

# Immunolocalization of MP70 in Lens Fiber 16–17-nm Intercellular Junctions

W. T. M. Gruijters,\* J. Kistler,\* S. Bullivant,\* and D. A. Goodenough‡

\*Department of Cell Biology, University of Auckland, Auckland, New Zealand;

‡Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, Massachusetts 02115

**Abstract.** Thin section electron microscopy reveals two different types of membrane interactions between the fiber cells of bovine lens. Monoclonal antibodies against lens membrane protein MP70 (Kistler et al., 1985, *J. Cell Biol.*, 101:28–35) bound exclusively to the 16–17-nm intercellular junctions. MP70 localization was most dramatic in the lens outer cortex and strongly reduced deeper in the lens. In contrast, the 12-nm

double membrane structures and single membranes were consistently unlabeled. In freeze–fracture replicas with adherent cortical fiber membranes, MP70 was immunolocalized in the junctional plaques which closely resemble the gap junctions in other tissues. MP70 is thus likely to be associated with intercellular communication in the lens.

LENS fiber cells in the mammalian eye are joined in extensive membrane junctional domains. In spite of their similar pentalaminar appearance in thin section electron microscopy, two classes of double membrane structures can be distinguished based on their different widths of 16–17-nm and 12-nm, respectively (14, 15, 18, 19). The first have generally been referred to as 16–17-nm lens fiber junctions, lens fiber intercellular junctions, or lens fiber gap junctions. These 16–17-nm intercellular junctions occur throughout the lens but are most frequent in the lens outer cortex where metabolic cooperation and reversible electrical coupling and uncoupling have been demonstrated (8, 16). By analogy with the gap junctions in other tissues, lens 16–17-nm junctions are thought to contain transmembrane pores and allow the passage of small molecules between lens fibers (7). Although not previously experimentally demonstrated, 16–17-nm junctions are most likely identical with the frequent junctional plaques of high particle density revealed by freeze–fracture electron microscopy of lens cortical tissue. These intramembrane particles have a central depression strongly suggestive of a transmembrane pore (13).

The other class consists of double membrane structures measuring 12 nm in width. These are rare in the lens cortex but abundant deeper in the lens (14). Their origin and function is uncertain.

The protein composition of the 16–17-nm lens fiber junctions has not yet been finally established. Although the major intrinsic polypeptide (MIP)<sup>1</sup> (apparent molecular weight 26,000) is widely believed to be a junctional component (1, 2, 4, 17, 18), MIP has also been localized in nonjunctional membranes and appears to be ubiquitous in fiber plasma

membranes (5, 15). Another lens fiber membrane protein MP70 (apparent molecular weight 70,000) has been localized in macular double membrane domains; however, MP70 could not be firmly assigned to either the 16–17-nm intercellular junctions or to the 12-nm double membrane structures (10).

Using monoclonal anti-MP70 antibodies and immunoelectron microscopy we now report new data on the distribution of MP70 both in isolated fiber plasma membranes and in membranes in situ. Anti-MP70 antibodies bound exclusively to lens fiber 16–17-nm intercellular junctions. They did not bind to 12-nm double membrane structures and did not bind to single membranes. Freeze–fracture replica immunogold labeling (FRIL) of lens fiber plasma membranes showed MP70 to be a component of the junctional plaques in the outer cortex. MP70 is thus likely to be associated with intercellular communication in the lens.

## Materials and Methods

### Immunoreagents

Anti-MP70 antibodies were drawn from a collection of monoclonal antibodies directed against sheep lens fiber plasma membranes (11). They are secreted in cultures of hybridoma line 6-4-B2-C6, have been subtyped as IgM, and their binding pattern to lens membranes has been characterized (10). On immunoblots of SDS PAGE separated lens membrane proteins, this antibody recognized MP70 and its somewhat variable 64,000-mol-wt cleavage product. We will refer to this antibody specificity simply as anti-MP70.

Immunogold was Auroprobe EM GAMIgM G5 from Janssen Pharmaceutica (Beerse, Belgium).

### Immunoelectron Microscopy of Isolated Membranes

Membrane isolation and immunocytochemistry were carried out on bovine lens membranes according to the procedures described by Paul and Goodenough (15). For the cortex/nucleus membrane separation, bovine eyes were chilled on ice until cold cataracts formed. The lenses were removed and the

1. *Abbreviations used in this paper:* FRIL, freeze–fracture replica immunogold labeling; MIP, major intrinsic polypeptide.

outer cortical layers of the lenses manually dissected free of the opaque nucleus. The cortex and nucleus were then handled separately, but identically, through the remainder of the protocol. Anti-MP70 antibody binding to the isolated membranes was detected with undiluted immunogold, which had been preabsorbed by three successive incubations with 100  $\mu$ g isolated whole lens membranes. The absorption was carried out as follows. 100  $\mu$ g of lens membranes were centrifuged to a pellet by centrifugation at 7,000 g for 10 min. The membrane pellet was resuspended in 200  $\mu$ l of concentrated Auroprobe with trituration, allowed to stand 15 min at room temperature, and was then recentrifuged at 7,000 g 10 min. The supernatant was carefully removed, and used to resuspend a fresh 100- $\mu$ g pellet of lens membranes. In all, three 100- $\mu$ g membrane aliquots were used to treat the Auroprobe reagent, with minimal dilution of the gold. The final membrane pellet did not appear red by eye.

### FRIL of Lens Plasma Membranes In Situ

FRIL is a novel technique designed for immunogold labeling freeze-fractured and replicated lens fiber plasma membranes. Lenses were extracted from the eyes of sheep generally <1-yr-old, chilled on ice during transport, and frozen at  $-90^{\circ}\text{C}$  1-3 h after animal death. Lenses were stored at this temperature for several months. For FRIL, a lens was thawed, decapsulated, and the anterior face touched briefly onto a dry piece of filter paper in order to remove epithelial cells and to rupture peripheral lens fibers. The lens was gently pressed with the same side down into a drop of glycerol on a piece of glass slide cleaned with hot chromic acid shortly before use. The lens attached to the glass was quick-frozen in nitrogen slush and transferred into liquid nitrogen. The lens was fractured off the glass leaving one or more membrane layers adsorbed to the glass surface. The glass slide was mounted in a large tunnel cold block while still under liquid nitrogen and platinum/carbon shadow casting from  $45^{\circ}$  and carbon evaporation from  $90^{\circ}$  was carried out according to Bullivant (3). The replica was not cleaned in the usual way but was floated off the glass onto PBS such that membranes remained attached to the replica. Transfer with a platinum loop onto hybridoma supernatant was followed by a 1-h incubation period, during which anti-MP70 antibodies could bind to their epitopes on the unfractured non-coated side of the membranes. Unbound antibodies were removed by transferring the replica successively onto several drops of PBS. Controls used pieces from the same replica and nonconditioned hybridoma medium. The replica was then incubated on 1/20 immunogold in PBS for 1 h, washed in PBS, and finally in water, and mounted onto collodion/carbon-coated electron microscopy grids. Specimens were examined with a Philips EM301 electron microscope operating at 80 kv with a 50- $\mu$ m objective aperture, and photographs recorded on Kodak 4489 electron microscope film or Kodak 5302 film.

## Results

### Thin Section Immunocytochemistry of Isolated Fiber Membranes

Monoclonal anti-MP70 antibodies have previously been demonstrated to bind to double membrane regions in isolated fiber plasma membranes from lens cortex (10). In this earlier study, no distinction was made between the 16-17-nm intercellular junctions and the 12-nm double membrane profiles. A more precise mapping of MP70 in isolated bovine fiber plasma membranes has now been achieved after adopting the following experimental modifications. (a) Colloidal gold conjugated with anti-mouse IgM antibodies rather than with anti-mouse general immunoglobulins was used for secondary membrane labeling. The enhanced specificity of this immunogold reagent to monoclonal anti-MP70 antibodies (which are of IgM subtype), allowed us to label immunopos-

itive membrane regions with suitably high gold densities. (b) Using the procedures of Paul and Goodenough (15) for immunoelectron microscopy, both 16-17-nm junctions and 12-nm double membrane structures were readily observed. Furthermore, both types of membrane interactions frequently occurred next to each other in the same membrane stretch.

In fiber membranes isolated from bovine lens cortex, anti-MP70 immunogold complexes consistently labeled the 16-17-nm intercellular junctions (Fig. 1 a). The extent of labeling was generally similar on both cytoplasmic faces of junctional membranes but may show considerable variability and nonuniformity along the length of any individual junction (Fig. 1, a and b). Anti-MP70 antibodies did not bind to the 12-nm double membrane structures (arrows in Fig. 1 a) and furthermore did not bind to single membrane regions.

The gold particles are seen separated from the surfaces of the lens fiber junctions by a fuzzy layer. This layer is composed of the complex of the primary and secondary antibodies, made visible by the tannic acid fixation protocol, as reported by Paul and Goodenough (15). Neither this fuzzy layer nor the associated gold particles are seen on the surfaces of control membranes treated using an experimental protocol in which the anti-MP70 is replaced with nonimmune culture supernatant (Fig. 1 c).

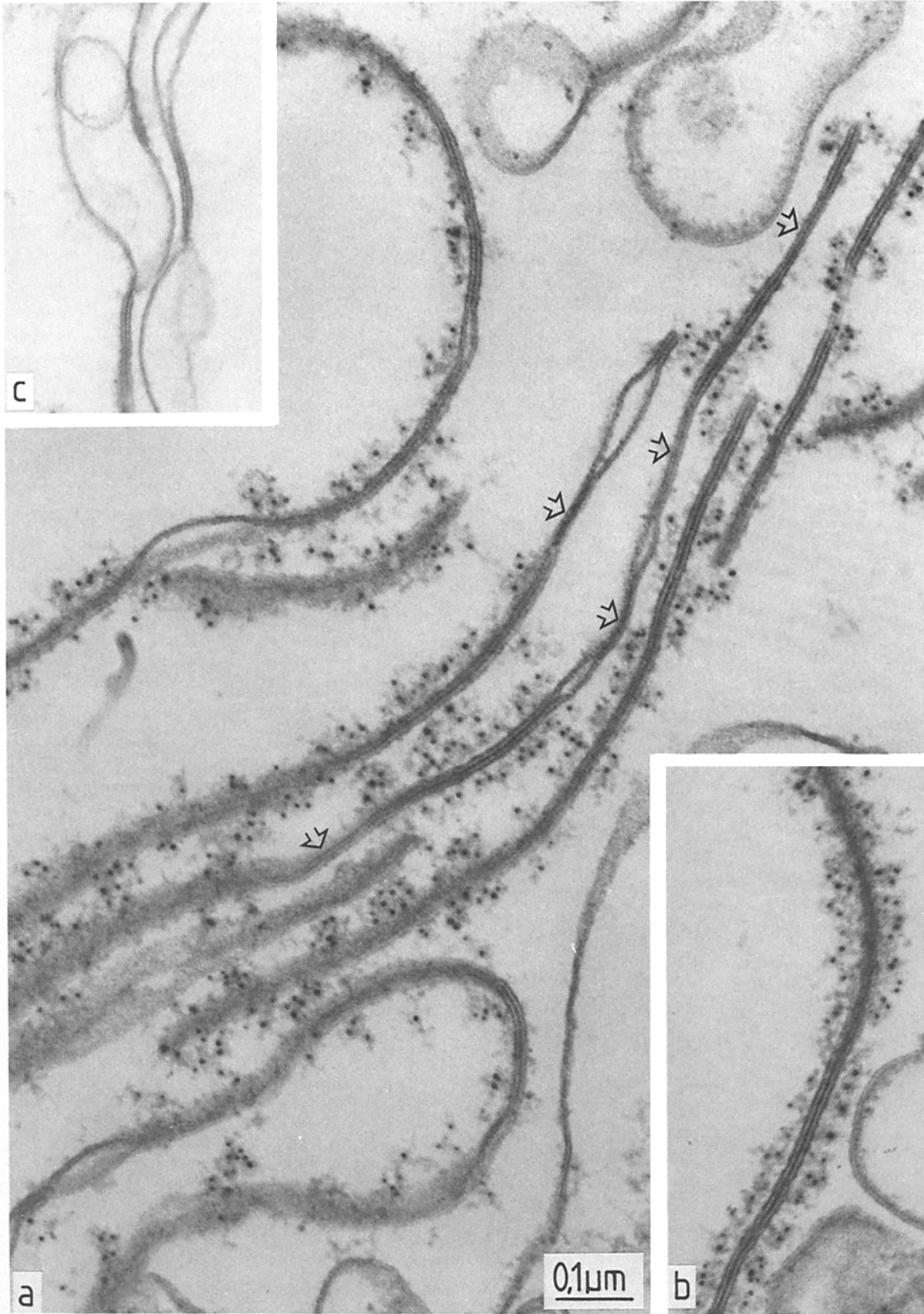
In experiments with membranes from whole lens, we have observed that not all 16-17-nm intercellular junctions labeled with anti-MP70 antibodies. When membranes were isolated separately from cortex and deeper lens regions (lens nucleus), 16-17-nm junctions were consistently labeled in the former membrane fraction but only rarely in the latter (Fig. 2, a and b). These data have not been quantitated since the cortex/nucleus dissection is difficult to control, and results in variable cross-contamination. It is noteworthy, however, that all 16-17-nm junctions in the same membrane sheet always show the same labeling pattern. We have never observed 16-17-nm intercellular junctions reactive with anti-MP70 antibodies side by side in the same fiber membrane with an unreactive junction of the same class. It is possible to find asymmetrically labeled 16-17-nm intercellular junctions. In many cases, this is probably due to inaccessibility of the immunolabeled surface to antibody gold complexes. Sometimes when there appears to be no problem with accessibility, 16-17-nm junctions are labeled asymmetrically (Fig. 2 c) and this may reflect the true situation in these cases.

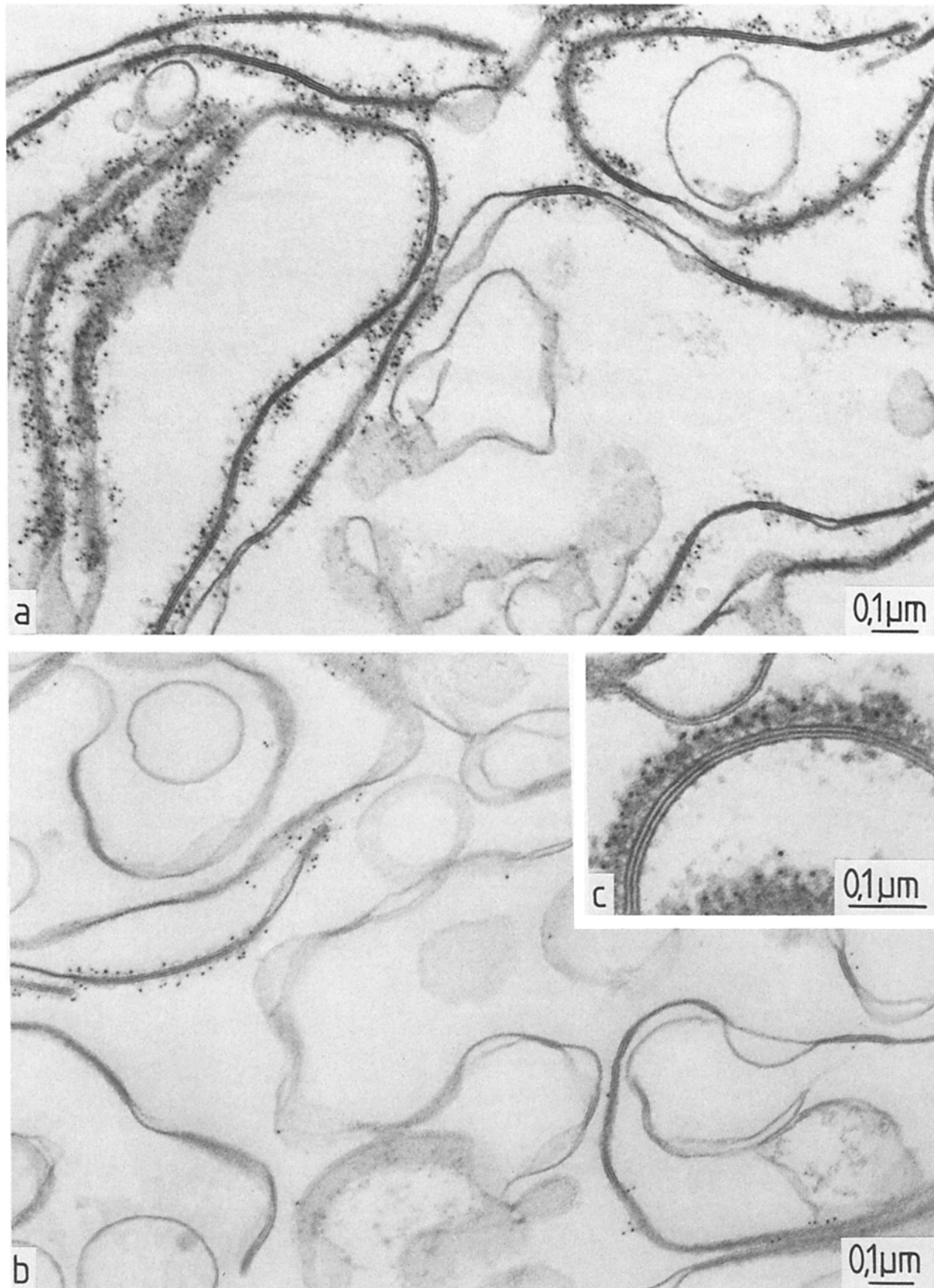
In summary, MP70 localization is most dramatic in the outer lens region and strongly reduced deeper in the lens. 16-17-nm intercellular junctions and 12-nm double membrane structures are immunologically distinct and MP70 is a component of the former.

### Localization of MP70 in Junctional Plaques

16-17-nm intercellular junctions are most abundant in thin sections of membranes isolated from lens outer cortex. Fiber plasma membranes of the same lens region studied in situ by

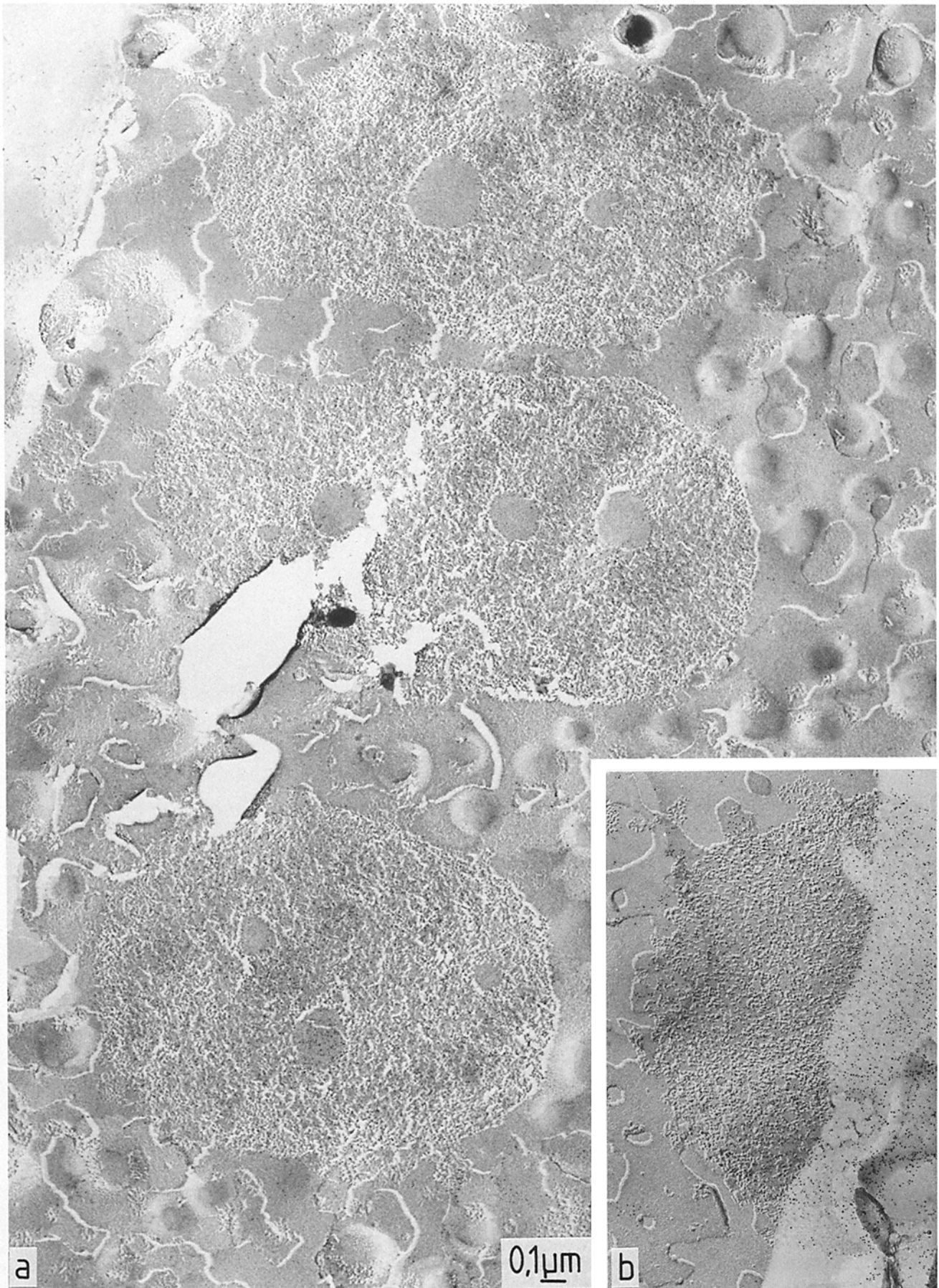
*Figure 1.* Anti-MP70 immunogold labeling of fiber plasma membranes isolated from bovine lens cortex. (a) 16-17-nm fiber junctions are extensively decorated with immunogold complexes. Anti-MP70 antibodies do not bind to the 12-nm double membrane profiles (open arrows) and do not bind to single membranes. (b) The labeling density with anti-MP70 immunogold is often similar on both cytoplasmic sides of cortical 16-17-nm junctions. (c) Control using nonconditioned hybridoma medium.



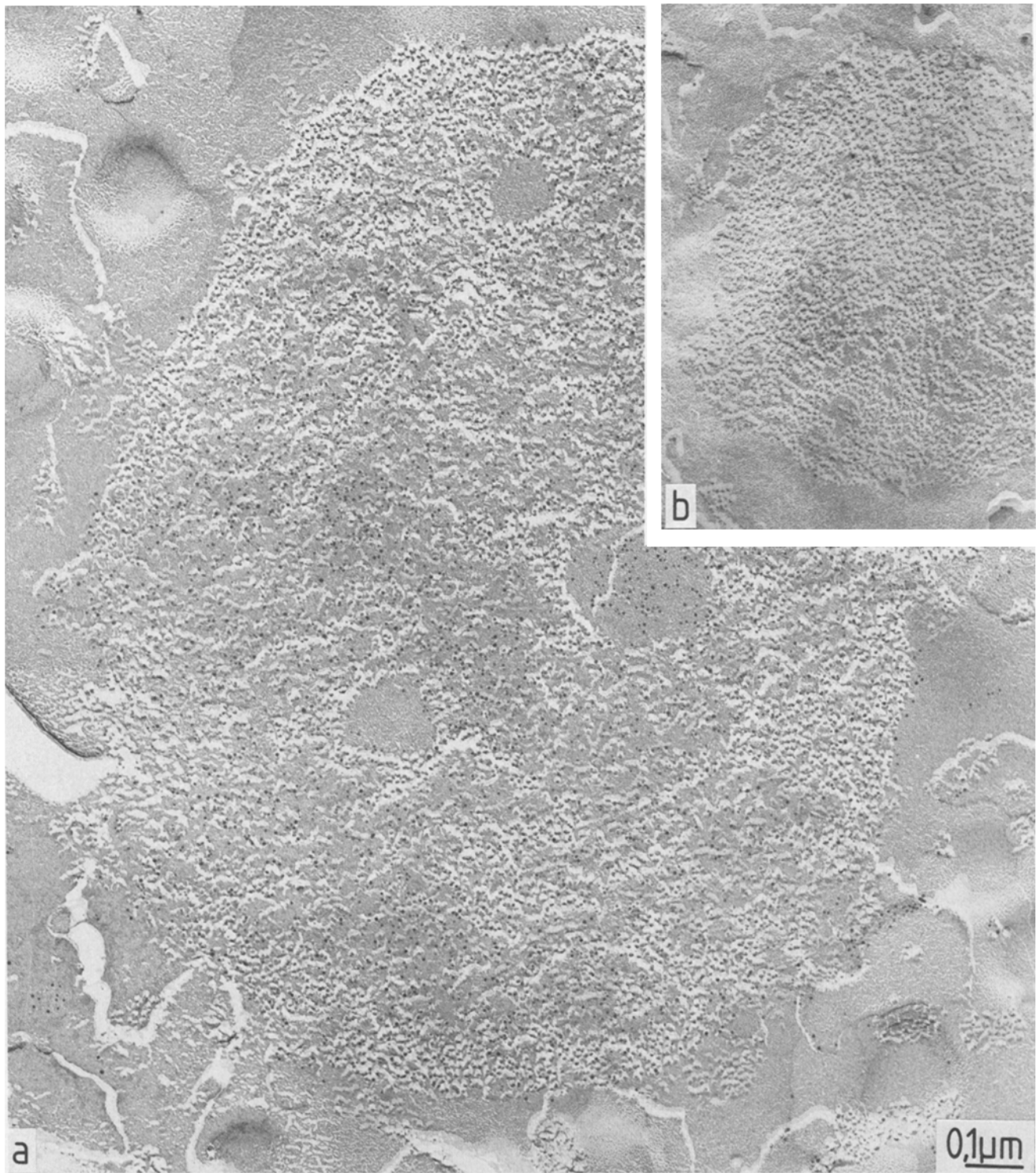


**Figure 2.** Comparative anti-MP70 immunocytochemistry of fiber plasma membranes isolated separately from bovine cortex and nucleus. (a) Anti-MP70 immunogold complexes bind abundantly to the cortical 16–17-nm intercellular junctions. (b) In the nucleus, binding of anti-MP70 antibodies is reduced and not all 16–17-nm junctions are labeled. (c) Nuclear 16–17-nm junctions appear occasionally asymmetrically labeled. Colloidal gold in the lower portion of this inset indicates that the unlabeled junctional face was accessible to antibodies.





**Figure 3.** Anti-MP70 FRIL of sheep lens outer cortex membranes. (a) Overview of three MP70 immunogold-labeled junctional plaques. The junction in the lower half is reproduced at higher magnification in Fig. 4. (b) Junctional plaque only partially shadowed with platinum clearly shows anti-MP70 immunogold labeling in the uncontrasted portion.



**Figure 4.** FRIL of sheep lens outer cortex membranes. (a) Higher magnification of the junctional plaque in the bottom half of Fig. 3a. Anti-MP70 immunogold particles are best visualized in junctional areas with smooth E-face aspect (see technical note in text). (b) Control with nonconditioned hybridoma medium; gold beads are rare in these replicas.

freeze-fracture electron microscopy also reveal frequent membrane contacts as plaque-like areas of high particle density reminiscent of gap junctions in other tissues (5, 7, 12). It is likely that 16–17-nm junctions in thin-sectioned lens material and junctional plaques in freeze-fractured fiber membranes are different views of the identical structure. To date, however, this had not been demonstrated experimentally.

The different aspects of thin sectioned and freeze-fractured 16–17-nm junctions can be reconciled using a novel immunocytochemical technique, FRIL. The basis of the technique is as follows. (a) One membrane half-leaflet, or a complete membrane plus a half-leaflet from the lens outer cortex attached to a glass slide can be fractured away from the bulk of the lens. (b) Membrane fracture faces adhered

firmly to the platinum/carbon replica when floated off the glass slide and protein epitopes on the unfractured, non-coated side of the membranes were thus accessible to immunochemical labeling.

Replicas of fiber broad sides were obtained which were suitable for immunogold labeling over areas often as large as several 1,000  $\mu\text{m}^2$ . Junctional plaques were visible as macular patches of particulate texture and were similar in size, structure, and arrangement to those described previously (5, 12). Three characteristic junctional plaques from a large number on a FRIL-prepared stretch of fiber broad sides are shown in Fig. 3 *a*. Anti-MP70 immunogold complexes bound consistently to these junctional plaques (Figs. 3 *a* and 4 *a*). In these plaques, 5-nm gold beads can readily be identified in regions of smooth E-face aspect and are more difficult to see though still plentiful among intramembrane particles. The positive labeling is clearly visible in the junctional portion not contrasted with platinum due to obstruction by a larger object nearby (Fig. 3 *b*). Using nonconditioned hybridoma medium instead of supernatant from line 6-4-B2-C6 for FRIL, gold beads were rarely found in the replicas (Fig. 4 *b*).

The FRIL technique has the advantage that it permits immunocytochemistry of junctional plaques and at the same time reveals their in situ arrangement in lens fiber membranes unaffected by distortions potentially associated with membrane isolation procedures. It should be noted, however, that the FRIL technique in this case necessitates slow freezing of unfixed tissue. Three phenomena appear to be associated with the resulting freezing damage. (*a*) Intramembrane particles outside the junctional plaques tend to aggregate. These aggregates are always small, have generally irregular outlines, and are therefore distinct from the large, oval-shaped junctional plaques (Fig. 3 *a*). They appear to be of nonjunctional origin, and consistent with this, are not labeled with anti-MP70 immunogold. (*b*) E-fracture faces are poorly differentiated from P-fracture faces, and E-face pits generally are not visible. (*c*) Smooth domains of particle-free fracture face are evident in the junctional plaques (Figs. 3 and 4), which generally are not labeled with anti-MP70. However, occasional smooth domains ( $\sim 10\%$ ) are seen to be labeled (see domain in Fig. 4 *a* which is partially intersected by the lower left corner of Fig. 4 *b*). The meaning of this labeling is not currently understood, and cannot be interpreted due to the quality of the freeze-fracture replicas.

The result that MP70 has been localized specifically in the 16–17-nm intercellular junctions in thin sections and in the junctional plaques in freeze-fracture replicas demonstrates that these structures are different aspects of the same membrane interaction.

## Discussion

A network of transmembrane pores maintains metabolic and electrical coupling between the fibers of the avascular vertebrate lens. Membrane junctions as potential sites for intercellular communication can readily be observed in thin sectioned material, however the presence of two junctional classes, occasionally in the same membrane sheet, is confusing. Both 16–17-nm and 12-nm double membrane structures have been labeled with monoclonal anti-MIP antibodies and it has been suggested that the two structures are related (18). In contrast, polyclonal anti-MIP antibodies bound to only

one side of 12-nm double membrane profiles and did not bind to the 16–17-nm junctions (15). This result, together with our finding that only the 16–17-nm junctions are labeled with anti-MP70, would indicate that the two membrane interactions are distinct from each other and in fact are due to different membrane proteins.

MP70 is a component of the 16–17-nm junctions which in freeze-fractured fiber plasma membranes have now been unequivocally identified as plaques of clustered intramembrane particles. The indication of a central pore in these particles (13) together with the great similarity between 16–17-nm membrane contacts in lens and gap junctions in other tissues suggest strongly that they are communicating junctions.

In deeper lens regions there are apparently intact 16–17-nm junctions that fail to bind anti-MP70 antibodies. Proteolysis of MP70 and other membrane proteins has previously been observed associated with lens fiber maturation and aging (11). We have preliminary evidence that junctional MP70 is cleaved to MP38 deeper in the lens, and that this cleavage leads to the loss of the epitope for monoclonal anti-MP70. No gross change in junction thickness appears to accompany this cleavage (still 16–17-nm, Fig. 2 *b*). Although it is presently unknown whether MP70 itself forms transmembrane channels, three pieces of evidence suggest that membrane permeability suddenly changes at a transition between outer and deeper cortex. (*a*) Bathing frog lenses in a solution containing 2,4-dinitrophenol caused electrical uncoupling in the peripheral cortex but had no measurable effect deeper in the lens (16). (*b*) In the galactosaemic rat lens (osmotic cataract) there is an abrupt change in the extent of cataract-associated tissue disruption at a transition  $\sim 200 \mu\text{m}$  from the lens periphery (9). This transition coincides with that observed after anti-MP70 immunofluorescence labeling (10). (*c*) Sodium-23 magnetic resonance imaging of the eye lens suggests that nucleus and cortex have significantly different ion concentrations (6). A functional assay must now be developed in order to relate particular protein(s) and permeability in the lens.

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