J. Membrane Biol. 74, 217–228 (1983)

The Journal of
Membrane Biology

Arrangement of MP26 in Lens Junctional Membranes: Analysis with Proteases and Antibodies

Peggy Keeling, Keith Johnson, Daryl Sas, Kathleen Klukas, Peter Donahue, and Ross Johnson
Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108

Summary. The major membrane protein of the bovine lens fiber cell is a 26-kilodalton (kD) protein (MP26), which appears to be a component of the extensive junctional specializations found in these cells. To examine the arrangement of MP26 within the junctional membranes, various proteases were incubated with fiber cell membranes that had been isolated with or without urea and/or detergents. These membranes were analyzed with electron microscopy and SDS-PAGE to determine whether the junctional specializations or the proteins were altered by proteolysis. Microscopy revealed no obvious structural changes. Electrophoresis showed that chymotrypsin, papain, and trypsin degraded MP26 to 21–22 kD species. A variety of protease treatments, including overnight digestions, failed to generate additional proteolysis. Regions on MP26 which were sensitive to these three proteases overlapped. Smaller peptides were cleaved from MP26 with V8 protease and carboxypeptidases A and B. Protein domains cleaved by these proteases also overlapped with regions sensitive to chymotrypsin, papain, and trypsin. Specific inhibition of the carboxypeptidases suggested that cleavage obtained with these preparations was not likely due to contaminating endoproteases. Since antibodies are not thought to readily penetrate the 2–3 nm extracellular gap in the fiber cell junctions, antibodies to MP26 were used to analyze the location of the protease-sensitive domains. Antisera were applied to control (26 kD) and proteolyzed (22 kD) membranes, with binding being evaluated by means of ELISA reactions on intact membranes. Antibody labeling was also done following SDS-PAGE and transfer to derivatized paper. Both assays showed a significant decrease in binding following proteolysis, with the 22 kD product showing no reaction with the anti-MP26 sera. These investigations suggest that MP26 is arranged with approximately four-fifths of the primary sequence "protected" by the lipid bilayer and the narrow extracellular gap. One-fifth of the molecule, including the C-terminus, appears to be exposed on the cytoplasmic side of the membrane.

Key Words membrane topography · ELISA · electrophoretic transfer · calf lens · gap junctions

Introduction

The lens of the vertebrate eye is a most unusual, intriguing tissue (Bloemendal, 1981). Of particular interest to us is the prominent protein species located in the plasma membranes of the terminally differentiated lens fiber cells. This major membrane protein, of approximately 26,000 daltons in the bovine and human species, often termed MP26 (Bloemendal et al., 1977; Friedlander, 1980), comprises almost half of the plasma membrane protein in these mammalian lenses (Broekhuyse & Kuhlmann, 1974; Benedetti et al., 1976).

MP26 has received additional attention (Goodenough, 1979; Friedlander, 1980; Maisel et al., 1981; Hertzberg, Anderson, Friedlander & Gilula, 1982), following the suggestion that it is a major component of the intercellular junctions linking lens fiber cells (Benedetti et al., 1976). These extensive specializations bear all the key structural features of gap junctions. However, the situation has been made more complex by a report, based on structural analysis of calf lens membrane fractions, that two junctional classes exist in the lens (Zampighi et al., 1982). To better understand the lens membrane differentiations, investigators are pursuing a wide variety of questions. For example, studies have dealt with the organization, development, and purification of the junctional structures (Benedetti, Dunia & Bloemendal, 1974; Benedetti et al., 1976; Kuszak, Maisel & Harding, 1978; Goodenough, 1979). In addition, the cell-to-cell permeabilities in the lens have been explored (Rae, 1979; Goodenough, Dick & Lyons, 1980; Peracchia & Peracchia, 1980). Furthermore, since substantial quantities of lens membranes can be readily isolated, it has been possible to begin characterizing lens membrane proteins (Broekhuyse & Kuhlmann, 1978; Horwitz & Wong, 1980; Nicholson, Hunkapiller, Hood & Revel, 1980; Takemoto, Hansen & Horwitz, 1981; Hertzberg et al., 1982), and to obtain antisera for analyzing and localizing MP26 (Waggoner & Maisel, 1978; Broekhuyse, Kuhlmann & Winkens, 1979; Hertzberg et al., 1982; Bok, Dockstader & Horwitz,

1982). It has also been reported that MP26 can be synthesized in an *in vitro* protein synthesis system (Ramaekers et al., 1980). A clearer understanding of the organization of MP26 within the membrane of the fiber cells is important to the interpretation of all these studies.

In the experiments reported here, we have used proteases and antibodies directed against MP26 to evaluate the arrangement of bovine MP26 within the membrane. The data support the idea that approximately one-fifth of the molecule (including the C-terminus) is exposed on the cytoplasmic surface of the membrane, where it is accessible to proteases and antibodies. The functional significance of this protein domain of MP26 remains to be determined. However, it is possible that it serves some regulatory role (Johnson & Johnson, 1982).

Preliminary reports of these investigations have appeared previously (Sas, Keeling, Johnson & Johnson, 1980; Sas, Wagoner & Johnson, 1981).

Materials and Methods

Protease Treatments

Proteolytic digestions were performed on purified junctions or urea-treated plasma membranes isolated from calf lenses by a previously reported procedure (Goodenough, 1979).

Digestions with α-chymotrypsin (Sigma type VII) were performed in 25 mM Tris-HCl, pH 7.8, 10 mM $CaCl_2$. Papain (Sigma type III) was activated for 30 min at 37 °C in 5 mM Tris-HCl, 5 mM cysteine-HCl, 0.06 mM 2-mercaptoethanol, and 1 mM EDTA at pH 7.0. The enzyme was then diluted and digestions were performed in 5 mM Tris-HCl, pH 7.8. Proteolysis with trypsin (Sigma type XI) was carried out in 25 mM Tris-HCl, pH 7.8, 10 mM $CaCl_2$. *Staphylococcus aureus* V8 protease (Miles 36-900-1) digestions were performed in 10 mM Tris-HCl, pH 7.8. Reactions with carboxypeptidase A (Worthington COAPMS) activated with LiCl and carboxypeptidase B (Worthington COBPMS and Sigma DFP) were in 30 mM Tris-HCl, pH 7.8.

Reactions involving chymotrypsin and trypsin were inhibited by the addition of 1–5 mM PMSF (Sigma); those with papain by neutralized 30–50 mM iodoacetamide (Sigma); and carboxypeptidases A and B with 20 μM benzyl succinic acid (Byers & Wolfenden, 1972) from Burdick and Jackson. In a few experiments, e.g., with V8 protease, membranes were pelleted out of the enzyme solution and washed, with each spin being 12,000 × *g* for 15 min. For sequential digestions, care was taken to insure that the first enzyme used was fully inhibited prior to the addition of the second enzyme.

Electrophoresis

Samples were solubilized for gel electrophoresis at room temperature in 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.003% bromphenol blue in 20 mM Tris-HCl buffer, pH 7.9 (Wong, Robertson & Horwitz, 1978). Electrophoresis was performed on 8–18% linear gradient or straight 12% acrylamide slab gels 1 mm thick and 12–13 cm in length (Laemmli, 1970). Following electrophoresis, the gels were fixed and stained (Fairbanks, Steck & Wallach, 1971) with Coomassie brilliant blue R-250 (BioRad). Gel densitometry was performed using a Helena Quick Scan R & D densitometer. Molecular weights were estimated by calibration of the gels with marker proteins (*see* Fig. 1).

Electron Microscopy

For conventional thin-section analysis, membrane pellets were fixed with glutaraldehyde followed by treatment with tannic acid and osmium tetroxide (Simionescu, Simionescu & Palade, 1975). Membranes were also stained in the block with uranyl acetate (Johnson & Sheridan, 1971) prior to embedment in Spurr's resin.

Immunolabeling

Antiserum was prepared against electrophoretically purified, SDS-denatured MP26. The MP26 band was cut from a regular SDS-gel and electrophoretically eluted or diced and homogenized. This material was mixed with an equal volume of Freund's adjuvant (Difco). The primary injections were with complete Freund's and subsequent boosts with incomplete Freund's at 3–4 week intervals. Several hundred micrograms of protein were used for primary immunizations and approximately 200 μg for booster injections. Antibody binding to native or urea-purified lens membranes and to trypsin-digested lens membranes was determined by the enzyme-linked immunosorbent assay, ELISA (Engvall & Perlmann, 1972), with adaptations of a microtiter procedure described previously (Voller, Bidwell & Bartlett, 1979). One μg of lens membrane protein in pH 9.6 carbonate buffer was added per well in 96-well Falcon microtiter plates. We have found that nearly all of the added membrane protein adheres to the well if the solvent is allowed to evaporate. Twofold dilution series of rabbit immune and preimmune sera were constructed in rows of microtiter wells. Dilutions from 1:2 to greater than 1:16,000 in 100 μl of PBS with 0.05% Tween 20 (Sigma) were incubated at 37 °C for at least one hour. Goat anti-rabbit Ig conjugated to horseradish peroxidase (Miles) was used at a dilution of 1:500 to probe for bound rabbit antibodies. The chromogen *o*-phenylenediamine (Sigma) was used to detect the presence of the peroxidase conjugate (Engvall, 1980). The enzyme reaction was stopped by addition of 50 μl of 2.5 M H_2SO_4. The plate was immediately photographed using Polaroid type 55 film. Reaction product was quantitated by densitometric scanning of the photographic negative with a Helena Quick Scan recording densitometer. We have observed an excellent correlation between these densitometric scans and the optical density of the microtiter well contents at 492 nm.

Proteins from 1 mm, 12% acrylamide slab gels (*see* above) were transferred to DBM paper (Schleicher & Schuell) or DPT paper (gift of Margaret Wheelock) in a BioRad Trans-Blot Cell. Prior to transfer, the gel was treated with four 20-min washings in 10 volumes of 25 mM sodium phosphate, 0.1% SDS at pH 6.5. Inactivation of the paper and detection of proteins bound to the paper were done essentially as described (Renart, Reiser & Stark, 1979).

Results

Untreated Junctional Fractions

A typical SDS-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of purified bovine lens junc-

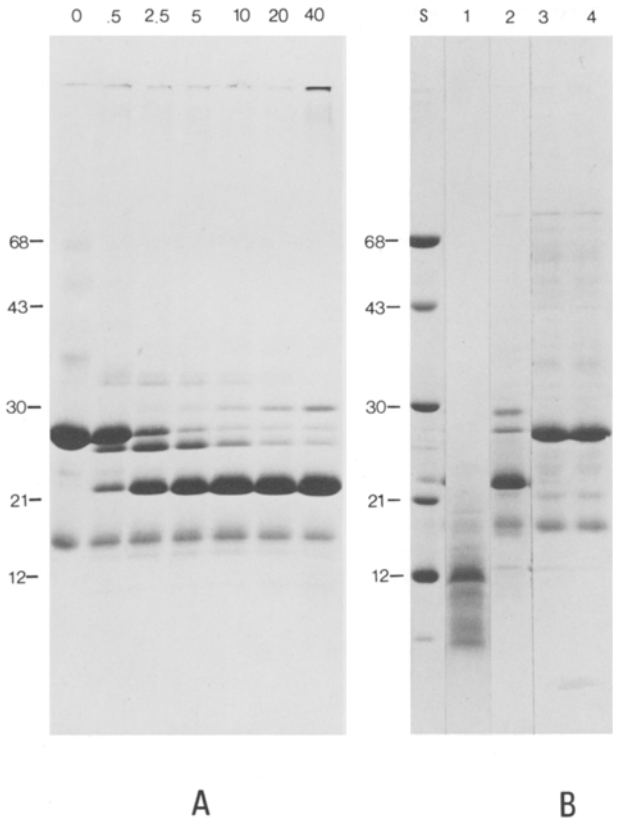

Fig. 1. (*A*): Time course for a chymotrypsin digestion at a 50:1 protein to enzyme ratio, with 17 μg of membrane protein per lane. Membranes were prepared with urea and detergents (*see* text). Times are noted in minutes. *0* refers to sample prior to addition of protease. Densitometric scans indicated that MP26 comprized about 60% of the total protein in the junctional fraction collected from sucrose step gradients following detergent treatment (deoxycholate and Brij). The major contaminating band ran at approximately 18 kD, as in other studies of bovine lens fractions (Benedetti et al., 1976; Broekhuyse & Kuhlmann, 1978; Goodenough, 1979; Friedlander, 1980; Horwitz & Wong, 1980). Inhibited with PMSF before solubilization. Note cleavage of MP26 to 22-kD products. (*See* Methods for description of gel, electrophoresis and staining.) (B): Controls for chymotrypsin. *Lane S:* Molecular weight standards include bovine serum albumen (68 kD), ovalbumen (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (21 kD), and cytochrome *c* (12 kD). For lanes 1–4, membranes incubated with chymotrypsin under various conditions. *Lane 1:* No PMSF added prior to SDS solubilization. *Lane 2:* PMSF added after 90 min incubation with enzyme, but before SDS added. *Lane 3:* PMSF added to enzyme prior to incubation with membranes. Note thorough inhibition, as in lane 4. *Lane 4:* PMSF (1 mM final) and enzyme added simultaneously to membranes

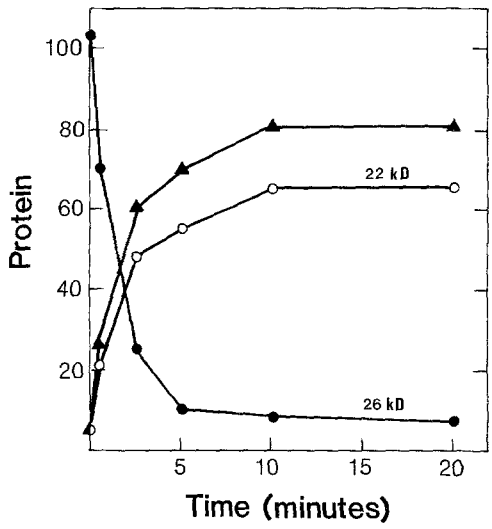

Fig. 2. Plot of gel scan results for the chymotrypsin time course. Densitometer scans of the 26- and 22-kD bands from the gel shown in Fig. 1 *A* are plotted for the first 20 min. The decrease in 26-kD staining (closed circles) parallels an increase in 22-kD staining (open circles). The closed triangles plot the 22-kD increase where a proportional adjustment has been made for the loss of 4 kD of protein. Note that about 20% of the 26-kD staining is not accounted for. This presumably includes MP26 found in the nonjunctional membrane

tions, prior to proteolysis, is shown in Fig. 1 (lane 0). The major component ran approximately as a 26-kD band, in agreement with a number of previous reports (Bloemendal et al., 1977; Broekhuyse & Kuhlmann, 1978; Goodenough, 1979). We will refer to this major membrane protein as MP26 (Bloemendal et al., 1977). However, it did migrate routinely as a slightly slower band on our gels (mean value of 27.5 kD).

Gel patterns of urea-treated plasma membranes which had not been exposed to detergents and to subsequent density gradient centrifugation (Fig. 3 *A*, lane 0) were similar to the patterns of the junctional preparations, with MP26 again the major protein component (45% of the staining per lane). Since the sensitivities to proteolysis of these membrane fractions and the more purified detergent-treated fractions were indistinguishable, the membrane fractions (no detergents) were used interchangeably with junctional preparations (detergent-treated) in the following studies.

Proteolysis with Chymotrypsin

The time course for proteolytic digestions of MP26 in junctional membrane fractions was followed with SDS-PAGE. Digestion with chymotrypsin (0 to 40 min, Fig. 1 *A*) gradually degraded MP26 to a 22-kD band, which resisted further degradation. There was a reciprocal relationship between the decrease in MP26 and the increase in the 22-kD band (Fig. 2).

No significant degradation of the 22-kD product was obvious over the 40-min digestion period. The 22-kD band was also stable during overnight

digestions and when treated with enzyme/substrate ratios as low as 1:2.5 (not shown). Thus, after removing approximately a 4–5 kD length, the protease was apparently unable to further degrade the junctional polypeptides. Yet chymotryptic activity remained after the digestion, since the 22-kD products were susceptible to extensive degradation upon addition of 1% SDS, if PMSF was omitted (Fig. 1 *B*, lane 1). This also indicates that the 22-kD products are not inherently resistant to chymotrypsin.

Since chymotrypsin can act in the presence of SDS, controls were always carried out to show that addition of 1 mM PMSF resulted in complete inhibition of chymotrypsin (Fig. 1 *B*). It was not possible to boil the sample in SDS as this leads to an aggregation of MP26 (Wong et al., 1978).

We have also incubated membranes at 37 °C without added enzymes for more than 24 hr and have not detected any degradation due to endogenous proteases in the membrane fractions (data not shown). Thus, it is reasonable to conclude that chymotrypsin cleaves MP26 to 22-kD polypeptides prior to solubilization of the membrane. A similar cleavage has been reported on eluted MP26 (Horwitz & Wong, 1980), but not on intact membranes.

Proteolysis with Papain

Similar experiments were conducted to determine if MP26 was susceptible to more extensive digestion in the presence of proteases, like papain, with different substrate specificities (Fig. 3).

The similarity to chymotryptic digestion was striking. The two proteases rapidly removed fragments of similar size, leaving 22 kD resistant regions. Omission of iodoacetamide, prior to SDS addition, results in extensive degradation with the majority of the cleavage fragments migrating near the dye front (Fig. 3 *B*, lane 2). These results suggest that papain, like chymotrypsin, encountered restrictions to further proteolysis after degrading MP26 in intact membranes to 22-kD fragments.

Proteolysis with Trypsin

Several earlier investigations found MP26 cleaved to 22-kD products by trypsin (Broekhuyse & Kuhlmann, 1980; Kistler & Bullivant, 1980; Takemoto et al., 1981). We also obtained 21–22 kD fragments (Fig. 7), which ran slightly ahead of the chymotrypsin and papain-generated peptides on our 8–18% gels. We found it necessary to pellet and wash the membranes to thoroughly eliminate tryptic activity prior to solubilization in SDS.

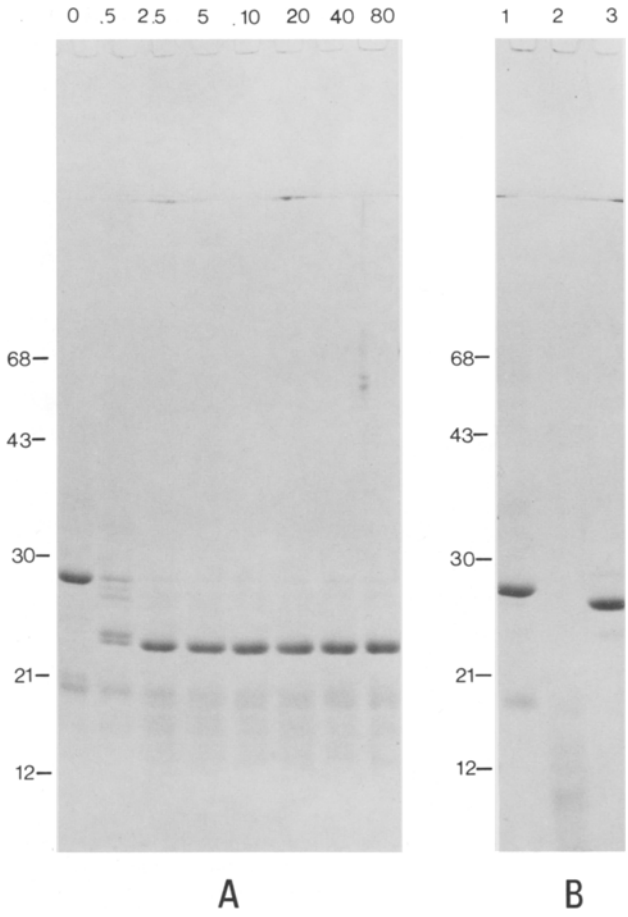

Fig. 3. (*A*): Time course for papain digestion at 45:1 protein to enzyme ratio, with 5 µg urea-treated membrane protein per lane. Times noted in minutes. '*0*' again refers to sample prior to addition of protease. Inhibited with iodoacetamide prior to solubilization with SDS. Note that MP26 is cleaved to stable 22-kD products. Several intermediates exist at 0.5 min. (*B*): Controls for papain. *Lane 1:* Iodoacetamide and papain added simultaneously to membranes and incubated 20 min prior to SDS solubilization. Note inhibition. *Lane 2:* 40-minute digestion with papain but no iodoacetamide added prior to SDS solubilization. *Lane 3:* Papain only, 4 µg

Proteolysis with S. aureus V8 protease

In contrast to papain and chymotrypsin, *Staphylococcus aureus* V8 protease (V8 protease) exhibits a narrow substrate specificity, (Drapeau, 1976). With V8 protease treatment of lens membranes for increasing periods of time, two different results were obtained. In a majority of experiments, MP26 was reduced to products migrating as peptides of 23–24 kD (Figs. 4 *A* and 9). In these experiments, there was a suggestion of a more gradual cleavage to 22-kd products, after extended proteolysis

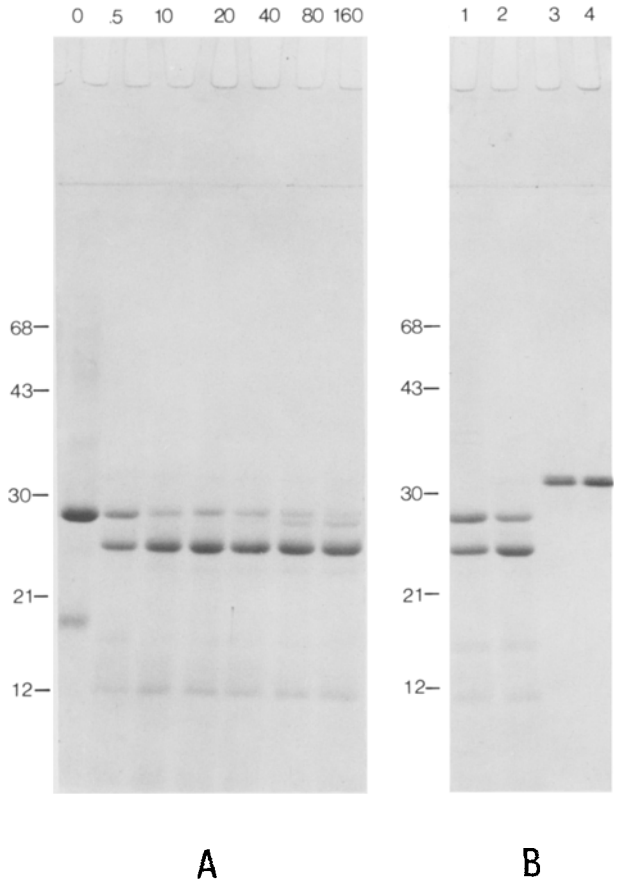

Fig. 4. (*A*): *Staphylococcus aureus* V8 protease time course at a 25:1 ratio of protein to enzyme. Urea-treated membranes were used (5 μg protein per lane). Times noted in minutes. Cleavage of MP26 to only 24-kD fragments is observed, with latter being stable. (*B*): V8 protease controls. *Lane 1:* PMSF (1 mM final) and V8 protease added simultaneously to membranes and incubated 20 min prior to solubilization with SDS. Some 24-kD products are obtained, as in lane 2. *Lane 2:* SDS solubilization buffer (1% SDS final) and V8 protease added simultaneously to membranes. *Lane 3:* V8 protease only, 5 μg

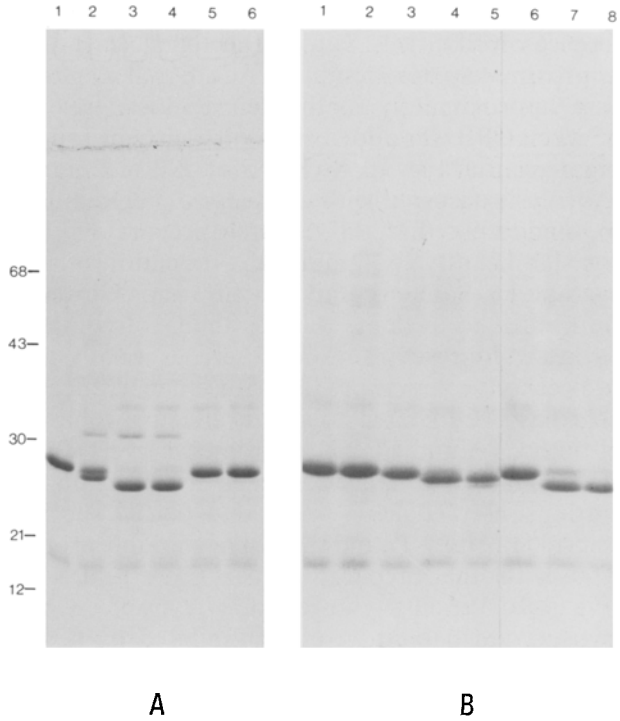

Fig. 5. (*A*): Proteolysis with carboxypeptidases A and B. Purified junctions (15 μg protein per lane) were incubated with 0.6 μg of CPA and/or CPB. *Lane 1:* Junctional protein. *Lane 2:* 60-min digestion with CPA. *Lane 3:* 120-minute digestion with CPA and CPB added simultaneously. *Lane 4:* 60-min digestion with CPA followed by 60-min digestion with CPB. *Lane 5:* 60-min digestion with CPB. *Lane 6:* 120-min digestion with CPB. (*B*): Controls for carboxypeptidases. *Lane 1:* Untreated lens membranes. *Lane 2:* Lens membranes after 60-min incubation at 37 °C without enzyme. *Lanes 3–5:* Membranes incubated with CPA (1:200) for 60 min at 37 °C, with benzyl succinic acid added at different times to yield 20 μm (lane 3, 0 min; lane 4, 30 min; lane 5, 60 min) before SDS solubilization. *Lanes 6–8:* Membranes incubated with CPA (1:200) and CPB (1:100) for 60 min at 37 °C with benzyl succinic acid added at different times (lane 6, 0 min; lane 7, 30 min; Lane 8, 60 min) before SDS solubilization

(80–160 min). This may be due to the action of other proteases found in the V8 preparation. However, in other experiments, substantial amounts of 22-kD products and fewer 24-kD products were obtained. This latter result resembles that of recent reports (Horwitz & Wong, 1980; Bloemendal, Hermsen, Dunia & Benedetti, 1982). The basis for these two different results is not yet clear. Nevertheless, the generation of 24-kD products is helpful in analyzing MP26 as one can compare the 22- and 24-kD products, for example with respect to antibody binding (*see* Fig. 9).

The action of V8 protease on lens membranes differs in another respect as well from that of the three above proteases. They all degrade MP26 in the presence of SDS to very small fragments, if not thoroughly inhibited. In contrast, uninhibited V8 protease simply hydrolyzes some of MP26 to 24-kD products (Fig. 4*B*, lanes 1 and 2). This also illustrates that 1% SDS is not an effective inhibitor of V8 protease. Thus, we found it necessary to pellet and wash the membranes to avoid any proteolysis upon solubilization.

Proteolysis with Carboxypeptidase A and B

Carboxypeptidase *A* (CPA) cleaves various C-terminal residues, with arginine and lysine being notable exceptions, while carboxypeptidase B (CPB) is able to remove these residues (Folk, 1971). We began by applying CPA to junctional fractions (Fig. 5*A*). This protease slowly generated

products running slightly ahead of MP26; the difference on SDS gels would reflect the cleavage of approximately ten residues. Additional degradation (approximately another ten residues) was noted when CPB was added simultaneously or following digestion with CPA (Fig. 5*A*). No meaningful hydrolysis occurred with CPB alone (Fig. 5*A*, lane 5), suggesting that this enzyme preparation was not significantly contaminated with endoproteases even at a 1:25 enzyme to protein ratio. However, we needed to examine the possibility of contaminating endoproteases more closely, especially with respect to the action of CPA. Since benzyl succinic acid has been shown to be an effective inhibitor of both CPA and CPB at micromolar levels (Byers & Wolfenden, 1972), we studied its effect on the cutting observed here. We found benzyl succinic acid to consistently and extensively inhibit proteolysis with the preparations of CPA and CPB (Fig. 5*B*). We also showed that trypsin, chymotrypsin, and papain were not inhibited by benzyl succinic acid at concentrations as high as 1 mM (data not shown). Presumably, if similar endoproteases contaminated the CPA and CPB preparations they would react in a similar manner. Thus, for the variety of reasons detailed below (*see* Discussion), we consider the proteolysis with CPA and CPB to be specific C-terminal cleavage.

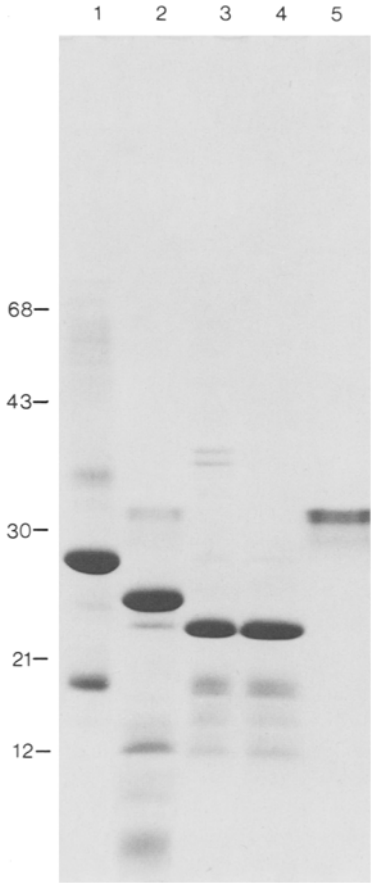

Fig. 6. Sequential addition of V8 protease followed by papain to urea-treated membranes (13 μg protein per lane). For thorough V8 inhibition, membranes must be washed free of enzyme. *Lane 1:* Lens membranes before addition of enzymes. *Lane 2:* After 40 min of V8 protease (1:10 protein/enzyme). *Lane 3:* As in lane 2, but followed by addition of papain (20-min digestion at 1:50) which was then inhibited with iodoacetamide before solubilization. *Lane 4:* Only papain added (20 min at 1:50). Note similarity of lanes 3 and 4

Sequential Digestions

We performed sequential protease digestions to determine whether the domains degraded by one protease overlapped with those of any or all of the other proteases. We initially took advantage of the fact that V8 protease often generated products that were noticeably larger than those generated by papain and chymotrypsin. Figure 6 shows the products obtained with V8 protease, papain, and with these two enzymes added sequentially. It appeared that the 24-kD polypeptides produced by V8 protease (lane 2) were further degraded to 22-kD products by papain (lane 3). The resulting 22-kD products were indistinguishable by SDS-PAGE from the 22-kD cleavage products obtained with papain alone (lane 4). These findings suggested that V8 protease and papain were active at overlapping sites on MP26.

We also carried out a number of other sequential digestions. For example, after papain digestion and inhibition with iodoacetamide, we treated with chymotrypsin or trypsin and solubilized for gel analysis (Fig. 7, lanes 3–7). In no instance did we find that MP26 was degraded to less than a 21–22 kD band. That is, we found that none of these three enzymes removed a significant portion of MP26 not cleaved by the other two.

The relationships between the carboxypeptidase-sensitive regions and those cleaved by the endoproteases were also examined in sequential experiments. In all cases, the products of the endoproteases alone were indistinguishable from those of the carboxypeptidases (A and B) followed by the various endoproteases (Fig. 7, lanes 8–15). Thus, these endoproteases all appear to attack regions at the C-terminus of MP26.

Immunolabeling Experiments

Antibody binding experiments were carried out in an attempt to localize at least some of the protease-

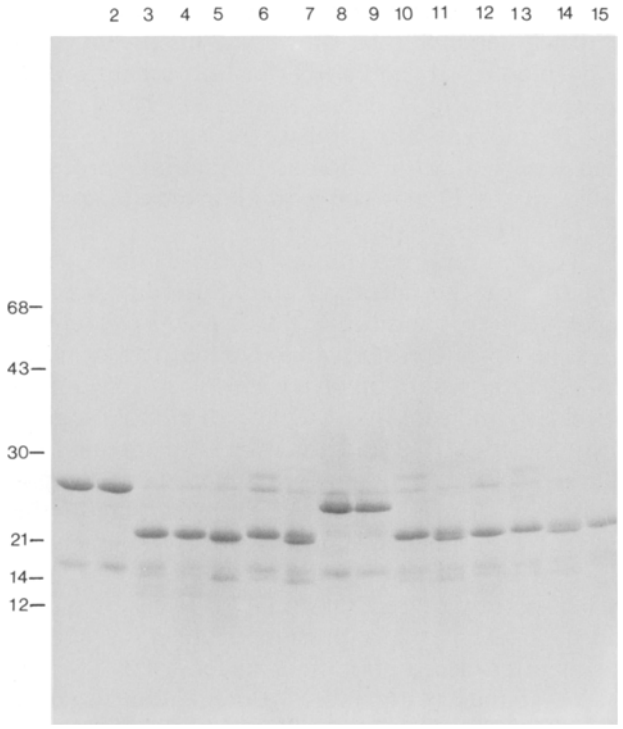

Fig. 7. Sequential digestion of urea-treated lens membranes (6 μg per lane) with carboxypeptidases and several endoproteases. The enzymes in the first digestion were inhibited before the second enzymes were added. Each digestion was for 30 min. In some digestions enzyme buffers were substituted as controls, so that other enzymes added subsequently could be shown to be effective. After the digestions all membranes were washed prior to solubilization in SDS. *Lane 1:* Untreated membranes. *Lane 2:* Membranes incubated for 60 min without enzymes. *Lane 3:* Incubated 30 min with papain and 30 min with the buffer for trypsin and chymotrypsin. *Lane 4:* Papain for 30 min, add iodoacetamide, chymotrypsin for 30 min. *Lane 5:* As in lane 4, but trypsin not chymotrypsin. *Lane 6:* 30 min in papain buffer, 30 min in chymotrypsin. *Lane 7:* As in lane 6, but trypsin not chymotrypsin. *Lane 8:* 30 min in CPA + CPB, add benzyl succinic acid, 30 min in buffer for trypsin and chymotrypsin. *Lane 9:* As in lane 8, but use papain buffer. *Lane 10:* 30 min in buffer for CPA + CPB, then benzyl succinic acid and 30 min in chymotrypsin. *Lane 11:* As in lane 10, but 30 min in trypsin. *Lane 12:* As in lane 10, but 30 min in papain. *Lane 13:* 30 min in CPA + CPB, then benzyl succinic acid and 30 min in chymotrypsin. *Lane 14:* As in lane 13, but 30 min in trypsin. *Lane 15:* As in lane 13, but 30 min in papain. Note that the products of the three endoproteases are identical to those generated by the carboxypeptidases in combination with the endoproteases. Therefore, the sites appear to overlap

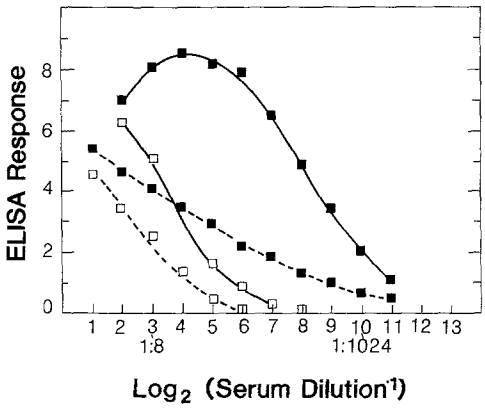

Fig. 8. Plot of ELISA response *vs.* antiserum dilution for control membranes containing MP26 (solid lines) and trypsinized membranes containing 22 kD (dashed lines). The closed squares represent immune values, the open squares preimmune values. One microgram of membranes was used for each test well. Antiserum was raised against electrophoretically purified 26-kD proteins. (*See* Methods section for ELISA details.) ELISA response assayed by densitometer scan of photographic negative. Trypsinization led to a significant decrease in antibody binding. At a 1:128 dilution, the difference in immune values is almost fourfold with the preimmune values being at background levels

sensitive domains within the junctional structure. We made three assumptions: (1) a significant fraction of MP26 resides in junctional membranes, (2) antibodies do not penetrate the lipid bilayer in intact membranes, and (3) components within the 2–3 nm gap would not be labeled in an indirect labeling experiment with a peroxidase conjugated immunoglobulin. An important observation, in this last regard, is that we did not find junctional structure altered by trypsin treatment when examined with electron microscopy.

Routine thin-section EM was carried out on pellets of membranes treated with tannic acid to enhance membrane detail. When trypsinized samples displaying a typical 22-kD band upon electrophoresis were evaluated, no significant structural alterations were detected. As with control junctions, a uniform intermembrane "gap" of 2–3 nm was detected. In addition, no discontinuities were noted within the lipid bilayer. Recently, similar observations have been made on extensively trypsinized bovine lens membranes (Kistler & Bullivant, 1980).

Thus, we considered antibodies to be effective probes for protein domains exposed on the cytoplasmic side of the junctional membrane. We carried out a series of ELISA experiments (*see* Methods) on intact junctional membranes, with and without trypsinization, using rabbit antisera directed against electrophoretically purified MP26. Trypsin treatment resulted in much less antibody binding, although some binding persisted (Fig. 8).

To further support the idea that the 22- and 24-kD protease products bound less antibody than undegraded MP26, these proteins were transferred to paper from SDS gels (Renart, Reiser & Stark, 1979) for labeling. In this case, treatment of membranes with three different proteases gave a strik-

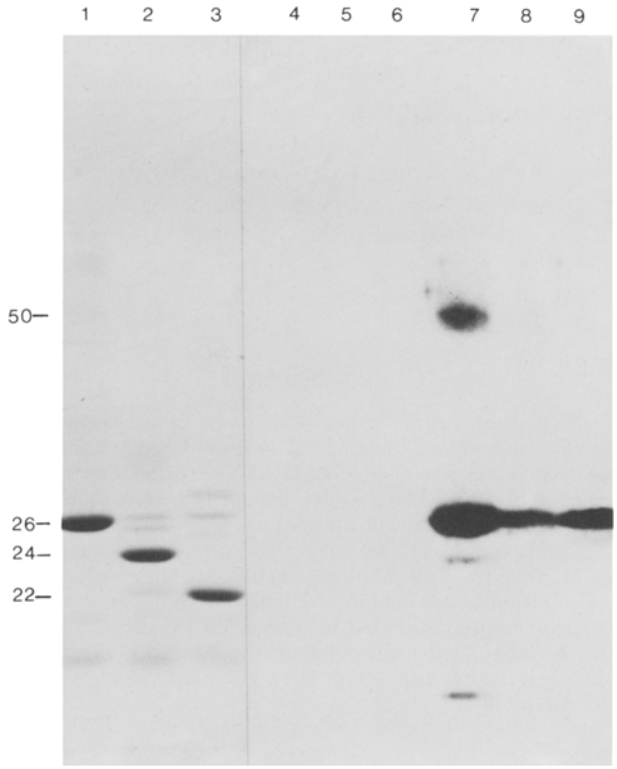

Fig. 9. Gel and autoradiograph of proteins electrophoretically transferred to paper and labeled with antisera to MP26 and with ^{125}I-protein A (*see* Methods). Three different samples were studied: undigested membranes (containing MP26), V8-digested membranes (with 24 kD products), and chymotrypsin-digested membranes (with 22 kD). The set of three lanes is repeated three times for Coomassie staining (lanes 1–3), preimmune serum (lanes 4–6), and immune serum (lanes 7–8). Serum was diluted 1:200. Note that the 24- and 22-kD bands are not labeled even though they contain a similar amount of protein to that at 26 kD. Preimmune serum gives no (significant) reaction. The band at approximately 50 kD is thought to be a dimer of MP26. MP26 has often been found to form oligomers (e.g., see Horwitz & Wong, 1980). The two lightly labeled bands running ahead of MP26 in the immune autoradiogram (lane 7), which are not seen with Coomassie, are thought to be degradation products of MP26, presumably cut at the N-terminal end, (*see* Sas et al., 1982). Note that some labeling persists at 26 kD following protease treatment

ing result (Fig. 9). Heavy labeling of MP26 occurred (lane 7), with only background labeling associated with a comparable amount of protein in the 22-kD band (lane 9 generated with chymotrypsin; trypsin results were identical, but are not shown). In addition, the V8 protease product at 24 kD was not labeled (lane 8). Preimmune serum was tested and yielded very low backgrounds. Thus, two lines of evidence indicate that proteolysis of intact junctional membranes removes antigenic determinants from MP26, presumably from the cytoplasmic side of the membranes.

The experiments involving protein transfer and antibody labeling also show that significant antibody binding at the 26-kD position remains after proteolysis (Fig. 9, lanes 8 and 9). Thus, some 26-kD antigens are protease resistant. We have also detected, with Coomassie staining, protease resistant 26-kD material with all proteases studied (*see*, e.g., Figs. 1–5).

To accurately interpret all of these immunological studies, the nature of the antiserum directed against electrophoretically purified 26-kD proteins must be considered. It is possible that two or more 26-kD antigens are involved. However, our analyses clearly indicate that we have an immunological response to MP26 and that we have no obvious marked contamination by a soluble, 26-kD component; e.g., a crystallin. It is stressed that in the present experiments, e.g., the effort to label the 22- and 24-kD products, an absolutely pure anti-MP26 is not required. Our analysis has shown that (1) a 50-kD band, a presumed dimer of MP26, labels after electrophoretic transfer when 22- and 24-kD bands with more protein are negative (Fig. 9); (2) supernatants from lens homogenates are negative on electrophoretic transfer; (3) when beta-crystallins and MP26 are separated on hydroxyapatite, the former is negative and the latter positive on electrophoretic transfer; (4) the pattern of labeling in Fig. 9 (lanes 7–9) is identical to that obtained with a monoclonal antibody specific for calf MP26 (Sas, Johnson, Menko & Johnson, 1982); and (5) when rabbit antibodies are bound and then eluted from antigens following electrophoretic transfer, studies show that the same antibodies label all four of the bands (two major, two minor) seen in lane 7 of Fig. 9. Thus, the four bands are antigenically related.

Discussion

The new findings on the membrane arrangement of MP26 derived from this study can be summarized as follows: (1) various conditions with a number of proteases generated cleavage products from MP26 that were no smaller than 21–22 kD, (2) all of the proteases cleaved overlapping domains, (3) carboxypeptidases digest MP26 within the intact membrane, and (4) antibody labeling indicates that protease-sensitive domains are found on the cytoplasmic side of the membrane.

Limits to Proteolysis

After treating lens membranes with a variety of proteases, specific for different amino acid resi-

dues, MP26 was degraded to products no less than 21–22 kD. The values reported here for the limits to proteolysis prior to SDS solubilization are consistent with other studies on bovine and human MP26 (Broekhuyse & Kuhlmann, 1980; Horwitz & Wong, 1980; Kistler & Bullivant, 1980; Takemoto et al., 1981). Yet these former studies typically provided data on only one or two enzymes utilizing one set of conditions. Consequently, questions did exist regarding actual limits to proteolysis. Our results were obtained in spite of applying a number of proteases, high enzyme concentrations (up to 400 μg/ml in the case of chymotrypsin), low enzyme to substrate ratios (down to 1:2.5), extended digestion times at 37 °C (up to 25 hr), the inclusion of additional enzyme during the course of various digestions, and the rapid cleavage of MP26 to 21–22 kD fragments. However, in the case of several endoproteases, the 21–22 kD peptides were readily degraded to much smaller fragments following addition of SDS, if the enzymes were not inhibited. Thus, under the conditions employed with intact membranes there are definite restrictions placed on the proteolysis of MP26.

In view of the SDS effect, the most obvious interpretation is that the 22-kD peptides are protected by the membranes from further degradation. This protection would be provided by the lipid bilayer and the close apposition of the two junctional membranes. The partial resistance of a variety of membrane proteins to proteolytic digestion is well documented (*see*, e.g., Strader & Raftery, 1980). In fact, such studies have attempted to clarify protein organization within the lipid bilayer through the use of proteases, as in the present investigation.

An alternate interpretation for the protease sensitivity of the 22-kD peptides following solubilization in SDS is that this denaturing detergent renders certain protein domains more susceptible to proteases. If this were true, it is possible that other denaturing conditions, e.g., 7 M urea, might also modify the results with proteolysis. Yet, we found resistant 22-kd peptides with isolation methods which ranged from thorough aqueous washings without subsequent urea and detergent treatment to application of both these agents. Detergents included NP-40, Brij 58 and deoxycholate. Therefore, we prefer the interpretation that implicates membrane organization in the protection of the 22-kD peptides.

Several observations argue forcefully that the 22-kD peptides are, indeed, products of MP26. First, we observed a reciprocal relationship between these components during the course of digestion with chymotrypsin (*see* Fig. 2). Second, bovine MP26 and 22-kD peptides (generated with trypsin) are similar in amino acid composition (Broekhuyse & Kuhlmann, 1980) and in peptide mapping (Horwitz & Wong, 1980; Takemoto et al., 1981). Furthermore, immunological cross-reactivity has been reported (Zigler & Horwitz, 1981). Sequencing studies, which are in progress (Nicholson et al., 1980), will provide more specific information on protein similarities.

Overlapping Sensitivites to Proteases

In our present experiments we studied four endoproteases and two carboxypeptidases. We have demonstrated with sequential digestions that these various proteases hydrolyze overlapping regions (e.g., Fig. 7). The only proteases which we did not test sequentially were trypsin, chymotrypsin, and V8 protease, all serine-site proteases. Nevertheless, these three proteases in combination cleave only to 22-kD fragments. Furthermore, in sequential experiments, they all cleave domains which overlap with papain. Thus, it appears reasonable to argue that all six of these proteases attack overlapping regions of MP26.

It is important to note that although our studies indicated that all the endoproteases cleaved C-terminal regions from MP26, we do not know whether cleavage is restricted to this end of the molecule. We await sequencing data on MP26 and the 22-kD products to clarify this issue.

MP26 Orientation within the Membrane

Carboxypeptidases consistently removed significant portions of MP26. In addition, carboxypeptidases A and B in combination produced more extensive cleavage than the A form alone. It is possible that the presence of a proline residue inhibited further hydrolysis. A major concern was that these cuts were made by contaminating proteases in the commercial carboxypeptidase preparations. However, several observations argued against the role of endoproteases in these observed cleavages. (1) Micromolar benzyl succinic acid inhibited the cutting obtained with carboxypeptidases A and B, as previously reported (Byers & Wolfenden, 1972). (2) Even millimolar benzyl succinic acid did not inhibit papain, chymotrypsin, and trypsin digestions. (3) CPB alone did not significantly degrade MP26, yet the cutting with CPA was increased by addition of CPB. (4) Only when we increased carboxypeptidase concentrations (e.g., to enzyme/substrate ratios of 1:25) or the length of incubations (a number of hours to overnight) did we produce

22-kD fragments with the carboxypeptidase preparations. However, most importantly, these cuts were not sensitive to micromolar benzyl succinic acid. Thus, only these more extensive cuts were presumably made by contaminating endoproteases. Therefore, we suggest that the C-terminal end of MP26 in intact calf membranes is accessible to, and specifically cut by, carboxypeptidases A and B.

It would also be useful to determine whether the carboxypeptidases and the endoproteases cleave MP26 at sites on the extracellular and/or cytoplasmic side of the membrane. The latter orientation has been suggested in a recent study (Broekhuyse & Kuhlmann, 1980), based on the isolation of 22-kD peptides from older fiber cells. The rapid and essentially complete cleavage of MP26 to 22-kD fragments analyzed here also indicates that proteolysis may occur on the readily accessible cytoplasmic side. However, since the protease studies alone cannot answer this question, we included immunolabeling experiments in the present study, both on intact membranes and on purified proteins.

The immunolabeling data firmly support the idea that protease-sensitive sites of MP26 are found on the cytoplasmic side. Antibodies against electrophoretically purified 26-kD proteins, which bind extensively to intact membranes rich in MP26, bind much less extensively to membranes containing the 22-kD fragments (Fig. 8). Since the antigen is detected in the ELISA with two antibody layers and an attached peroxidase on the second antibody, it seems unlikely that significant numbers of these complexes could be found within the 2–3 nm extracellular "gap". Our efforts rely on the conclusion that proteolysis does not alter membrane apposition (*see* above; Kistler & Bullivant, 1980). In addition, the labeling of MP26 after transfer to paper supports our argument since there was essentially no labeling of either the V8-generated 24-kD band or the 22-kD band generated by either trypsin or chymotrypsin. We suggest, therefore, that significant portions of the protease-sensitive domains are found on the cytoplasmic side of the junctional membrane. In addition, because of the V8 result with this particular rabbit serum, the important antigenic determinant(s) appears to be on a 2-kD portion including the C-terminus of MP26.

Comparisons with Other Systems

It was suggested several years ago that MP26 is the major protein found in the extensive, intercellular junctions of the lens (Benedetti et al., 1976). The strongest support for the localization of MP26 in these junctions comes from a recent immunolabeling study at the EM level (Bok et al., 1982). As noted above, these specializations resemble gap junctions seen in a variety of other tissues.

One of our purposes in initiating this study was to compare the protease effects on MP 26 within the membrane with those on the major proteins of gap junction fractions from mammalian hepatocytes. There was fair agreement over the idea that trypsin yielded approximately 10 kD products from the major 26-kD band in the liver fractions (Henderson, Eibl & Weber, 1979; Finbow, Yancey, Johnson & Revel, 1980). Therefore, we sought to determine whether there were conditions under which MP26 could be proteolytically degraded to less than the 20–22 kD values reported in the literature. This study has shown that under conditions similar to those used for several mammalian hepatocyte junctional fractions, calf MP26 is not degraded to fragments resembling those in the liver preparations. Furthermore, in preliminary experiments, we have also obtained this result with the major lens membrane protein in the chicken.

In view of a variety of studies, these results on the lens membranes are not surprising. Peptide mapping of liver junctional protein and MP26, as well as partial sequencing, have not demonstrated significant homology (Hertzberg et al., 1982; Nicholson et al., 1980). In addition, antigenicity appears to vary. Antibodies to lens MP26 in the calf are reported to not react with 26-kD hepatocyte junctional protein (Hertzberg et al., 1982). Moreover, myocardial gap junction fractions have recently been reported to contain a major protein component at 28 kD, which on peptide mapping differs from both MP26 in the lens and the 26-kD protein from liver gap junctions (Nicholson, Gros & Revel, 1982).

Yet within a cell type, considerable conservation may exist. Peptide maps of the major lens membrane protein from several vertebrate lenses, including MP26 in the calf, display homologies (Takemoto et al., 1981). Moreover, there seems to be cross-reactivity in terms of antigenicity, since antibodies to calf lens MP26 will react with membranes from other vertebrate lenses (Friedlander, 1980; Hertzberg et al., 1982; Sas et al., 1981; Bok et al., 1982; however, *see* Alcala & Maisel, 1978). We have also recently developed a monoclonal antibody that recognizes both the calf and chicken lens proteins (Sas et al., 1982).

Thus, the existing data, although sketchy, indicate that for three different cell types one appears

to obtain three rather different junctional proteins. It would be interesting to develop the idea that the major proteins found in the various gap junctions of a particular organism constitute a family of proteins (Hertzberg et al., 1982; Nicholson et al., 1980). Yet, it will first be necessary to determine whether junctional proteins contain any "constant" as well as "variable" domains.

Presumably, molecular structure will be found to reflect the specific roles played by these junctional specializations in a variety of cell types. Some of the most interesting biological questions relate to what these roles actually are in different cells. It is generally agreed that gap junctions contain hydrophilic channels which link adjacent cytoplasms and provide for small molecule exchanges. Yet, it is likely that the specific roles vary considerably with cell type. It has even been suggested, since lens junctions differ in extent and protein composition from liver, that MP26 and the lens junctions might serve a role other than intercellular communication (Hertzberg, 1980) Others have raised questions about lens junctions based on structural variations in lens membrane fractions (Zampighi et al., 1982). Consequently, some investigators have hesitated to apply the term "gap junction" to the junctions in the lens. However, we consider the term to refer to structures satisfying a morphological definition. Therefore it is appropriate to apply the term "gap junction" in an objective manner to structures throughout animal tissues which fit the morphological definition. We recognize that in most of these systems it will be a long time before we gain an idea of chemical composition and biological function. However, we remain hopeful about the prospects for better understanding these parameters in model systems, such as the lens, in the not-so-distant future.

We sincerely thank Judson Sheridan, Sue Menko, and Irving Liener for reviewing this manuscript, Rod Kuehn for help with the electron microscopy, Kris Kohn for the artwork, and Kae Ebling for typing the manuscript. This work was supported by the University of Minnesota Graduate School, an Institutional Grant from the American Cancer Society, and Grant CA28548 from the National Cancer Institute.

References

Alcala, J., Maisel, H. 1978. Specific antiserum to the main intrinsic polypeptide of chick lens fiber cell plasma membranes. *Exp. Eye Res.* **26**:219–221

Benedetti, E.L., Dunia, I., Bentzel, C.J., Vermorken, A.J.M., Kibbelaar, M., Bloemendal, H. 1976. A portrait of plasma membrane specializations in eye lens epithelium and fibers. *Biochim. Biophys. Acta* **457**:353–384

Benedetti, E.L., Dunia, I., Bloemendal , H. 1974. Development of junctions during differentiation of lens fibers. *Proc. Natl. Acad. Sci USA* **71**:5073–5077

Bloemendal, H. 1981. Molecular and Cellular Biology of the Eye Lens. Wiley-Interscience, New York

Bloemendal, H., Hermsen, T., Dunia, I., Benedetti, E.L. 1982. Association of crystallins with the plasma membrane. *Exp. Eye Res.* **35**:61–67

Bloemendal, H., Vermorken, A.J.M., Kibbelaar, M., Dunia, I., Benedetti, E.L. 1977. Nomenclature for the polypeptide chains of lens plasma membranes. *Exp. Eye Res.* **24**:413–415

Bok, D., Dockstader, J., Horwitz, J. 1982. Immunocytochemical localization of the lens main intrinsic polypeptide (MIP26) in communicating junctions. *J. Cell Biol.* **92**:213–220

Broekhuyse, R.M., Kuhlmann, E.D. 1974. Lens membranes. I. Composition of urea-treated plasma membranes from calf lens. *Exp. Eye Res.* **19**:297–302

Broekhuyse, R.M., Kuhlmann, E.D. 1978. Lens membranes. IV. Preparative isolation and characterization of membranes and various membrane proteins from calf lens. *Exp. Eye Res.* **26**:305–320

Broekhuyse, R.M., Kuhlmann, E.D. 1980. Lens membranes. XI. Some properties of human lens main intrinsic protein (MIP) and its enzymatic conversion into a 22,000 dalton polypeptide. *Exp. Eye Res.* **30**:305–310

Broekhuyse, R.M., Kuhlmann, E.D., Winkens, H.J. 1979. Lens membranes. VII. MIP is an immunologically specific component of lens fiber membranes and is identical with 26K band protein. *Exp. Eye Res.* **29**:303–313

Byers, L.D., Wolfenden, R. 1972. A potent reversible inhibitor of carboxypeptidase A. *J. Biol. Chem.* **247**:606–608

Drapeau, G.R. 1976. Protease from *Staphylococcus aureus*. *In:* Methods in Enzymology. L. Lorand, editor. Vol. 45, pp. 469–475. Academic Press, New York

Engvall, E. 1980. Enzyme immunoassay ELISA and EMIT. *Methods Enzymol.* **70**:419–439

Engvall, E., Perlman, P. 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* **109**:129–135

Fairbanks, G., Steck, T.L., Wallach, D.F.H. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**:2606–2617

Finbow, M., Yancey, S.B., Johnson, R., Revel, J.P. 1980. Independent lines of evidence suggesting a major gap junctional protein with a molecular weight of 26,000. *Proc. Natl. Acad. Sci. USA* **77**:970–974

Folk, J.E. 1971. Carboxypeptidase B. *In:* The Enzymes. P. Boyer, editor. Vol. 3, pp. 57–79. Academic Press, New York

Friedlander, M. 1980. Immunological approaches to the study of myogenesis and lens fiber junction formation. *Curr. Top. Dev. Biol.* **14**:321–358

Goodenough, D.A. 1979. Lens gap junctions: A structural hypothesis for nonregulated low-resistance intercellular pathways. *Invest. Ophthalmol. Vis. Sci.* **18**:1104–1122

Goodenough, D.A., Dick, J.S.B., II, Lyons, J.E. 1980. Lens metabolic cooperation: A study of mouse lens transport and permeability visualized with freeze substitution autoradiography and electron microscopy. *J. Cell Biol.* **86**:576–589

Henderson, D., Eibl, H., Weber, K. 1979. Structure and biochemistry of mouse hepatic gap junctions. *J. Molec. Biol.* **132**:193–218

Hertzberg, E.L. 1980. Biochemical and immunocytological approaches to the study of gap junctional communication. *In Vitro* **16**:1057–1067

Hertzberg, E.L., Anderson, D., Friedlander, M., Gilula, N.

1982. Comparative analysis of the major polypeptides from liver gap junctions and lens fiber junctions. *J. Cell Biol.* **92**:53–59

Horwitz, J., Wong, M.M. 1980. Peptide mapping by limited proteolysis in sodium dodecyl sulfate of the main intrinsic polypeptides isolated from human and bovine lens plasma membranes. *Biochim. Biophys. Acta* **622**:134–143

Johnson, K.R., Johnson, R.G. 1982. Bovine lens MP26 is phosphorylated *in vitro* by an endogenous cAMP-dependent protein kinase. *Fed. Proc.* **41**:755 (*abstr.*)

Johnson, R.G., Sheridan, J.D. 1971. Junctions between cancer cells in culture: Ultrastructure and permeability. *Science* **174**:717–719

Kistler, J., Bullivant, S. 1980. Lens gap junctions and orthogonal arrays are unrelated. *FEBS Lett.* **111**:73–78

Kuszak, J., Maisel, H., Harding, C.V. 1978. Gap junctions of chick lens fiber cells. *Exp. Eye Res.* **27**:495–498

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685

Maisel, H., Harding, C.V., Alcala, J.R., Kuszak, J., Bradley, R. 1981. The morphology of the lens. *In:* The Molecular and Cellular Biology of the Eye Lens. H. Bloemendal, editor. pp. 49–71. Wiley-Interscience, New York

Nicholson, B., Gros, D., Revel, J.P. 1982. Tissue specificity in the gap junctional protein. *J. Cell Biol.* **95**:104*a* (*abstr.*)

Nicholson, B.J., Hunkapiller, M.W., Hood, L.E., Revel, J.-P. 1980. Partial sequencing of the gap junctional protein from rat lens and liver. *J. Cell Biol.* **87**:200*a* (*abstr.*)

Peracchia, C., Peracchia, L.L. 1980. Gap junction dynamics: Reversible effects of hydrogen ions. *J. Cell Biol.* **87**:719–727

Rae, J.L. 1979. The electrophysiology of the crystallin lens. *In:* Current Topics in Eye Research. J.A. Zadunaisky and H. Davson, editors. pp. 37–90. Academic Press, New York

Ramaekers, F.C.S., Selten-Versteegen, A.M.E., Benedetti, E.L., Dunia, I., Bloemendal, H. 1980. *In vitro* synthesis of the major lens membrane protein. *Proc. Natl. Acad. Sci USA* **77**:725–729

Renart, J., Reiser, J., Stark, G.R. 1979. Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: A method for studying antibody specificity and antigen structure. *Proc. Natl. Acad. Sci. USA* **76**:3116–3120

Sas, D., Johnson, K., Menko, S., Johnson, R. 1982. A monoclonal antibody specific for chicken and bovine lens gap junctional proteins. *J. Cell Biol.* **95**:106*a* (*abstr.*)

Sas, D., Keeling, P., Johnson, K., Johnson, R. 1980. Characterizing gap junction proteins from calf lens: Questions of glycosylation and protease sensitivity. *J. Cell Biol.* **87**:96*a* (*abstr.*)

Sas, D., Wagoner, M.J., Johnson, R. 1981. Monoclonal antibodies and rabbit antisera to gap junction-rich fractions of bovine lens fiber cell membranes. *J. Cell Biol.* **91**:119*a* (*abstr.*)

Simionescu, M., Simionescu, N., Palade, G. 1975. Segmental differentiations of cell junctions in the vascular endothelium. *J. Cell Biol.* **67**:863–885

Strader, C.D., Raftery, M.A. 1980. Topographic studies of Torpedo acetylcholine receptor subunits as a transmembrane complex. *Proc. Natl. Acad. Sci. USA* **77**:5807–5811

Takemoto, L.J., Hansen, J.S., Horwitz, J. 1981. Interspecies conservation of the main intrinisc polypeptide (MIP) of the lens membrane. *Comp. Biochem. Physiol. B* **68**:101–106

Voller, A., Bidwell, D.E., Bartlett, A. 1979. The enzyme linked immunosorbent assay (ELISA). Independent publication available from Dynatech Lab., Inc., Alexandria, VA.

Waggoner, P.R., Maisel, H. 1978. Immunofluorescent study of a chick lens fiber cell membrane polypetide. *Exp. Eye Res.* **27**:151–157

Wong, M.W., Robertson, N.P., Horwitz, J. 1978. Heat induced aggregation of the sodium dodecyl sulfate-solubilized main intrinsic polypeptide isolated from bovine lens plasma membrane. *Biochem. Biophys. Res. Commun.* **84**:158–165

Zampighi, G., Simon, S., Robertson, J., McIntosh, T., Costello, M. 1982. On the structural organization of isolated bovine lens fiber junctions. *J. Cell Biol.* **93**:175–189

Zigler, J.S., Horwitz, J. 1981. Immunochemical studies on the major intrinsic polypeptides from human lens membrane. *Invest. Ophthalmol. Vis. Sci.* **21**:46–51

Received 25 January 1983

Note Added in Proof

After treatment of intact lens membranes with trypsin, protein sequencing studies have shown that only five amino acid residues are removed from the N-terminus of MP26 (Nicholson, B.J., Takemoto, L.J., Hunkapiller, M.W., Hood, L.E., Revel, J.-P. 1983. Differences between liver gap junction protein and lens MIP26 from rat. *Cell* **32**:967–978). Thus, when MP26 is degraded to a 22-KD product, essentially all of the cleavage occurs at the C-terminal end.