linked immunosorbent assay (ELISA) technique (17, 18). 1 μg of total membrane protein in 0.05 M Na_2CO_3 buffer, pH 9.6, was added to each well of a 96-well, polystyrene microtiter plate, and the solvent allowed to evaporate. Wells were washed 3 times with PBS-Tween (0.05% Tween, Sigma Chemical Co., St. Louis, MO). For labeling, 100 μl of PBS-Tween was added to each well, followed by 50 μl of the hybridoma medium to be examined. The plate was incubated at 37°C for 1 h and washed three times with PBS-Tween. Rabbit-anti-mouse IgG conjugated to horseradish peroxidase (Miles Laboratories, Elkhart, IN) was diluted 1:500 in PBS-Tween, added to each well (100 μl), and incubated for 1 h at 37°C. The plate was washed as before. The peroxidase activity was detected with an o-phenylenediamine substrate, and recorded as previously reported (18).

All hybridomas in this series were screened against both chicken and bovine lens membranes, and only those hybridomas that cross-reacted were cloned. Wells giving the strongest positive reactions were cloned twice by a serial dilution technique (19) in 50% conditioned medium. Clones were screened by ELISAs as described for uncloned hybridomas.

Western Transfer Analysis: The molecular specificities of the antibodies produced by the twice-cloned hybridomas were determined using transfer of antigens from SDS gels to derivatized diazophenylthioether (DPT) paper. Lens proteins, solubilized in SDS (20), were resolved on Laemmli 12% SDS

polyacrylamide gels (21). The DPT paper (22) and SDS gel were prepared for transfer according to manufacturer's instructions (Schleicher and Schuell, Inc., Keene, NH). Transfer, in a Bio-Rad Trans-Blot electrophoretic transfer cell, was affected for 3 h at 0.9 A (~35 V) for a 14 × 14 cm gel. At the end of the transfer period, the gel was stained with Coomassie Brilliant Blue R-250, to check on efficiency of transfer and the remaining active groups on the paper were inactivated by incubating in 10% ethanolamine (23).

Antibody labeling was done essentially as reported (22), using cloned hybridoma media diluted from 1:5–1:50, followed by 0.2–0.5 μCi ^{125}I-goat (or sheep) anti-mouse Ig (Amersham Corp., Arlington Heights, IL). Controls included normal mouse serum, unconditioned hybridoma medium, and unrelated mcAbs.

The majority of bound mcAb B2 could be successfully eluted from the paper, without altering antibody specificity, by treating it with 1% Triton X-100 in saline (pH 3) for 30 min with occasional sonication.

Prior to electrophoresis and transfer, some isolated membranes were treated with *Staphylococcus aureus* V8 protease (Worthington Biochemical Corp., Freehold, NY), chymotrypsin (type VII, Sigma Chemical Co., St. Louis, MO) or carboxypeptidase A and B (Worthington Biochemical Corp.) as detailed elsewhere (18).

Hydroxylapatite Chromatography: SDS hydroxylapatite chromatography was performed as described (24) with modifications. Bovine lens membranes washed only with 1 mM $NaHCO_3$, 5 mM EDTA, pH 8 were solubilized in 8.8% SDS, 8.8% β-mercaptoethanol, 3.5 mM EDTA at 37°C. The sample was dialyzed against starting buffer (100 mM sodium phosphate, 1.0 mM EDTA, 0.1% SDS, 0.1 mM dithioerythritol, pH 6.5) (25) overnight. The solubilized membranes were clarified by centrifugation in a Beckman microfuge (Beckman Instruments, Inc., Palo Alto, CA) and loaded onto a hydroxylapatite column (Bio-Rad HTP, 1.5 × 15 cm) equilibrated in starting buffer. Gradient elution was with 100–600 mM sodium phosphate in starting buffer. 2-ml fractions were collected and portions dialyzed against 20 mM Tris-HCl pH 7.0, 1.0 mM EDTA, 0.1% SDS, 0.1 mM dithioerythritol.

Immunofluorescence: Cultures of 10-d-old chicken embryo lens cells (14) were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 7–10 min. To permeabilize, the cells were incubated with 0.1% Triton X-100 in PBS for 7–10 min. The cells were incubated with monoclonal, rabbit anti-mouse, and fluorochrome-conjugated anti-rabbit antibodies diluted in PBS for 1 h each with PBS washes between incubations. Cultures were then washed with PBS, distilled water, mounted in Elvanol, and viewed using a Zeiss microscope equipped with phase-contrast and fluorescence optics. Alternatively, sections were stained in the same manner after the culture was fixed in formaldehyde, dehydrated in ethanol, embedded in Epon in situ, and thick sectioned (1–2 μm). The Epon resin was removed by treatment in sodium ethoxide (26). Specificity of binding was checked by substituting normal mouse serum, unconditioned hybridoma medium, or an irrelevant mcAb preparation for the primary antibody.

Ultrastructural Localization of Antibodies: The localization of antibodies at the ultrastructural level was done using a modified version of the ELISA technique (see *Screening Assay*). Lens membranes were plated as for the ELISA except that a Falcon 96-well, flat-bottomed, polyvinyl-chloride microtiter plate (Becton Dickinson Labware, Oxnard, CA) was used instead of a polystyrene plate. Washes and antibody dilutions were done with the Western transfer buffer (23), which was found to reduce background. The monoclonal antibody was used at dilutions of 1:5–1:50. A monoclonal antibody towards ovalbumin (a gift from Randy Strobel in Murray Rosenberg's lab), myeloma cell conditioned medium, and unconditioned hybridoma medium containing 10% FCS (DME·FCS) were used as controls. Goat-anti-mouse IgG antibodies conjugated to colloidal gold particles (GAM·gold) (Janssen Pharmaceutica, Beerse, Belgium) were used at dilutions of 1:5–1:40. Goat-anti-mouse IgA, IgG, and IgM antibodies conjugated to horseradish peroxidase (GAM·HRP) (Cappel Laboratories, Malvern, PA) were used at a dilution of 1:200 and detected with 0.05% diaminobenzidine in the presence of 0.01% H_2O_2 (27).

The membranes were fixed with 2.5% glutaraldehyde, stained with 1% tannic acid, postfixed with 1% osmium tetroxide, stained with 0.5% uranyl acetate, dehydrated in a graded ethanol series, and embedded in situ with Epon. After Epon polymerization, the polyvinyl well could be peeled away and the embedded membranes mounted for thin sectioning. The membranes were sectioned perpendicular to the bottom of the well. Sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi 600 electron microscope.

Quantitative Analysis of Ultrastructural Labeling: For quantitative analysis of the labeling of junctions in the peroxidase procedure, five categories were established. Those junctions that were labeled so that an even deposit of reaction product at least doubled the thickness of the junctions were classified as having "continuous, heavy label." Those junctions that had an even deposit of reaction product that was significant, but did not double the thickness of the junction, were classified as having "continuous, moderate

label." Infrequently, junctions were seen where part of the length of the junction would be heavily or moderately labeled, but areas of that same junction would be unlabeled. Such junctions were classified as having "discontinuous label." Junctions exhibiting small, light clumps of apparent label along their lengths were classified as having "limited, patchy label." When virtually no label above background could be detected, as in the controls noted above, the junctions were classified as having "no significant label."

RESULTS

Monoclonal antibody production specific for chicken and bovine lens membranes was screened with ELISA methods described above. ELISAs were also used to check retention of antibody production by hybridomas during cloning. As reported by others involved in mcAb production (28), some hybrids lose the ability to produce antibodies during the cloning steps. Of 20 original wells that were cloned, five retained antibody production, and one of these antibodies prepared against chicken lens membranes, which we have designated "B2," continued to cross-react in ELISAs with both chicken and bovine lens membranes.

Electrophoretic Transfer Analysis

To determine the molecular specificity of our mcAbs, the electrophoretic transfer procedure, or Western, was modified for working with MP26 and MP28, which are hydrophobic, aggregation-prone, integral membrane proteins. When freshly solubilized embryonic chicken lenses were electrophoresed on SDS gels, transferred to DPT paper, and probed with mcAb B2, binding was limited to a single band with an M_r of 28,000 (Fig. 1 a). Despite high concentrations of other protein bands, no other labeling was detected (Fig. 1 a), even following extended exposures. The same result was seen when membranes isolated from embryonic chick lenses were analyzed by Westerns (data not shown). Control preparations, e.g., unconditioned hybridoma medium (see Materials and Methods), were routinely run and were negative, being comparable to lanes 9–10 in Fig. 3.

Electrophoretic transfer experiments were also carried out on urea-treated fiber cell membrane fractions from adult chicken and calf lenses. The cross-reactivity of mcAb B2 with bovine lens MP26 (Fig. 1 b), seen originally in ELISAs, was confirmed. The main intrinsic protein from the chicken lens migrates somewhat more slowly than that of the bovine lens, MP26, in our gel system. Consequently, we have termed the chicken form MP28. We also found that B2 labeled two minor bands with M_r of approximately 24,000 and 16,000 from both chicken and calf lenses. Binding to the major and two minor protein bands was obtained consistently. Since ~0.5 μg of protein is sufficient to saturate the DPT paper in the area of a single band (unpublished observations), and ~5 μg of MP28 is present to be transferred (assuming 50% of the 10 μg total lens membrane protein loaded is MP26), the ratio of major to minor bands was much greater than the Westerns appeared to indicate.

Labeling of the 24,000- and 16,000-M_r bands had not been reported before, likely due to the low concentrations of these proteins in the calf lens membrane preparations. We thought it was likely that the minor bands represented degradation products of MP28 and MP26. To confirm the antigenic relatedness of the 24,000- and 16,000-M_r proteins to MP26, mcAb B2 was eluted (see Materials and Methods) from the MP26 band on a DPT paper strip containing calf lens membrane protein. The eluted B2 was then allowed to bind to a second DPT paper strip to which lens membrane proteins
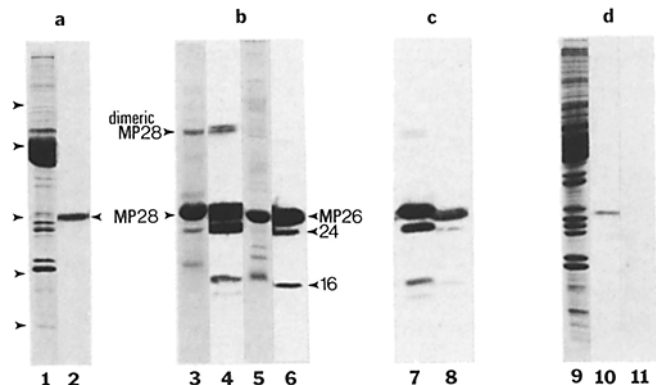

FIGURE 1 Western analysis of B2 binding to MP28 from whole lens, isolated membrane, and lens cell culture. To determine the molecular specificity of the mcAb, the following samples were solubilized in SDS, electrophoresed on 12% SDS gels, transferred to DPT paper, and probed with mcAb B2 or stained with Coomassie Brilliant Blue R-250, as indicated. (a) Whole 10-d embryonic chick lens, stained with Coomassie (lane 1) or labeled with mcAb B2 (lane 2). Out of the numerous protein bands present, B2 specifically binds a single band of M_r 28,000. (b) ~10 μg urea-washed adult chicken (lanes 3 and 4) or bovine (lanes 5 and 6) lens fiber cell membranes stained with Coomassie (lanes 3 and 5) or labeled with mcAb B2 (lanes 4 and 6). The pattern of binding of B2 to the bovine lens MP26, its dimer and degradation products is remarkably similar to the pattern of binding to the chicken lens proteins used as the source of antigen for immunization. (c) Urea-washed adult chicken (lane 7) or bovine (lane 8) lens membranes labeled with mcAb B2 that had been eluted from the MP26 band of another DPT paper strip after transfer from a preparative SDS gel. The antigenic relatedness of the minor bands with MP26 and MP28 is confirmed since B2 eluted specifically from the MP26 band binds in a pattern identical to that of unfractionated B2 (compared with lanes 4 and 6). (d) Chick embryo lens (CEL) cultures before the development of lentoids (lane 11) or following differentiation into lentoids (lanes 9 and 10) were reacted with Coomassie (lane 9) or B2 (lanes 10 and 11). The profile of proteins present in the cultures containing lentoids is remarkably similar to that present in whole embryonic lens (compare with lane 1). Although B2 binds to a single band co-migrating with the embryonic and adult chicken lens MP28, no detectable binding is seen in CEL cultures before the development of lentoids. Molecular weight markers indicated along the left edge are BSA, (68,000 M_r), ovalbumin (43,000 M_r), α-chymotrypsinogen (25,700 M_r), β-lactoglobulin (18,400 M_r), and cytochrome c (12,300 M_r). 24, 16, mol wt × 10^{-3}.

from chicken and calf had been transferred. As shown in Fig. 1, lanes 7 and 8, the eluted B2 bound to all three proteins (16,000, 24,000, and MP26 [or MP28]), as well as to apparent dimers (29). These results suggest that MP28, MP26, the lower molecular weight proteins, and the higher molecular weight dimers are related antigenically. The lower molecular weight bands are most likely degradation products of MP28 and MP26.

Although immunoreactive degradation products may be present in isolated bovine and chicken lens membranes, we have found that the B2 antigenic site on MP26 and MP28 was removed by treating isolated membranes with various exogenous proteases. Fig. 2 shows that mcAb B2 failed to react with the 24,000-M_r product of bovine MP26 resulting from V8 protease digestion, the protein products generated by extensive treatment with carboxypeptidases A and B, and the 22,000-M_r chymotrypsin product (18). Only a small amount of undigested material at 26,000-M_r was bound by this monoclonal antibody. Similar results were obtained with

MP28 and chicken lens membranes (data not shown). A previous study has indicated that these proteases cleave overlapping regions, as they remove up to 5 kd of the protein, including the carboxy-terminus (18), from intact membranes. Taken together, these data suggest that the antigenic determinant for mcAb B2 resides within a terminal 2-kd region, presumably found at the C-terminus of MP26. If this is the case, it would indicate that the apparent degradation products of M_r 24,000 and 16,000 noted above would involve cleavage near the N-terminus, since they retain the B2 antigenic site.

Since calf MP26 aggregates on boiling in SDS (30), we used this feature to examine the idea that B2 was binding to the major lens MP26, and not to another 26,000-M_r protein. When calf lens membranes were solubilized at 100°C, B2 labeling of material with an M_r of 26,000 was substantially reduced (Fig. 3 a). In addition, B2 labeled apparent dimers of the junctional protein (Fig. 3, lane 11) which have been reported previously (29, 30). Other studies were also performed to support the idea that monoclonal B2 is specific for MP26 and MP28. Supernatants containing soluble calf lens proteins were analyzed and found to be negative in Westerns utilizing mcAb B2 (Fig. 3, lane 9). Furthermore, when calf membranes were fractionated on hydroxylapatite columns the fractions containing β-Bp crystallins were negative and those containing MP26 were positive (Fig. 3, respectively, lanes 10 and 11). This is important as these crystallins and MP26 migrate at almost indistinguishable rates on our SDS gels. Finally, a cyanogen bromide fragment of MP26 (generously provided by Larry Takemoto, Kansas State University, Manhattan, KS) was studied with Western transfers and found to react strongly with mcAb B2 (Fig. 3, lane 12).

Localization of Monoclonal B2 at the Ultrastructural Level

To localize MP26 within calf membranes, labeling studies were initiated at the electron microscope level. With the

FIGURE 3 Western analysis of other lens fractions. To substantiate that mcAb B2 was binding to the major MP26, and not to an M_r 26,000 contaminant, various lens fractions were prepared and stained with Coomassie (lanes 1, 2, 5-8) or probed in Westerns with B2 (lanes 3, 4, 9-12). (a) 2 µg bovine lens fiber cell membranes were solubilized in SDS, and optionally boiled for 4 min (lanes 2 and 4), then electrophoresed, transferred, and stained with Coomassie (lanes 1 and 2) or B2 (lanes 3 and 4). MP26 has been shown to aggregate upon heating in the presence of SDS (30); B2's antigen also aggregates under these conditions, as can be seen by the decrease in binding at M_r 26,000. (b and c) Calf lens homogenate 10^6 g-min supernatant (lanes 5 and 9), bovine lens β-crystallins purified by hydroxylapatite chromatography in the presence of SDS (lanes 6 and 10), MP26 eluted from hydroxylapatite (lanes 7 and 11), or the M_r 15,000 cyanogen bromide fragment of MP26 (CB-1, see reference 36, lanes 8 and 12) were processed for Western analysis with B2 or stained with Coomassie after SDS-PAGE. Notice that no soluble proteins, including the enriched βbp-crystallin having an M_r of ~26,000, are bound by mcAb B2.
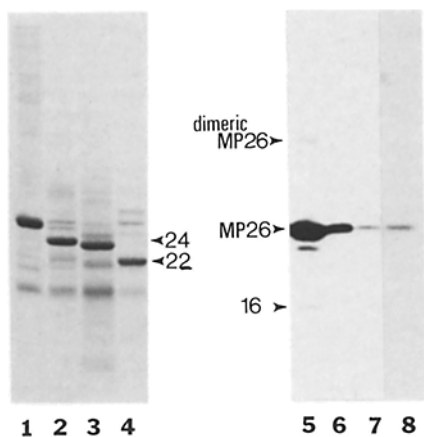
FIGURE 2 Susceptibility of the B2 binding site to exogenous proteases. To determine which domain on the presumed transmembrane MP26 protein is bound by mcAb B2, ~10 µg urea-treated bovine lens fiber cell membranes were treated with the following proteases, processed in Westerns, and reacted with Coomassie (lanes 1-4) or mcAb B2 (lanes 5-8): no protease (lanes 1 and 5); Staphylococcus aureus V8 protease (lanes 2 and 6); carboxypeptidases A and B (lanes 3 and 7); chymotrypsin (lanes 4 and 8). All of these proteases appear to act on overlapping regions of MP26 (18). Note that small amounts of residual MP26, but none of its experimentally generated proteolytic products, are bound by mcAb B2. 24, 22, 16, mol wt × 10^{-3}.
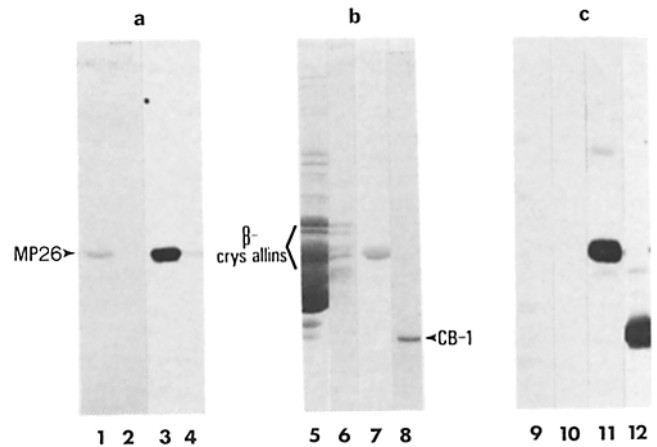
extensive data on labeling conditions derived from the ELISA experiments, we sought to utilize the ELISA approach in this effort. The embedment of labeled membranes from ELISA plates, as described in Materials and Methods, avoids multiple centrifugation steps that increase membrane aggregation and background staining. Also, the color changes that occur with diaminobenzidine labeling allow direct examination and facilitate the identification of optimal conditions, without detailed electron microscopy efforts.

Three separate experiments utilizing a secondary antibody labeled with peroxidase were analyzed. Each indicated a significant labeling of the cytoplasmic surface of lens junctional membranes with B2 (Fig. 4a). Comparisons of varied samples demonstrated that the labeling of junctions was uniform throughout the preparations, e.g., at different levels of multilayered membranes. Control experiments with unconditioned hybridoma medium containing 10% FCS (DME·FCS), myeloma conditioned medium, and a monoclonal antibody to chicken ovalbumin were negative (Fig. 4b). To provide objective support for this interpretation, a detailed quantitative analysis of different sample regions (four each, control and experimental) in one of these experiments was carried out. Using the criteria listed under methods, over 400 junctions in the B2 and the control samples (DME·FCS) were scored (Table I). With the five labeling categories used, 85% of the B2 labeling on junctions was in the highest category of labeling, while 79% of the labeling of control junctions was in the lowest category. This suggested that peroxidase reaction product was specifically associated with the lens junctions.
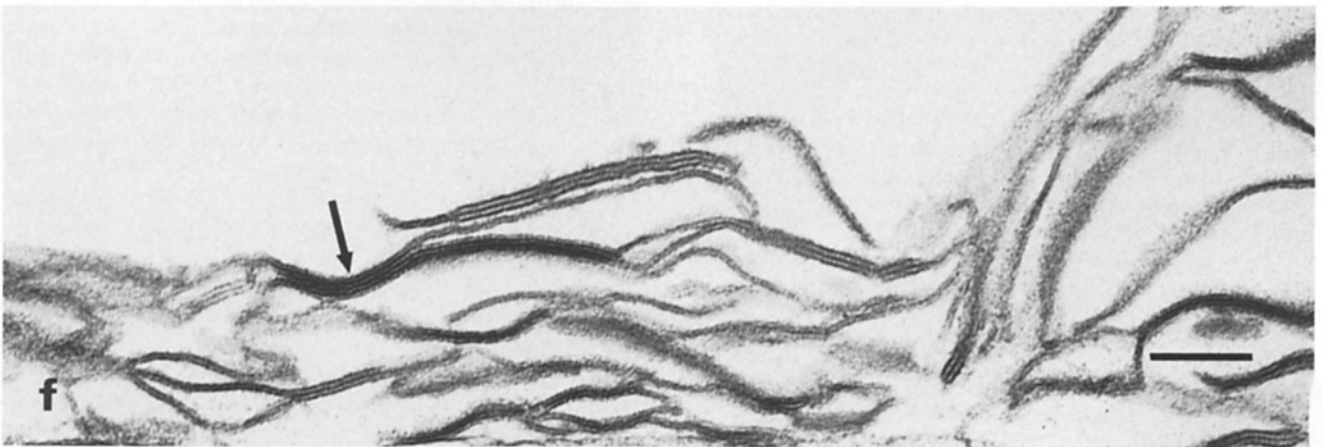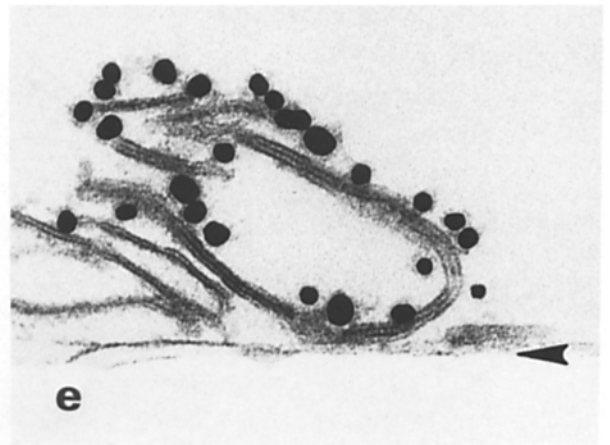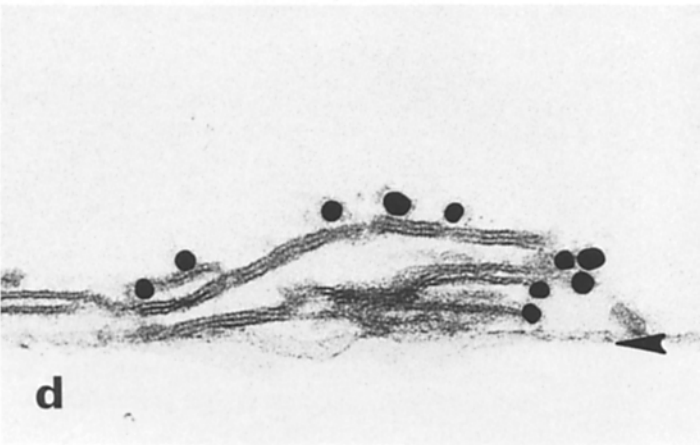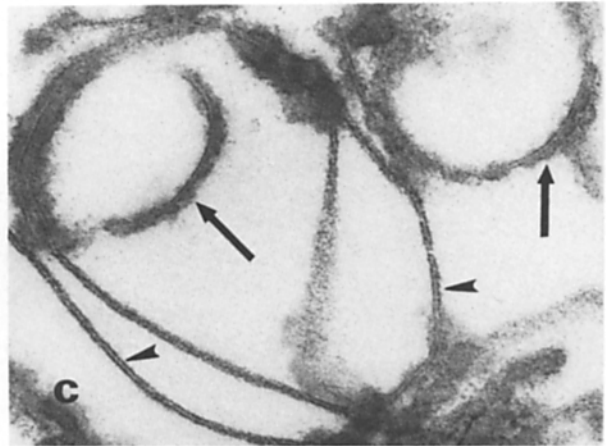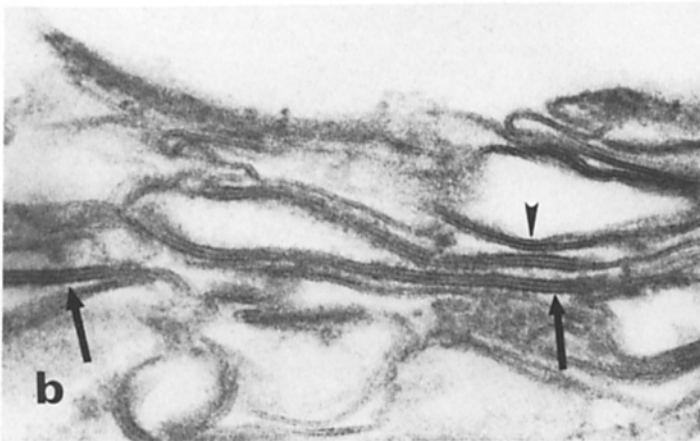
To guard against the possibility that reaction products were migrating and displaying a marked affinity for junctional membranes, colloidal gold-conjugated secondary antibodies were used in other indirect labeling experiments. These results were consistent with the peroxidase investigations. Both junctional and nonjunctional membranes were specifically labeled (Fig. 4, d and e). The especially low background labeling with the colloidal gold strengthened this interpretation (Fig. 4f). These observations provided further support for the presence of MP26 in the bovine lens junctions.

It was important to address the thickness of the junctions in these preparations, since an earlier report had indicated that antibodies specific for MP26 labeled only a thinner junctional population, measuring 12–14 nm in cross-sectional thickness (11). Therefore, based on thickness (~12 vs. 16 nm) and the presence of a denser central layer in the thinner junctions, as noted earlier (11, 12), we classified 140 junctions in our control preparations. Approximately 80% of the junctions were found to be of the thicker form. The same membrane isolates were used in the B2 labeling studies. Unfortunately, in our more extensive studies employing B2 and the peroxidase marker, it is not possible to classify the labeled junctions as thick or thin, due to the presence of the reaction product. However, since essentially all junctions were labeled in the peroxidase experiments, our data indicate that MP26 resides in both junctional forms. That is, of the 175 junctions labeled with mcAb B2, we would estimate that 140 were of the thicker junctional variety, not labeled in the earlier study (11). The data in Table I strongly support the labeling of the thicker junctions. Moreover, we would estimate that the thinner junctions, labeled in the previous study (11), amounted to ~35 of the junctions reacted with B2. The labeling of these junctions also appears likely based on our quantitative analysis, since only 4% of the junctions were not labeled. The more limited investigations carried out with the colloidal gold markers support this interpretation.

The labeling of nonjunctional membranes was also analyzed. Although many such membranes in the peroxidase experiments were clearly negative (Fig. 4, a and c), in some instances moderate labeling was observed (Fig. 4c). The reason for these differences is not presently understood. The colloidal gold studies provided even more support for the presence of antigen in nonjunctional membranes. In this case,

the discrete nature of the label would readily permit quantitative analysis of antigen distribution, especially given the very low background levels encountered (Fig. 4f). However, the size of the gold, particularly that used here, would interfere in a steric manner with a thorough labeling of antigen in a protein-rich junctional membrane. Thus, a quantitative evaluation of junctional vs. nonjunctional MP26 is not appropriate, based on the present labeling data.

Studies on Cultured Chick Embryo Lens Cells

Further support for the intracellular localization of the B2 binding site comes from immunofluorescence experiments on cultured chick embryo lens (CEL) cells. These experiments take advantage of highly differentiated regions in the cultures, termed lentoids (31). When cultures containing lentoids were fixed and permeabilized, mcAb B2 binding was visualized by intense fluorescence of the lentoids, as shown in Fig. 5a. However, if normal mouse serum, unconditioned hybridoma

FIGURE 4 Ultrastructural localization of B2 binding sites. Bovine lens membranes adsorbed to polyvinyl-chloride microtiter wells were exposed to mcAb B2 or to DME·FCS (for control purposes) at dilutions of 1:12. Next they were exposed to either GAM·HRP at a dilution of 1:200, or to GAM·gold at a dilution of 1:5. A substrate solution containing diaminobenzidine and H_2O_2 was added to those membranes that had been treated with GAM·HRP. The membranes were then processed for electron microscopy as described in Materials and Methods. All magnifications are × 138,000. (a) Lens membranes labeled with B2 and GAM·HRP. The insoluble reaction product approximately doubles the normal width of the lens junctions by depositing on both cytoplasmic sides of the junctions (arrows). Unlabeled nonjunctional membranes may also be seen in this section (arrowheads). Note that the labeling penetrates through the multilayered plating of membranes. (b) Lens membranes exposed to DME·FCS and GAM·HRP, showing low background labeling levels. Several of the more numerous 16-nm thick junctions may be seen (arrows), as well as nonjunctional membranes and one thinner, 12-nm junction (arrowhead). (c) Similar to a, showing labeled junctions, unlabeled nonjunctional membranes (arrowheads), and nonjunctional membranes labeled on one side of the membrane (arrows). (d and e) Membranes labeled with B2 and GAM·gold. Lens junctions are clearly labeled with colloidal gold particles. The arrowheads mark the edge of the thin-section, which was formerly adjacent to the bottom of the ELISA well. The orientation is the same for all micrographs in this figure. (f) Membranes exposed to DME·FCS and GAM·gold. This illustrates the low backgrounds associated with the technique. Typically, only 1–3 gold particles were observed along a 200 μm long thin-section. Since plated ELISA membranes were cut in cross-section, this represents membrane profiles distributed over 200 μm of the ELISA well. For comparative purposes, the length of the colloidal gold control sample in f corresponds to <2 μm. In addition, no clumping of gold particles was observed in the control samples. The arrow points to several closely apposed membranes which may result from the plating of the membranes onto the ELISA plate. Bar, 0.1 μm.
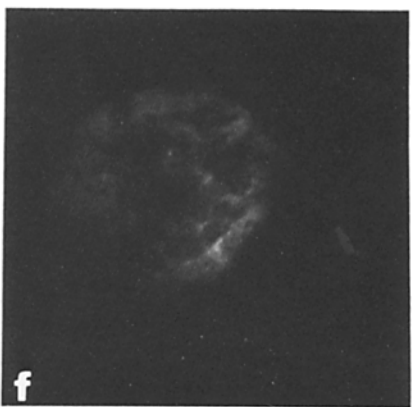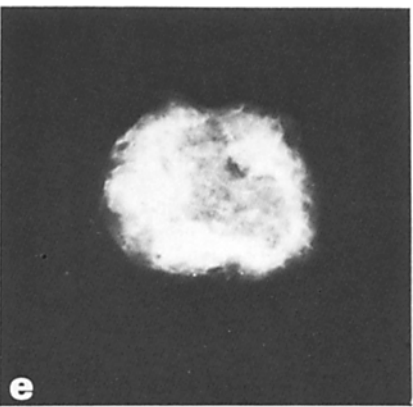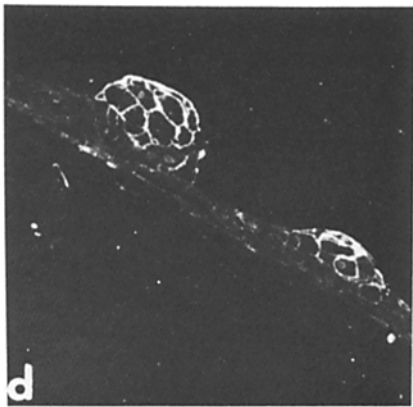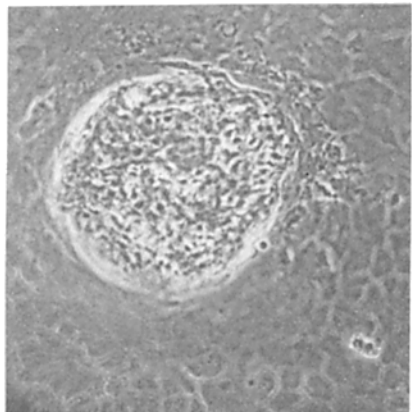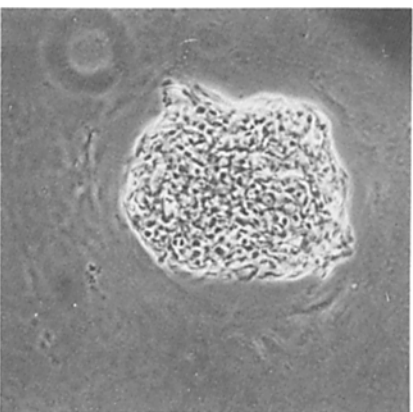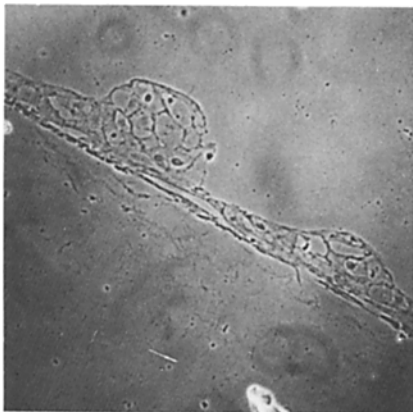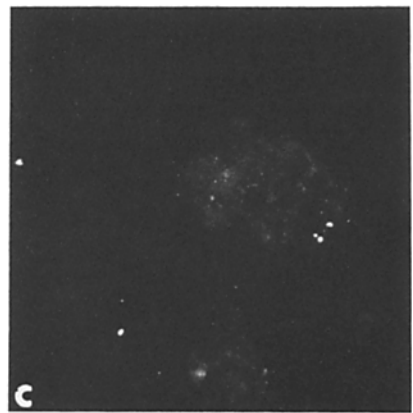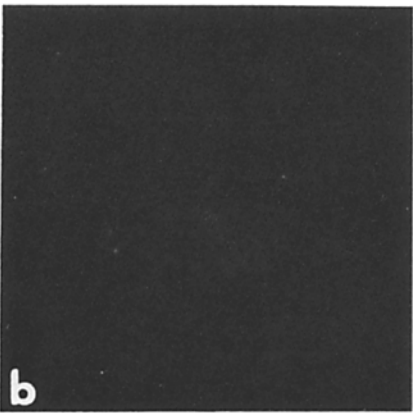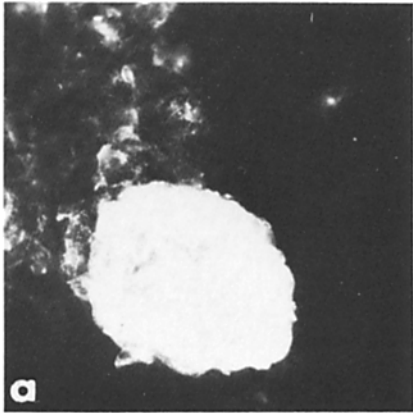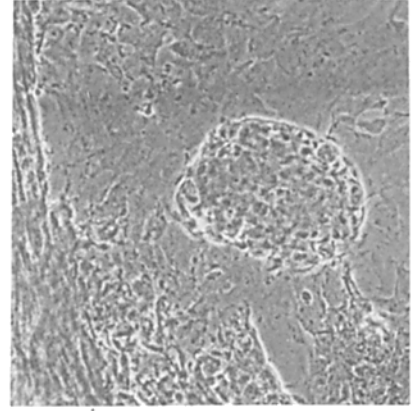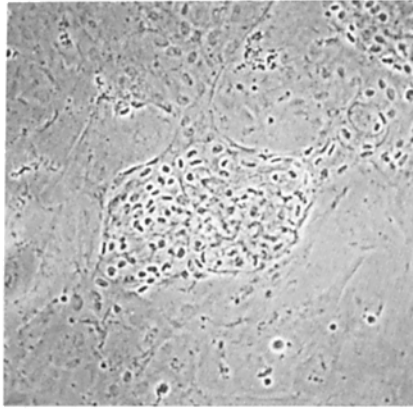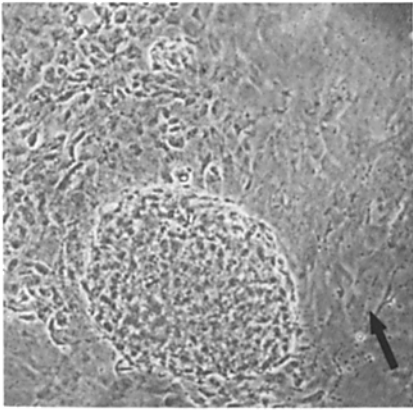
medium, or an irrelevant mcAb preparation was substituted for B2 medium, only background staining was detected (Fig. 5b). When the cells were fixed but not permeabilized (compare Fig. 5, b and c), no fluorescence above background levels (Fig. 5b) was visible. Comparison of the phase contrast and fluorescent images (Fig. 5) shows that the epithelioid cells (arrow) surrounding the lentoids do not possess significant numbers of B2 binding sites. We stress that the fluorescence appears to be concentrated at the cell membrane. Thick sections of CEL cultures cut perpendicular to the plane of the cell layer, after embedding in Epon, also showed the B2 binding to be localized at the cell membrane (Fig. 5d). Evaluation of thick sections and face-on views of whole cultures indicated that binding specific for MP28 was present on the free surface of cells, as well as in areas of cell-cell apposition (Fig. 5).

B2 binding to lentoids was not altered by preincubating the mcAb with a membrane preparation from sheep red blood cells (Fig. 5e). However, B2 binding was reduced to near background levels by preincubating the mcAb with an excess of chicken lens junctions (Fig. 5f), purified according to Takemoto et al. (32). The procedure for preparing these junctions, involving Sarkosyl extraction, is reported to yield fractions where 95% of the membrane profiles display junctional associations.

When differentiated CEL cultures were analyzed with Westerns (see Materials and Methods), mcAb B2 was found to label a single band, which co-migrated with MP28 (Fig. 1d). With Coomassie staining, these cultures displayed 50–100 bands. Early CEL cultures that had not yet exhibited signs of differentiation into lentoids demonstrated no significant labeling with mcAb B2 in Western analysis (Fig. 1, lane 11). Thus, this method, along with immunofluorescence, gives us a valuable approach for evaluating changes in MP28 as lens fiber cells differentiate.

DISCUSSION

While other labs have reported the use of MP26-specific rabbit antisera (9, 11, 33) for immunolabeling of this major lens membrane protein, the present publication is the first to characterize the production of a monoclonal antibody toward this protein in the bovine lens, and its counterpart in the chicken. McAbs provide several significant advantages. McAbs provide assurance of monospecificity, for example, in

the case of distinct polypeptides having the same molecular weight. Another advantage of mcAbs is the ability to obtain an antibody specific for a single antigenic determinant on a macromolecule that contains several unique determinants. A less recognized feature is the ability to select a particular antibody specificity out of a large number of antibodies with less interesting binding characteristics. For example, we have selected for mcAbs that recognize a conserved determinant, found in both the chicken and bovine proteins. Finally, although monoclonal antibodies may not bind after denaturation of the antigen, the use of a mcAb avoids a situation as might occur with rabbit serum, in which several antibodies are raised against native antigens, yet only one binds after denaturation. This apparent "monospecificity" would be misleading. This concern is relevant here, as it is necessary to use SDS to separate MP26 from other lens membrane proteins and thoroughly characterize its antigenicity.

Specificity of Monoclonal Antibody B2

McAb B2, though produced against chicken lens MP28, was found to cross-react with the analogous protein from bovine lens. Cross-reactivity between the bovine lens MP26 and the chicken lens MP28 was not an unexpected finding. We had observed cross-reactivity in ELISAs using three different rabbit antisera (34). Another study has also demonstrated the immunological relatedness of the major junctional proteins from these two species (9). In addition, the two proteins appear similar in protease sensitivity and two-dimensional peptide mapping (35). B2 has also bound to proteins with M_r of ~26,000 from rat, mouse, and frog lenses (unpublished results).

Several lines of evidence have assured us that mcAb B2 is binding to the major bovine lens MP26 protein and not to a 26,000 M_r contaminant. (a) MP26 has been found to form large aggregates upon heating in the presence of SDS (30); mcAb B2's antigen is also heat aggregated (Fig. 3a). (b) MP26 has been found in multimeric forms, dependent on its concentration in SDS solutions (29); mcAb B2 binds a protein band with the molecular weight of a dimer of MP26 (Fig. 1b). (c) McAb B2 binds to an M_r 15,000 cyanogen bromide fragment of MP26 (provided by L. Takemoto, Fig. 3), which appears to include the original C-terminal portion of MP26 (36). (d) B2 binds no proteins present in supernatant fractions of lens homogenate. In addition, B2 binding at an M_r of

FIGURE 5 Immunofluorescence studies on cultured chick embryo lens cells. Cultures containing numerous well-developed lentoids were prepared for immunofluorescence microscopy as in Materials and Methods. Each pair of photographs includes a phase contrast micrograph of a lentoid and surrounding epithelioid cells, as well as the corresponding B2 fluorescence image. (a) The cells were fixed and then permeabilized with 0.1% Triton X-100 to allow the antibodies access to the cell interior; the phase contrast micrograph shows many epithelioid cells (arrow), which do not label with mcAb B2. × 160. (b) Background levels of fluorescence visible on fixed and permeabilized cells incubated with the same dilution of unconditioned hybridoma medium containing 10% FCS as an antibody control × 160. (c) The cells were fixed, but not permeabilized before addition of antibodies; the same absence of specific binding is seen when the cells are not fixed, and the antibody reactions are carried out at 4°C (results not shown). × 160. (d) Cultures were sectioned (see Materials and Methods) perpendicular to the surface of the culture dish. After removing the plastic from the sections (27), antibody labeling steps were carried out. Uniform fluorescence is visible on several layers of plasma membrane in the lentoids, including the outermost, free surface. Similar results are seen if the cells are labeled with antibody and postfixed before embedding and sectioning ×400. Notice that nonlentoid, epithelioid cells are not significantly labeled by mcAb B2. (e) CEL cultures were fixed and permeabilized as in a; after B2 antibodies were preincubated with sheep erythrocyte membranes, the membranes and any bound antibodies were pelleted in a microfuge and the supernatant used as the source of primary antibodies in this immunofluorescence experiment. B2 does not bind to this antigen control × 160. (f) Similar to e, except 20 μg of Sarkosyl-extracted chicken lens membranes were preincubated with 8 μl of B2 culture medium. The Sarkosyl-enriched lens junctions compete successfully with the lentoids for B2 binding × 160.

26,000 on Westerns is not increased when the antibody is used to examine very crude membrane fractions, which contain much higher levels of crystallins, for example, than the urea-washed membranes (not shown). (e) After separation of lens proteins on hydroxylapatite, B2 binds MP26, but not components of crystallin fractions (Fig. 3).

Localization of MP26

The localization of the B2 antibody in the present ultra-structural study strongly supports the idea that MP26 is a component of the bovine lens junction. Not only did this work rely on the specificity of a monoclonal antibody, but it applied two antibody markers to detect the B2 binding. The different markers were found to be complementary, with HRP-labeled antibodies penetrating membrane layers best and colloidal gold being most discrete. In addition, the study employed tannic acid in clearly visualizing the features of the antigen-bearing structures. This latter point is critical in view of recent reports on two junctional classes in isolated lens membranes (11, 12).

Since both the 12–14- and 16–18-nm junctions likely bind monoclonal B2, the precise relationship between these structures is of interest. It is noteworthy that in the original study (12), which indicated that most junctions were of the thinner variety, some lenses were obtained from a tissue supply house. In the present study, all lenses were homogenized and diluted with buffer within 1 h of the death of the animal. It is possible that the thinner junctions are derived by alteration or degradation of the thicker class. In the other study, which drew attention to two junctional thicknesses (11), only the 12–14-nm type was thought to contain MP26. If the 12–14-nm junctions are derived from the 16–18-nm form, different conformational states could lead to the generation of antibodies with specificity for only one conformation. A precedent for this feature of membrane proteins involves the α-subunit of the acetylcholine receptor (37), where antigenic features are apparently altered with a "conformational maturation." Since in the earlier study antibodies to MP26 were affinity-purified (11), it is possible that these investigators selected for an antibody specific for a particular conformation.

Several experiments have been carried out in this laboratory to examine a possible conversion of the thicker junctional forms into the thinner forms. To date, such a conversion has not been clearly demonstrated. With the large number of variables involved (e.g., nature of the tissue, isolation procedures, and electron microscope methods), a substantial effort may be required to resolve this matter.

The fact that monoclonal B2 also labels nonjunctional membranes (as in references 9–11), suggests that unaggregated junctional precursors may exist. If MP26 forms cell-to-cell channels, a series of preliminary experiments is consistent with the idea of junctional precursors. The experiments show that dye transfer occurs between two cultured lentoids manipulated into contact (T.-F. Liu, unpublished observation). An alternate interpretation of nonjunctional MP26 in isolated membranes implicates the dispersal of junctional components during the purification of membranes. Significant changes in the structure of lens junctions during the course of isolation and purification would not be unexpected. Until recently, a procedure for separating membranes in isolated lens junctions had not been reported. However, it has now been shown that a low pH treatment is sufficient to split lens junctions (38).

Thus, caution must be applied in evaluating the relative stability of isolated lens junctions.

Applications of Monoclonal B2

The C-terminal portion of MP26 is thought to extend from the cytoplasmic side of the lens membrane (18, 36, 39). This domain, which is ~5 kd long, is removed from intact membranes by a variety of proteases. However, there is very little additional information regarding the arrangement of MP26 within the membrane. This report of B2 binding to peptides of M_r 24,000 and 16,000 suggests that these components, likely degradation products of MP26, have retained their intracellular carboxy-terminal domain while losing up to ~10 kd from their amino terminus. Preliminary observations indicate that the generation of the 24,000- and 16,000-M_r peptides is a phenomenon related to the age of the lens. Extracellular proteases might hydrolyze nonjunctional MP26 molecules or MP26 at the edges of junctional appositions, over extended periods of time, as steric considerations may prohibit access to the space between junctional membranes. Native degradation products of MP26 with an M_r of 22,000 have already been reported in older human lenses (29, 42). However, mcAb B2 fails to bind to any native 22,000 M_r bovine lens protein or to 22,000-M_r products of MP26 generated experimentally. Presumably, the highly immunogenic intracellular C-terminus is removed by endogenous or exogenous proteases yielding a 22-kd protein. Additional studies will be necessary to determine the source and relationships of these various MP26 breakdown products.

A recent publication describes a similar result with antibodies to the proteins of mouse liver gap junctions (43). The major junctional protein of M_r 26,000 is degraded to immunoreactive products of 24,000 and 16–18,000 (as well as a 10,000-M_r peptide) upon storage of junctional plaques for 2–4 wk at 4°C. However, as in our studies of the lens, a 21,000-M_r species was negative on reaction with the anti-26,000 serum. Therefore, although existing peptide mapping and amino acid sequence data show no homology between the liver and lens 26,000-M_r proteins (40, 41), other features may be similar in these two proteins. In addition, a recent report indicates that antibodies eluted from the SDS-denatured 26,000 M_r liver protein cross-react slightly with MP26 from the lens (44).

With the indication that MP26 is a lens junctional protein, mcAb B2 becomes a valuable probe for exploring whether these lens junctions contain cell-to-cell channels, as expected of gap junctions. For example, intercellular permeability can be studied after injection of B2 into cultured lens cells. Such experiments are important in view of the sequence of MP26, which strongly suggests that this molecule can form a trans-membrane, hydrophilic channel in the fiber cell membrane (45).

Developmental Aspects

The piles of differentiated cells, termed lentoids (31), which develop in our chick embryo lens cultures, display a number of characteristics in common with the fiber cells of the intact lens. They contain few cytoplasmic organelles, undergo limited DNA synthesis, are rich in δ-crystallin, and in both thin-section and freeze-fracture electron microscope analyses have been shown to contain extensive lens junctions (14). We have also found rapid movement of the fluorescent tracer dye,

Lucifer yellow, between cells within the lentoid (46), like that reported between fiber cells in the lens (47). Additionally, MP28 has been located by mcAb B2 in the lentoids, but not in the monolayer epithelioid cells. The epithelioid cells may be analogous to the undifferentiated epithelial cells of the anterior lens, which have been shown to contain gap junctions (48), but which have not been reported to be labeled by antibodies prepared against bovine MP26 (11, 33). The ability of B2 to recognize its determinant in the native conformation in CEL cultures will permit an analysis of the distribution and dynamics of MP28 during the course of lentoid differentiation.

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