

A Lens Intercellular Junction Protein, MP26, Is a Phosphoprotein

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Abstract. The major protein present in the plasma membrane of the bovine lens fiber cell (MP26), thought to be a component of intercellular junctions, was phosphorylated in an *in vivo* labeling procedure. After fragments of decapsulated fetal bovine lenses were incubated with [³²P]orthophosphate, membranes were isolated and analyzed by SDS PAGE and autoradiography. A number of lens membrane proteins were routinely phosphorylated under these conditions. These proteins included species at M_r 17,000 and 26,000 as well as a series at both 34,000 and 55,000. The label at M_r 26,000 appeared to be associated with MP26, since (a) boiling the membrane sample in SDS led to both an aggregation of MP26 and a loss of label at M_r 26,000, (b) the label at 26,000 was resistant to both urea and nonionic detergents, and (c) two-dimensional gels showed that a phosphorylated M_r 24,000 fragment was derived from MP26 with V8 protease. Studies with proteases also provided for a

localization of most label within ~20 to 40 residues from the COOH-terminus of MP26. Published work indicates that the phosphorylated portion of MP26 resides on the cytoplasmic side of the membrane, and that this region of MP26 contains a number of serine residues. The same region of MP26 was labeled when isolated lens membranes were reacted with a cAMP-dependent protein kinase prepared from the bovine lens. After the *in vivo* labeling of lens fragments, phosphoamino acid analysis of MP26 demonstrated primarily labeled serines, with 5–10% threonines and no tyrosines. Treatments that lowered the intracellular calcium levels in the *in vivo* system led to a selective reduction of MP26 phosphorylation. In addition, forskolin and cAMP stimulated the phosphorylation of MP26 and other proteins in concentrated lens homogenates. These findings are of interest because MP26 appears to serve as a protein of cell-to-cell channels in the lens, perhaps as a lens gap junction protein.

HYDROPHILIC channels connect the cytoplasm of a variety of animal cells, providing pathways for the direct intercellular movement of small molecules (6). Hexamers of gap junction protein, forming channels in each apposed membrane, are thought to meet head to head and create the direct cytoplasmic connections (72). It has been suggested that these channels serve significant functions in differentiation (74), growth control (43), and the response of cells to extracellular signals (41).

Given the importance of this cell communication system, precise mechanisms for regulation of the junctions would be expected. However, such mechanisms have been examined in only a limited way. For example, covalent modifications of junctional protein have not been firmly established *in vivo*, nor have any accessory proteins or membrane lipid controls been detected. Yet a variety of interesting studies indicate that these cell-to-cell channels are gated (66). In particular, increases in the cytoplasmic concentrations of calcium and hydrogen ions have been reported to reduce junctional permeability (60, 71). In addition, the reported half-life of liver gap junction protein is less than that of other plasma membrane proteins in the liver (15, 78). Thus, the turnover of junctional protein is probably regulated as well. Finally, the assembly of

gap junctions from protein monomers might be regulated (34).

Since protein phosphorylation and dephosphorylation are instrumental in regulating various activities and structures in the cell (61), we sought to determine whether lens junctional proteins were phosphorylated *in vivo*. Work on the phosphorylation of MP26 in isolated membranes with exogenous protein kinases (19, 33, 44) reinforced the need for such studies. The alteration of protein conformation by phosphorylation has been implicated in the closing of junctional channels (72). In fact, a number of intriguing papers describe the importance of phosphorylation in regulating different membrane channels in neurons (49) and myocardial cells (59).

Specifically relating to cell-to-cell channels, short-term effects of dopamine and cAMP metabolism have been reported on permeable junctions that link horizontal cells in the retina (40, 55). The intracellular injection of cAMP was found to reduce dramatically and reversibly the electrical coupling between horizontal cells, with a time course of minutes (40). Similar studies with intracellular injections of heart cells have implicated cAMP in junctional permeability changes (13). Other observations relate to longer term effects of cyclic

nucleotides on junctions and possible phosphorylation events. In these cases, treatments of cultured cells designed to increase the intracellular levels of cAMP have been reported to alter junctional permeability and structure (5, 37, 57). In addition, exogenous protein kinase (the catalytic subunit of the cAMP-dependent enzyme) has been implicated in "correcting" the reduced junctional permeability of certain cells in culture (75).

We report here that MP26 is phosphorylated within the bovine lens. We chose to examine the possible phosphorylation of lens junctional protein for a variety of reasons considered below (see Discussion). The work focuses on the phosphorylation of membrane proteins, after fragments of fetal lenses are incubated in [³²P]orthophosphate. In addition, we show that with lens fragments, decreases in cytoplasmic calcium lead to a reduction in MP26 phosphorylation, and that with concentrated homogenates, forskolin and cAMP stimulate the phosphorylation of MP26. Finally, these results are compared with those from experiments in which isolated membranes were phosphorylated with a cAMP-dependent protein kinase purified from bovine lenses (33). The implications of these results are discussed in view of an *in vitro* labeling of MP26 by protein kinase C (39) and other work on cell-to-cell channels. Preliminary reports of this work have already appeared (31, 32).

Materials and Methods

Tissue Labeling

Lenses were dissected from fetal calf eyes, cleaned of most of the adherent vitreous and pigmented material, and decapsulated, and the core of the lens was removed with a cork borer. Routinely, the cortices from several lenses were cut into small pieces (several millimeters in diameter) which were suspended in medium. In a few experiments, core samples were retained and analyzed in parallel with the cortical samples. For phosphorylation studies, the medium used was phosphate-free, serum-free, Hepes-buffered Dulbecco's modified Eagle's medium. Labeling with methionine was done in methionine-free Hank's balanced salts supplemented with amino acids and vitamins.

The tissue pieces were suspended in medium, then allowed to settle, and the supernatant was removed. After resuspension in 5–10 vol fresh medium, 1-ml aliquots of the suspension were distributed into wells of a 24-well tissue culture plate (Falcon Labware, Oxnard, CA) and placed at 37°C in an atmosphere containing 5% CO₂. Individual wells were labeled with 0.25–0.5 mCi of [³²P]phosphoric acid (New England Nuclear, Boston, MA) or 50 μCi [³⁵S]-methionine (Amersham Corp., Arlington Heights, IL), for up to 6 h. In some experiments, after a 30-min initial incubation, wells were incubated with the following: 7.5 μM A23187 (29), 20 or 50 μM forskolin (Calbiochem-Behring Corp., La Jolla, CA); 50 μM isobutylmethylxanthine 1 mM dibutyl cAMP, and 50 μM cAMP (Sigma Chemical Co., St. Louis, MO), and 5 mM EGTA.

Incubations with [³²P]phosphate were terminated by diluting the entire incubation mixture into 5 ml ice-cold homogenization buffer (20 mM sodium phosphate, 4.0 mM EDTA, 100 mM sodium fluoride, pH 6.5) in a Corex tube. The tissue was disrupted by sonication (sonifier [Branson Sonic Power Co., Danbury, CT] with a microtip) and diluted with another 5 ml homogenization buffer. The homogenate was centrifuged (20,000 g, 10 min) to obtain buffer-washed membranes. For further purification, the buffer-washed membranes were sequentially resuspended in and pelleted from homogenization buffer (2 × 10⁵ g-min), TEC buffer (5 mM Tris-HCl, 1 mM EDTA, 1 mM CaCl₂, pH 9.0, 2 × 10⁵ g-min), 7 M urea in TEC buffer (4 × 10⁵ g-min), and water (2 × 10⁵ g-min). The final pellets, called urea-washed membranes, were resuspended in water and frozen at -70°C. These washes served to remove the soluble lens proteins and the bulk of the extrinsic membrane proteins.

Incubations with [³⁵S]methionine were terminated by placing the incubation mixture on ice, sonicating the tissue, and freezing at -70°C. Incorporation of ³⁵S into protein was assayed by spotting aliquots of the sample onto filter disks. The disks were dropped into ice cold 10% trichloroacetic acid (TCA). They were then sequentially washed with boiling 10% TCA, cold 10% TCA (twice), ethanol, and ether. After being allowed to air dry, the disks were counted in a toluene-based scintillant.

For the phosphorylation studies in lens homogenates, 1 g lens cortical material was homogenized in 1 ml of 20 mM imidazole buffer at pH 7.5. The incubation medium contained 1.0 mM isobutylmethylxanthine, 3.0 mM MgCl₂ and 0.1 mM γ-[³²P]ATP (~3 μCi). EGTA, CaCl₂, forskolin, and cAMP were added as indicated in Fig. 7. Incubation was for 15 min at 37°C.

Preparation of Purified Membranes and Lens Kinase

Urea-washed bovine lens plasma membranes were processed through the first sucrose gradient as described (22) and resuspended to ~2 mg protein per ml with water. Fractions enriched in the lens cAMP-dependent protein kinase holoenzyme and its catalytic subunit were prepared as described elsewhere (33). In brief, the procedure to prepare the holoenzyme fraction from soluble lens proteins consisted of acid precipitation, batch treatment of the supernatant with DEAE cellulose, and a hydroxylapatite chromatography step. Additional steps to effect a 5,000-fold purification of the catalytic subunit involved chromatography on carboxymethylcellulose and hydroxylapatite (33).

Phosphorylation of Purified Membranes

Aliquots of lens membranes were incubated in 25 mM 2-(*N*-morpholino)ethane sulfonic acid pH 7.0, 10 mM MgCl₂, and 100 μM ATP (supplemented with γ-[³²P]ATP [Amersham Corp.] to 1,000 to 3,000 dpm/pmol), with either the catalytic subunit of the protein kinase or the holoenzyme plus 5 μM cAMP. Reactions were terminated either by pelleting the membranes in an Eppendorf microfuge (12,000 g) or by adding SDS gel solubilization buffer (38).

Electrophoresis and Autoradiography

One-dimensional SDS PAGE (38) was performed in 8–18% or 10–20% linear gradient gels or in straight 12% gels. Unless otherwise stated, samples were solubilized at room temperature to prevent the aggregation of MP26 (10, 77). Standard two-dimensional gels were run as described (51, 80). Isoelectric focusing in the presence of buffers and zwitterionic detergent will be described in greater detail elsewhere. In brief, this procedure involved substituting a mixture of buffers for the ampholines to generate the pH gradient (56). For the first dimension, samples solubilized in zwitterionic detergent were run in tube gels, followed by standard SDS PAGE for the second dimension. The pH gradient of the first dimension focusing gels was determined by cutting the tube gel into 5-mm lengths and soaking them in 2 ml degassed distilled water. Gels were stained with either Coomassie R-250 (69), Coomassie G-250 (26), or silver (47, 48). Autoradiography of stained, dried gels was done using Kodak XAR or BB film and a Dupont Cronex intensifying screen.

Phosphoamino Acid Analysis

Phosphoamino acid analysis was essentially as reported (28). Membranes were purified from phosphorylated lens fragments as above (90 min ³²P incubation, no additives). Lens membrane proteins (200 μg) were electrophoretically fractionated on a 1-mm 12% gel, and the MP26 band was identified by soaking in 4 M sodium acetate (25). The MP26 band was excised, recast in a 5% stacking gel, and electroeluted into 0.5% SDS, 30% glycerol (46). TCA was added to 25% concentration, and after 1 h at 4°C the precipitate was collected at 12,000 g for 10 min. The precipitate was dissolved in 200 μl water and immediately reprecipitated by the addition of 800 μl 25% TCA. After 1 h the precipitate was collected by centrifugation and hydrolyzed in 6M HCl under argon for 2 h. The HCl was then removed by lyophilization.

Nonradioactive phosphoamino acid standards (2 μg each/lane) were added to the samples. Phosphoamino acid analysis was performed at pH 1.9 (water/acetic acid/formic acid, 897:78:25) and pH 3.5 (water/acetic acid/pyridine, 945:50:5) in one dimension at 1.0 kV on Whatman No. 3 paper (Whatman Chemical Separation Inc., Clifton, NJ). Phosphoamino acid spots were detected by spraying with ninhydrin.

Proteolysis of Membrane Proteins

Proteolysis of intact lens membranes was conducted as described (36) with slight modifications. Chymotrypsin (Sigma Type VII), trypsin (Sigma Type XI), and *Staphylococcus aureus* V8 protease (Worthington Biochemical Corp., Freehold, NJ) were the proteases used.

Proteolysis of proteins resolved in SDS polyacrylamide gels was performed as described (9) with the following modifications. Lanes from first dimension (mini) slab gels (0.5 cm by 5 cm by 1 mm) were equilibrated in stacking gel buffer (125 mM Tris-HCl, pH 6.8, 0.1% SDS) containing 1% β-mercaptoethanol and placed on top of the stacking gel of a second dimension gel (1.5-mm thick, 2-cm stacking gel). The first dimension lanes were covered with stacking gel buffer containing 20% glycerol. An appropriate amount of protease in 0.2

ml stacking gel buffer containing bromphenol blue and 5% glycerol was layered on the 20% glycerol solution just before the start of electrophoresis. Current was regulated at 5 mA/cm² for 2 h and 2 mA/cm² thereafter.

Protein Assay

Samples were assayed for protein using an SDS solubilization technique (45) after N-ethyl maleimide treatment (27). For small samples, the fluorescamine assay was used with prior solubilization in SDS (7). In each case, bovine serum albumin (Sigma Chemical Co.) was used as the standard.

Results

Phosphorylation in Tissue Fragments

To determine whether the phosphorylation of MP26 is a physiological event in the lens fiber cell, a reliable system for labeling was sought. Tissue pieces were prepared as described in Materials and Methods to provide cells with reasonable access to the label. Several lines of evidence suggest that the procedures applied here produce tissue appropriate for phosphorylation studies.

First, several membrane proteins were consistently shown to be labeled when tissue pieces were incubated with [³²P]-orthophosphate (Fig. 1). Since the phosphate must first have been incorporated into a donor ATP pool, the tissue must have been capable of ATP synthesis and related processes. Second, the incubations with [³⁵S]methionine showed increasing incorporation of ³⁵S into TCA-precipitable material with time, being linear over the first 3–4 h (not shown). Phosphorylation patterns over the first several hours were comparable to those at up to 6 h. Third, the concentration of extracellular protein stayed relatively constant throughout the course of the experiments. Since lenses are remarkably rich in protein (400 mg/ml [12]), one might expect cell damage during the incubation period to result in an increase in protein in the incubation solution. Although a significant amount of protein was found in the supernatant after the preparation of the

tissue fragments, the level stayed roughly constant throughout the incubation period. Fourth, vimentin appears to be labeled in our phosphorylation system. This is consistent with reports that isolated lens vimentin can be phosphorylated (68) and that vimentin is phosphorylated in other cells (50, 67).

Several labeled bands were consistently seen in the lens fragment preparations. We will refer to them as *M_r* 17,000, 26,000, 34,000, and 55,000 species (Fig. 1), although it was evident that several components were present in the latter two molecular weight classes. Minor bands at higher molecular weights were noted, perhaps relating to proteins such as spectrin-like lens elements (2).

In Fig. 1, *A* and *B* the components present in urea-washed (lane 1) and buffer-washed membranes (lane 2) are shown after isolation from tissue that had been incubated with [³²P]orthophosphate. Differences in the amount of crystallins in the two preparations were evident from the Coomassie-stained gels (Fig. 1*A*). The corresponding autoradiogram (Fig. 1*B*) demonstrated that labeling patterns in the two membrane samples were similar, although some differences occurred in the *M_r* 55,000 cluster.

Incorporation of ³²P into specific bands on gels increased with time, reaching a constant level after 60–90 min in culture. Other studies have shown that the specific radioactivity of ATP in hepatocytes (1) and in heart slices (11) began to plateau after 30–60 min in the presence of [³²P]phosphate.

Analysis of MP26 Phosphorylation

Urea-washed membranes from labeled tissue fragments were analyzed to determine whether the labeling of the *M_r* 26,000 species was associated with MP26 (Fig. 1, *C* and *D*). When samples were boiled in SDS, MP26 was found to aggregate (Fig. 1*C*, lane 6) as previously reported (10, 77). This also led to a loss of label at the *M_r* 26,000 position (Fig. 1*D*, lane 6). In addition, when labeled membranes were extracted with

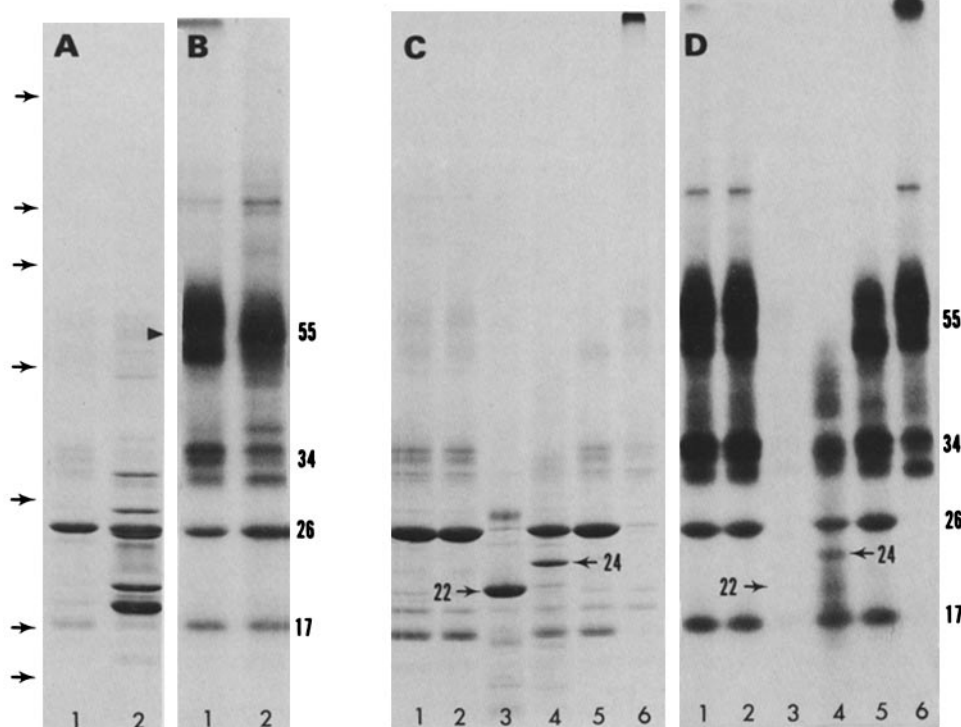


Figure 1. Analysis of membranes phosphorylated in lens fragments for 120 min. The Coomassie-stained gel (*A*) and corresponding autoradiogram (*B*) of urea-washed (lane 1) and buffer-washed (lane 2) membranes isolated from labeled lens fragments are shown. There is label in the *M_r* 55,000 cluster (lane 2), which is removed by urea (corresponding to arrowhead in lane 2). Urea-washed membranes isolated from the labeled fragments were further examined with Coomassie stain (*C*) and in a corresponding autoradiogram (*D*). Lane 1, an aliquot of the membranes. Lane 2, an aliquot of membranes incubated at 37°C and pelleted as a control for the protease digestions. Lanes 3–5, aliquots of membranes digested with chymotrypsin, V8 protease, and V8 protease (10% the amount used in lane 4). Lane 6, membranes solubilized in SDS at 100°C for 5 min. Both autoradiograms (*B* and *D*) are the result of 24-h exposures with intensifying screens.

0.5% Nonidet P-40 or 7 M urea, the label at M_r 26,000 was resistant (not shown). The apparent phosphorylation of MP26 was further analyzed with proteases, which can cleave this membrane protein to fragments of M_r 22,000 and 24,000 (36). These studies offered the added advantage of being able to localize label on MP26. Although MP26 was stable in the absence of exogenous proteases (Fig. 1C, lane 2), it was degraded to an M_r 22,000 species by chymotrypsin and an M_r 24,000 species by V8. The former fragment had lost label, whereas the V8 fragment retained label (Fig. 1D, lanes 3 and 4). Thus, most label in MP26 appeared to be found on a terminal 20-residue long portion of the M_r 24,000 species (see Discussion).

However, since the membranes labeled in the tissue showed considerable radioactivity in regions of $M_r > 26,000$ we further tested these membranes to determine clearly which proteins gave rise to the labeled M_r 24,000 species after V8 digestion. We used a one-dimensional peptide mapping procedure in which proteases were co-electrophoresed with proteins from entire lanes of first dimension gels (9). A stained control gel and its corresponding autoradiogram are shown in Fig. 2, A and B, demonstrating that the protein and label

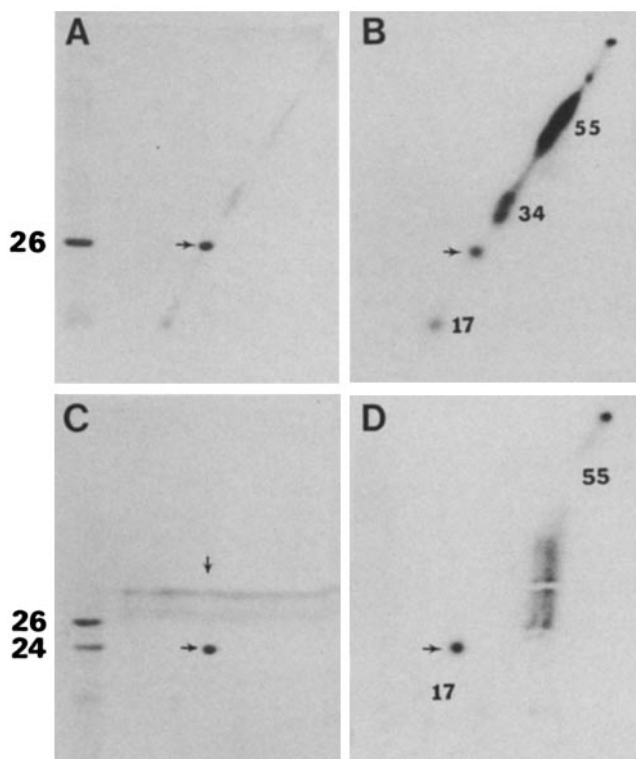


Figure 2. Two-dimensional proteolysis. Aliquots of membranes labeled in lens fragments (from the same experiment as Fig. 1, A and B) were run on first dimension slab gels (1 mm, 12% acrylamide, 5-cm resolving gel). Lanes were cut from these gels and equilibrated in stacking gel buffer (see Materials and Methods). They were then placed on second dimension gels (1.5 mm, 12% acrylamide) and co-electrophoresed with V8 proteases. A and C are Coomassie stained; B and D are corresponding autoradiograms (exposure of 24 h with screen). A and B show a control sample run using the procedure, without proteases. C and D show the results when 10 μ g V8 protease was co-electrophoresed with the membrane proteins. The bands across (C) are V8 protease. Note that in A and C unlabeled membranes are run at the left to mark clearly the location of MP26 (A) and the M_r 24,000 fragment (B).

ran on the diagonal in the absence of proteolysis. Digestion of the membrane proteins with V8 protease produced only one M_r 24,000 species. This labeled fragment was derived from an M_r 26,000 band in the first dimension (Fig. 2, C and D). Treatment with V8 produced no other MP26-related bands detectable with Coomassie or with autoradiography. Use of one-tenth the amount of V8 protease produced similar results, although less MP26 was digested to the M_r 24,000 level (not shown). In this case especially, it was clear that the M_r 24,000 fragment was derived from MP26. These studies strongly indicate that the labeling at M_r 26,000 is associated with MP26. Therefore, this lens junctional protein appears to be a phosphoprotein.

To evaluate the phosphorylation of MP26 within different regions of the lens, *in vivo* labeling was carried out with tissue fragments from both lens cortices and cores (Fig. 3). Cortical preparations often displayed a greater degree of labeling.

Characterization of Membranes Phosphorylated *In Vitro*

We also sought to determine whether the phosphorylation seen in lens tissue fragments was comparable to that obtained with purified lens membranes and added lens cAMP-dependent protein kinase. We hoped that a detailed analysis of this type would ultimately lead to an identification of the kinase system involved in the apparent phosphorylation of MP26.

Previously, we obtained purified preparations of a lens cAMP-dependent protein kinase (a type I kinase) and showed,

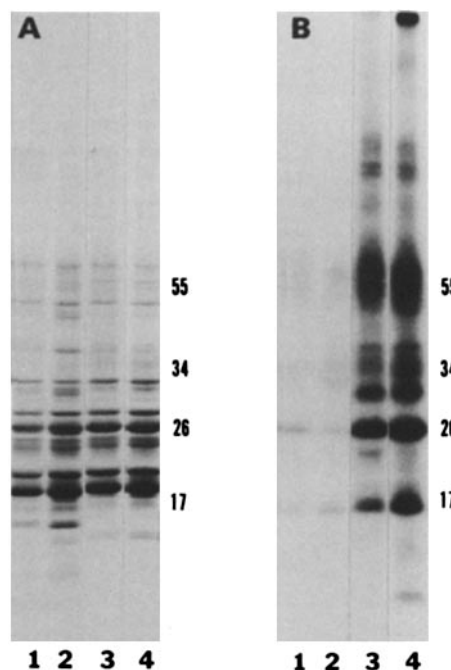


Figure 3. Aliquots of buffer-washed membranes from labeled lens fragments were stained with Coomassie (A) and then autoradiographed (B, 2-d exposure with screen). Lanes 1 and 2 in each case were obtained from the lens core, lanes 3 and 4 from the cortex. Note the much more extensive phosphorylation found in the cortical preparations. The lens fragments involved in lanes 2 and 4 were treated for 30 min at the end of a 6-h incubation in 32 P with several additives (1 mM dibutyl cAMP, 50 μ M isobutylmethylxanthine, and 50 μ M forskolin), in an unsuccessful attempt to enhance cAMP-dependent phosphorylation. In other experiments, the additives were included at different times with similar results. Also see Fig. 7.

with the holoenzyme, a phosphorylation of isolated lens membranes that was cAMP dependent and sensitive to the Walsh inhibitor preparation (33). To compare labeling patterns for the purified enzyme with respect to those observed with the tissue fragments, we used a preparation of the catalytic subunit in the present study. The resulting phosphorylation pattern was similar to that seen with the holoenzyme but was cAMP-independent as anticipated (Fig. 4, *A* and *B*, lane 2).

Since crystallin components migrate in the M_r 26,000 region on SDS gels, several properties of the major lens membrane protein were again used to verify that the label seen at M_r 26,000 was associated with MP26. Heating solubilized samples to 100°C caused MP26 to aggregate (10, 77) and label to appear at the top of the resolving gel (data not shown; result as in Fig. 1, *C* and *D*, lane 6). In addition, MP26 was found to be resistant to extraction with urea and nonionic detergents (22), as was the associated label (not shown). After phosphorylating membranes with the catalytic subunit, membranes were treated with proteases as in the above studies with tissue fragments. The 24,000 M_r band was again labeled; the 22,000 M_r forms were not (Fig. 4*B*, lanes 4–6). If membranes were first digested and then incubated with the catalytic

subunit (Fig. 4*B*, lanes 7–9), the M_r 24,000 form was a substrate whereas the M_r 22,000 fragments were not. A minor labeled band at somewhat less than 22,000 (Fig. 4*B*, lane 7) migrated ahead of the M_r 22,000 fragment derived from MP26. Thus, proteolytic digestion of labeled membranes defined a 2,000-D portion of the molecule (~20 residues) that contained a site or sites phosphorylated by the lens cAMP-dependent protein kinase, as found above with tissue labeling.

Phosphoamino Acid Analysis

An attempt was made to characterize and identify further the kinase system(s) involved in the *in vivo* phosphorylation system by means of phosphoamino acid analysis (Fig. 5). Phosphoamino acids were analyzed at pH 1.9 to separate clearly phosphoserine and phosphothreonine. Most of the label was associated with phosphoserine, with only 5–10% on phosphothreonine, as detected by examination of the autoradiogram. Phosphoamino acid analysis at pH 3.5 revealed no phosphotyrosine (not shown).

Modulation of Membrane Phosphorylation

Different aliquots of tissue fragments in a single experiment

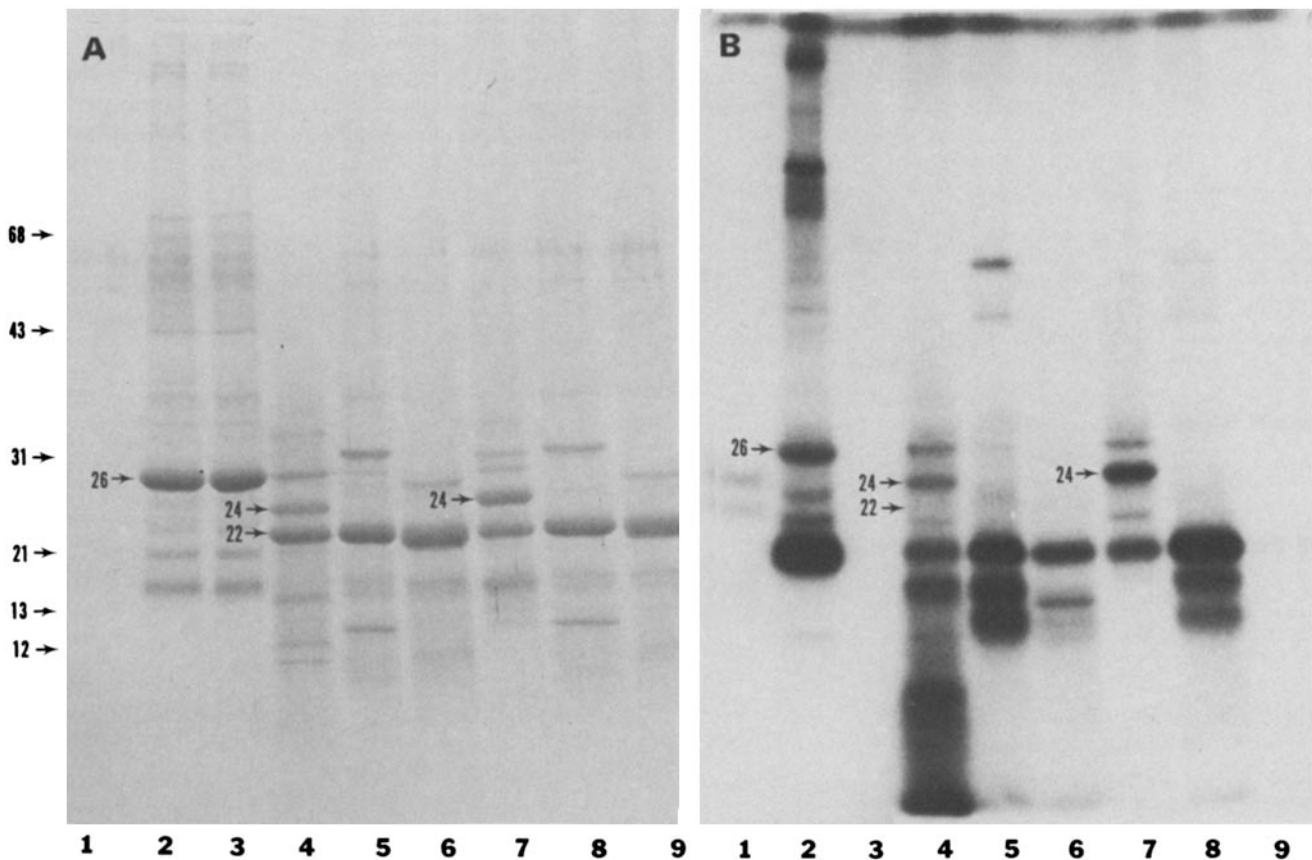


Figure 4. Phosphorylation of lens membranes with the catalytic subunit of the cAMP-dependent lens kinase. Lens membranes (15 μ g) were incubated with the catalytic subunit and γ -[32 P]ATP for 20 min and not treated with proteases (lanes 2 and 3). Other membranes were phosphorylated before (lanes 4–6) or after (lanes 7–9) digestion of the membranes with proteases. The Coomassie-stained gel (*A*) and its autoradiogram (*B*, exposure of 12 h with screen) are shown in the figure. Lane 1, aliquot of the catalytic subunit preparation, with the amount of enzyme examined being ten times that used for the membrane phosphorylations. Lane 2, lens membranes incubated with the catalytic subunit. Lane 3, lens membranes incubated without the catalytic subunit. Lanes 4–6, digestion of labeled membranes (as shown in lane 2) with V8 protease, chymotrypsin, and trypsin, respectively. Lanes 7–9, digested membranes (V8 protease, chymotrypsin, and trypsin, respectively) incubated with the catalytic subunit. Molecular weight standards are marked: bovine serum albumin (M_r 68,000 or 68), ovalbumin (43), carbonic anhydrase (31), soybean trypsin inhibitor (21), ribonuclease (13), and cytochrome *c* (12). In *B*, the minor band in lane 7, at M_r ~22,000, migrates significantly ahead of the M_r 22,000 fragment of MP26.

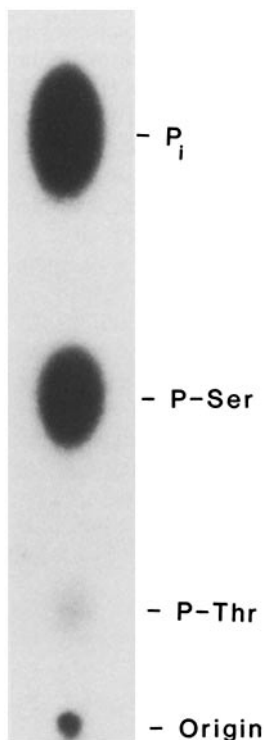


Figure 5. Phosphoamino acid analysis of MP26 phosphorylated *in vivo*, after a 120-min incubation with ^{32}P . Phosphoamino acid analysis was performed at pH 1.9 on MP26 eluted from SDS gels and hydrolyzed as in Materials and Methods. The positions of ninhydrin positive spots corresponding to phosphoserine and phosphothreonine are indicated. Autoradiographic exposure was for 3 d with an intensifying screen.

were found to yield nearly identical degrees and patterns of phosphorylation. Therefore, different effectors were added to the *in vivo* incubation mixtures in order to modulate phosphorylation (Fig. 6). Since calcium levels influence the activity of the lens adenylate cyclase (29) and protein kinase C (39), lens fragments were treated in various ways to examine the effect of intracellular calcium on phosphorylation patterns. Although treatment with the ionophore A23187 had no effect when it was added alone, in combination with extracellular EGTA it led to reduced phosphorylation (Fig. 6). Labeling was slightly reduced overall and markedly reduced at MP26. Since EGTA also had no effect when added alone, the EGTA effect with A23187 probably represents the result of decreased cytoplasmic calcium. Forskolin was also examined and found to not have a significant effect on labeling (Fig. 6). Moreover, individually and in combination, forskolin, dibutyryl-cAMP, isobutyl methylxanthine, and cAMP were added to samples that had been incubated for various periods (from 15 min up to 6 h) in [^{32}P]phosphate. We saw no consistent alteration in incorporation into any band as a result of adding this second group of agents (Fig. 3).

The lack of a forskolin effect on the lens fragment system could be interpreted in several ways, with one view emphasizing difficulties related to reagent penetration. Therefore, a series of experiments was performed in which lenses were homogenized in a one-to-one ratio (wt/vol) with imidazole buffer. This should facilitate penetration but not extensively dilute critical cellular components. With this approach, the addition of forskolin and ^{32}P -labeled ATP resulted in an enhanced phosphorylation of several proteins, including MP26 (Fig. 7). Thus, forskolin appears to be stimulating the lens adenylate cyclase, thereby activating the cAMP-dependent kinase. The effect was significantly greater in the presence of calcium than with EGTA, consistent with our studies indicating that lens adenylate cyclase activity is calcium dependent (29). When only cAMP was added with labeled ATP,

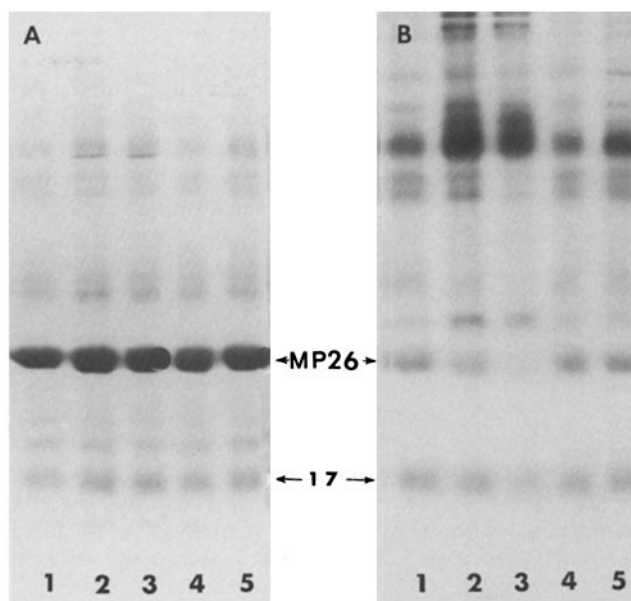


Figure 6. Effects of calcium on membrane phosphorylation *in vivo*. Lens fragments were phosphorylated as in Materials and Methods. If indicated, EGTA (5 mM), A23187 (7.5 μM), and forskolin (20 μM) were added 30 min into the 120-min incubation with 0.4 mCi ^{32}P . The Coomassie Blue-stained 12% polyacrylamide gel (A) shows an even loading of protein in the five lanes ($\sim 25 \mu\text{g}$ protein per lane). The corresponding autoradiogram (B, 1-d exposure with screen) shows: lane 1, a standard incubation mixture; lane 2, addition of EGTA to the incubation media; lane 3, addition of EGTA and A23187; lane 4, addition of A23187; and lane 5, addition of A23187 and forskolin.

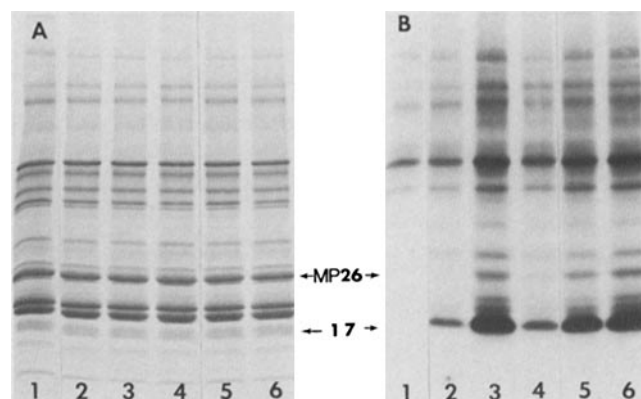


Figure 7. Effects of forskolin and cAMP on phosphorylation in lens homogenates. All samples received 0.1 mM [^{32}P]ATP after a 1:1 (wt/vol) homogenization in 20 mM imidazole buffer (at pH 7.5). After incubation for 15 min at 37°C, membranes were buffer washed and subjected to SDS PAGE with a 10–20% gradient gel. Coomassie Blue staining (A) and corresponding autoradiogram (B, a 24-h exposure with screen) are shown. Lanes 1–3 were incubated with 1 mM EGTA, lanes 4–6 with 0.1 mM calcium. In addition, lanes 2 and 5 included 30 μM forskolin and lanes 3 and 6 had 10 μM cAMP added. Lanes 1 and 4 were controls.

either in the presence of calcium or EGTA, a similar pattern of phosphorylation was obtained (Fig. 7). In this situation, added cAMP is probably activating the endogenous cAMP-dependent kinase directly, bypassing the calcium-sensitive adenylate cyclase. These results are discussed below.

Analysis of Phosphorylated Proteins on Two-Dimensional Gels

To analyze more thoroughly the various labeled species, the membrane samples were analyzed on two-dimensional gels. Two different systems were used. Results with a conventional system (51) are found in Fig. 8, *A* and *B*, those with buffer focusing in Fig. 8, *C* and *D* (see Materials and Methods). In several instances, proteins resolved in one system were not resolved in the other.

We had previously developed the alternative method of isoelectric focusing with solubilization in a mixture of zwitterionic and nonionic detergents, because we could not focus MP26 in the conventional system (32). With buffer focusing and subsequent running in a standard SDS system, MP26 is resolved into at least three protein spots (Fig. 8*C*). An acidic variant of these components had a higher specific radioactivity than the other forms (Fig. 8*D*). The apparent pI's of the three forms of MP26 were 7.2, 7.3, and 7.5. We show elsewhere

that normal calf lens membranes exhibit these focusing variants and that they are all recognized by a monoclonal antibody directed against MP26 (63). Thus, it appears that MP26 can exist as several forms varying in charge and that this charge heterogeneity is due, at least in part, to phosphorylation. Therefore, this provides a very different set of data, which indicates that MP26 occurs naturally as a phosphoprotein.

The two-dimensional systems also provide data on other lens proteins of interest, indicating their phosphorylation in lens fiber cells. Most of the label in the *M*, 55,000 class of buffer-washed membranes, which focused at a rather low pI, was noteworthy (Fig. 8*B*). When the isoelectric focusing step was performed with a more acidic range of ampholytes, these species were resolved into several focusing variants with pI's of 5.2–5.4 and apparent molecular weights of 54,000. Analysis of urea washed membranes showed that these species were largely absent (not shown). Thus, these species which have been phosphorylated in the tissue resemble lens vimentin,

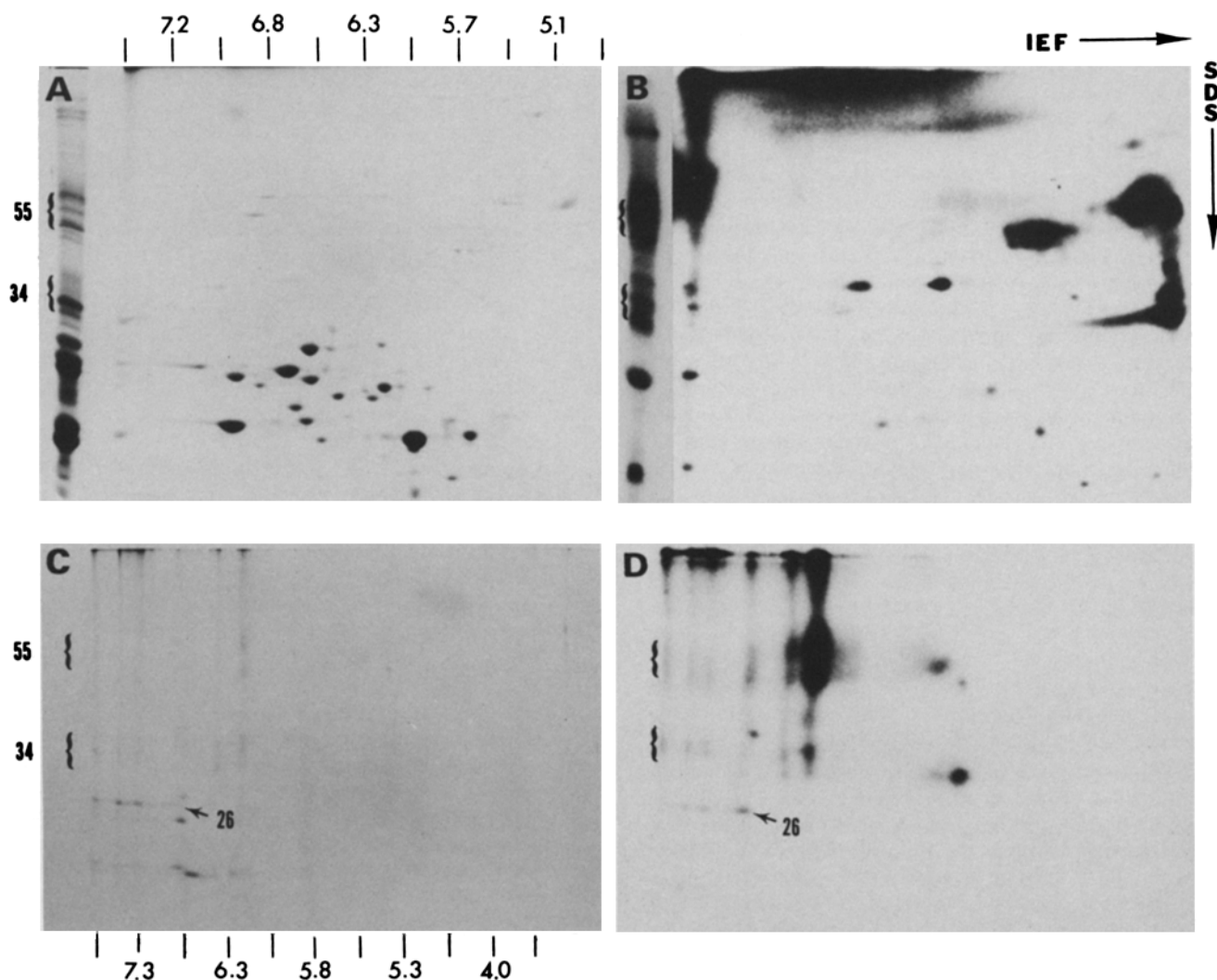


Figure 8. Two-dimensional analysis of proteins after a 120-min *in vivo* labeling. Buffer-washed membranes (*A* and *B*) and urea-washed membranes (*C* and *D*) were run on two-dimensional gels. The buffer washed membranes were separated using conventional isoelectric focusing in the first dimension (51). The urea-washed membranes were analyzed using buffer focusing in the presence of zwitterionic and nonionic detergents as the first dimension (see reference 56). The Coomassie-stained gels (*A* and *C*) and corresponding autoradiograms (*B* and *D*, both 24-h exposures with screen) are shown. The arrows indicate the most acidic of several *M*, 26,000 forms; this form has the highest specific activity. Since all of these forms bind a monoclonal antibody for MP26 (63), they are considered charge variants of MP26.

which has also been reported to label in the isolated form (68). That is, lens vimentin is membrane associated and extracted with urea (20), and bovine lens vimentin migrates at M_r 54,000 and exhibits several focusing variants with pI 's 5.36–5.50 (42). Vimentin from other tissue sources has been shown to exhibit focusing variants, due in part to different phosphorylated forms. Both cAMP-dependent (50) and calcium/calmodulin-dependent protein kinases (67) have been shown to phosphorylate vimentin from these other tissues.

Discussion

To show that the phosphorylation of a particular protein is a physiologically relevant event, one must demonstrate the phosphorylation of this protein in the cell. In this report, we provide strong evidence for the phosphorylation of MP26 within the bovine lens fiber cell. Two separate lines of evidence indicate MP26 is a phosphoprotein: MP26 was phosphorylated in our *in vivo* phosphorylation system, and in our buffer-focusing system MP26 migrated as three isoelectric variants, probably indicating different degrees of phosphorylation. The observation of MP26 phosphorylation is consistent with two preliminary reports describing the phosphorylation of liver junctional proteins (62, 76). However, MP28 has been reported not to label in the chicken lens (30).

We chose to examine the possible phosphorylation of MP26 in the lens for various reasons. First, the *in vitro* phosphorylation of MP26 by isolated protein kinases has been demonstrated (19, 33, 44). Second, using a monoclonal antibody for this major membrane protein, we recently gained strong support for the localization of MP26 within lens junctions (63). This was consistent with an earlier study (8). The lens junctions, resembling gap junctions seen in other tissues, are the structures most likely to contain (35, 53; however, also see reference 52) the cell-to-cell channels observed in the lens (58, 65). Third, the sequence of MP26 is known (23) and an understanding of it will contribute to interpretations of phosphorylation effects. Fourth, the channel properties of lens membrane components are now being evaluated in artificial membrane systems and offer an opportunity to analyze the effect of phosphorylation on the nature of these channels (21, 24).

The case for the *in vivo* phosphorylation of bovine MP26, in contrast to another M_r 26,000 species, was made by showing that the label resisted extraction with urea and detergents, was heat aggregated along with MP26, and was associated with an M_r 24,000 fragment of MP26. Two-dimensional gels confirmed that this labeled fragment, which was generated with V8 protease, was derived from MP26 (Fig. 2).

For the interpretation that MP26 is a phosphoprotein, the generation of the labeled M_r 24,000 fragment from MP26 is a critical observation. A number of published studies provide impressive support for a precursor-product relationship between these two species. It is known that proteases cleave overlapping regions of MP26, with the end result being an M_r 22,000 fragment (36). This fragment, which displays sequence homology with MP26 (70), binds some monoclonal antibodies that also recognize MP26 (64). Since the M_r 22,000 tryptic species lacks only five NH_2 -terminal residues, the remaining residues (~35 residues) must be lost from the COOH-terminus of MP26. Because V8 protease removes overlapping residues (36), most of these residues must also come from the COOH-

terminus. Consistent with this, a monoclonal antibody specific for the COOH-terminus of MP26 fails to bind to the M_r 24,000 fragment, as well as to that of M_r 22,000 (63). However, a monoclonal antibody specific for a site in the middle of MP26 binds both of these fragments (64).

Due to the labeling of the M_r 24,000 species (Figs. 1 and 2) and the lack of labeling at 22,000 (Fig. 1), most of the phosphorylation sites on MP26 can be mapped. These sites close to the COOH-terminus can also be localized to the cytoplasmic side of the membrane, based on antibody labeling at the electron microscope level (63).

Although the experiments reported here were simply described as indicative of phosphorylation in the lens, the organization of the lens permits a more refined localization of this activity. There are basically two cell types found in the lens, with the lens fiber cells being derived from a population of lens epithelial cells, which forms a single-cell layer on the anterior surface of the lens (54). Partially differentiated fiber cells, where MP26 is first found in detectable quantities, are found around the cortex of the lens, whereas samples derived from the core are strictly well-developed fiber cells. Since we detected phosphorylation of MP26 in both cortical and core samples (Fig. 3), phosphorylation does not appear to be restricted, to differentiating cells, for example. However, we often observed significantly more phosphorylation in membrane preparations derived from the labeling of cortical fragments (Fig. 3, compare lanes 1 and 2 with lanes 3 and 4). The increased phosphorylation levels, possibly related to synthesis and/or differentiation, were noted with MP26 as well as other components. These interesting questions require a more detailed analysis.

With the indication that MP26 is phosphorylated in the lens fiber cell, it will be important to determine which protein kinase or kinases are involved in the phosphorylation process. We have pursued the cAMP-dependent protein kinase system because a good case can be made for its involvement: (a) a Type 1 cAMP-dependent kinase is found in the lens (33); (b) this kinase phosphorylates MP26 in isolated membranes in the same region (Fig. 4) as labeled in lens tissue fragments (Fig. 1); (c) this labeled region, indicated by the difference in the M_r 22,000 and 24,000 proteolytic fragments, contains four serines and no threonines (23); (d) two of these serines in particular (229 and 235) occur in short amino acid sequences that resemble those recognized by cAMP-dependent protein kinases (73); and (e) cAMP has been reported to have both short-term (13, 40) and long-term (5, 37, 58) effects on junctional properties. Phosphoamino acid analysis further supports the involvement of the cAMP-dependent system. Not only did *in vitro* phosphorylation of MP26 in isolated membranes by this protein kinase yield only phosphoserines (19, 39), but in the *in vivo* phosphorylation reported here 90–95% of the label was found in phosphoserine. Moreover, studies of the modulation of MP26 phosphorylation are instructive. They showed a decreased labeling of MP26, apparently in conjunction with a reduced cytoplasmic level of calcium. This is consistent with work demonstrating that lens adenylate cyclase is stimulated by calcium in a calmodulin-dependent fashion (29). In addition, work with concentrated lens homogenates showed an increased labeling of MP26 with forskolin that was calcium dependent.

The existing data also suggest that protein kinase C may be involved in the phosphorylation of MP26. This could be in

addition to, or instead of, the cAMP-dependent system. It is known that (a) protein kinase C activity is found in the lens (39), (b) MP26 is phosphorylated in isolated membranes by this kinase (39), (c) most of this label on MP26 is found between residues 220 and 240, as with the cAMP kinase labeling, and (d) in vitro phosphorylations result in 75% of the MP26 label in phosphoserine and 25% in phosphothreonine (39). This could help explain the observation that 5–10% of the in vivo MP26 labeling is in phosphothreonine (Fig. 5). The label could be found on the threonines within the 12 most COOH-terminal residues of MP26 or on other threonines, for example, position 72 on a proposed cytoplasmic loop (23). In terms of serine labeling, serine 240 is found in a Gly-Ser-Arg sequence, which has been shown using synthetic peptides (16) to be labeled by protein kinase C.

The involvement of protein kinase C could also be reflected by the calcium effects on the modulation of in vivo MP26 labeling. Furthermore, the differential effect of calcium on MP26 labeling, with respect to other proteins, could represent the function of multiple kinases in the lens. Not only are the data consistent with protein kinase C and cAMP-dependent phosphorylation, but calmodulin-dependent protein kinases could also be involved. The dual regulation of cell-to-cell channels by cAMP-dependent kinases and protein kinase C has been suggested previously (18), based on studies with activators of these kinase systems (14, 17, 79). Future studies with such activators will be necessary to more clearly define the roles of different kinases within the lens.

The demonstration of MP26 phosphorylation emphasizes the need for exploring phosphorylation-dephosphorylation effects as one considers the regulation of cell junctions in different types of normal cells. In fact, these issues may also be important in altered cell systems. With infection by Rous sarcoma virus, for example, changes in junctional permeabilities have been noted (3, 4). In this case, phosphorylation by the transforming protein of the virus may be involved. Clearly, based on this work and the studies noted above (13, 40, 55, 57, 62, 76), the influence of phosphorylation on the permeability of cell-to-cell channels must be thoroughly addressed.

We are grateful to Richard Shope for supplying fetal calf eyes, Vicky Iwanij for helpful discussions, Michael Atkinson for beneficial comments on this manuscript, and Kae Ebling for typing the manuscript.

This work was supported by a National Institutes of Health (NIH) predoctoral traineeship to K. R. Johnson, American Cancer Society postdoctoral fellowship PF-2517 to P. D. Lampe, NIH grant EY05684 to C. F. Louis, and NIH grant CA28548 to R. G. Johnson.

Received for publication 3 June 1985, and in revised form 2 January 1986.

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