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Distribution of Gap Junctions and Square Array Junctions in the Mammalian Lens

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The morphology of membrane specializations of the cortex and nucleus of bovine lenses has been analyzed for both isolated membrane fractions and intact tissue fragments. Fractions of fiber cell membranes isolated from the outer cortex and the inner nucleus of lenses have been compared using x-ray diffraction, electron microscopy, SDS polyacrylamide gels and Western blots. Each fraction has distinctive structural characteristics. In x-ray experiments, the cortical fraction gives no sharp equatorial reflections (from the plane of the membrane), whereas the nuclear fraction gives sharp equatorial reflections which index on a square lattice of 6.6 nm. In thin-section electron micrographs, the cortical fraction is composed primarily of closed vesicles and flat membrane sheets, some of which contain pentalamellar structures similar in appearance to the 16–18 nm thick gap junctions found in other tissues. The nuclear fraction contains mostly undulating membrane pairs which often show 11–14 nm pentalamellar profiles and occasionally thicker junctions. In freeze-fracture images the cortical membranes display irregular clusters of intramembrane particles which resemble gap junctions, whereas the nuclear membranes contain numerous large square arrays with a 6.6 nm repeat and few irregular clusters or individual intramembrane particles. Images of fragments of intact lenses used in the membrane isolations give similar results; in the cortex the area covered by gap junctions is over 50 times the area covered by square lattices, whereas nuclear fiber cell membranes contain large square arrays. Thus, cortical and nuclear fiber cell membranes have quite different morphologies. In particular, the size of the square arrays of protein increases as the fiber cells mature. SDS polyacrylamide gels from cortical and nuclear fractions are similar in that they both contain MP26 as the major band. However, Western blot analysis shows increasing quantities of lower molecular weight, 25 kD and 22 kD, cleavage products as one progresses from the cortex to the nucleus. These data indicate that MP26 and/or its cleavage products form square crystalline arrays in nuclear fibers. The morphology of these arrays suggests a role for MP26 in cell-to-cell adhesion. Invest Ophthalmol Vis Sci 30:975–989, 1989

The mammalian eye lens is composed of many concentric layers of elongated cells, called fiber cells. The plasma membranes of these cells contain extensive areas of membrane junctions that appear morphologically similar to the gap junctions or communicating junctions found in other tissues.1–6 The presence of gap junctions is consistent with the observation that the fiber cells are electrically coupled7 and that protein isolated from the fiber cell membranes forms channels in planar lipid films8 and reconstituted lipid vesicles.9–12

However, a morphologically distinct membrane specialization, containing a square array of proteins in the membrane plane, has also been identified in fiber cells.1,2,13,14 Zampighi et al15 showed that the square arrays are more common than gap junctions in fiber cell membranes isolated from whole lenses, and Dunia et al16 have found extensive regions of square arrays in membranes from the lens nucleus. In addition, Lo and Kuck17 have recently observed square arrays in membranes from developing Emory mouse cataract. Lo and Harding18 and Costello et al19,20 have described several unusual properties of junctions containing the square arrays. In particular, the square arrays are often found on the undulating membranes of the tongue-and-groove interdigitations which are characteristic of the inner cortex and nucleus of the lens.18,21,22 Moreover, square crystalline regions alternate with noncrystalline regions within each membrane of an undulating pair, so that crystalline regions of one membrane are often paired with noncrystalline regions of the apposing membrane across the extracellular space.18–20 These areas, with
an asymmetrical arrangement of protein molecules in the membrane pair, are unlikely to participate in intercellular communication.\(^{18,20}\)

Several key questions remain concerning these membrane specializations. For instance, what is the relative distribution of gap junctions and square arrays in the young and old cells of the lens? How does the molecular organization of the fiber cell membranes change with aging? What are the roles of these junctions with regard to maintaining the small extracellular space found in the lens and the unique surface topography of the nuclear membranes? These questions can be addressed by comparing membranes from the cortex and nucleus of the lens. In this regard, Kistler and Bullivant\(^2\) recently analyzed by SDS gel electrophoresis membrane preparations made separately from the cortex and nucleus of sheep lenses. They found that the protein profiles from these regions have distinctive differences. That is, although the major intrinsic protein of the lens with molecular weight of 26 kD (MP26) is the most abundant membrane protein in both cortex and nucleus, they find that a lens-specific 70 kD protein (MP70) is much more prominent in the outer cortex than deeper in the lens.

In this paper, we determine for the first time the relative abundance of membrane specializations which resemble communicating junctions (in this paper referred to for convenience as gap junctions) and square array junctions from both the oldest fibers of the lens in the nucleus, and from the more recently formed fibers in the cortex. A combination of x-ray diffraction and electron microscopic analyses shows that the cortex contains primarily gap junctions and some small square arrays, whereas in the nucleus the square array is the predominant membrane specialization. That is, in the cortex gap junction plaques cover a large percentage of the membrane surface; small patches of square arrays coexist with the gap junctions, but are a minor component. On the other hand, in the nucleus few gap junctions are found, and the square arrays are large, have a characteristic curvature, and are commonly integral parts of tongue-and-groove interdigitations.\(^{18,20}\) SDS polyacrylamide gels and corresponding Western blots using anti-MP26 show that the major intrinsic membrane protein, MP26, is the predominant membrane protein in both the cortex and nucleus. In agreement with Kistler and Bullivant,\(^2\) we find that MP70 is more abundant in the cortex than in the nucleus. The relative abundance of these membrane specializations—typical gap junctions, located primarily in the cortex, and square arrays, located primarily in the nucleus—may be related to the natural aging of fiber cell membranes. Moreover, these morphological studies imply that these two structures may have different functional roles in the lens. That is, the gap junctions probably promote cell-to-cell communication, whereas the square arrays probably determine membrane curvature in tongue-and-groove interdigitations and may hold adjacent cells tightly together. The uniform packing of fiber cells is thought to be necessary for lens transparency,\(^24,25\) and the square arrays might serve to maintain an extremely narrow extracellular space. These results have been presented in abstract form.\(^27\)

**Materials and Methods**

**Membrane Isolation**

Bovine eyes were obtained from a local slaughterhouse and brought to the laboratory on ice. About 25 eyes were used in a typical preparation. Lenses were dissected from the eyes and the capsule was removed. The dimensions of a typical lens were about 19 mm by 12 mm. Each lens was then immediately divided into three parts by the following procedure. The outer soft layers of the cortex, approximately 2 mm in thickness, were removed with a scalpel. This part of the preparation is referred to as "cortex" throughout this paper. The inner hard core of the lens was obtained by removing a plug from the center of the remainder of the lens with a 7 mm cork borer, and trimming away cortical tissue from the ends of this plug. This part of the preparation, which consists of the embryonic, fetal, and a small portion of the adult nucleus, is called "nucleus" in this paper. The remaining layers, consisting of inner cortex and outer nucleus, were saved and labelled as the "intermediate" fraction. The membranes from each of these three lens fractions were then isolated by the same procedure described in Zampighi et al.\(^1\) This procedure avoids the usage of detergents or extrinsic proteases. In brief, each of the three fractions was suspended in about 100 ml of buffer A (2 mM NaHCO\(_3\), 3 mM EDTA, and 100 /\mu M phenylmethylsulfonyl fluoride, adjusted to pH 8.0 with NaOH). The preparations were homogenized by hand with a Teflon pestle, diluted to about 0.6 1 with buffer A, filtered through four layers of surgical gauze, and then passed through two layers of 50 mesh nylon. The following steps were all performed at 4°C. Each suspension was centrifuged at 2000 g for 15 min. Each of the pellets was then resuspended in buffer A, centrifuged again at 2000 g for 15 min. Each of the pellets was centrifuged in buffer B (4 mM Tris, 5 mM EDTA, 1.5 mM Na\(_2\)PO\(_4\), pH 8.0) and centrifuged at 2000 g for 20 min. In some preparations, 1 mM phenylmethylsulfonyl fluoride was added to buffer B; no differences were observed in SDS-PAGE, x-ray...
diffraction or electron microscopic results. Each pellet was resuspended in 4 M urea in buffer B and centrifuged at 17,000 g for 15 min. These pellets were suspended in 7 M urea in buffer B and centrifuged at 64,000 g for 100 min. The white portions of these pellets were resuspended in buffer B and a sucrose concentration of 47% was obtained by adding 67% sucrose in buffer B. A discontinuous density gradient of 41/25/8% sucrose, all in buffer B, was layered on top and the tubes were centrifuged at 97,000 g for 30 min. The material at the 25/41% interface was collected, diluted with solution B and centrifuged at 20,000 g for 20 min. The resulting pellets were immediately used for x-ray diffraction, electron microscopy or gel electrophoresis. Any remaining sample was resuspended in buffer B and stored at 4°C.

X-ray Diffraction

Specimens were prepared and x-ray diffraction patterns were recorded by the same procedure for the cortex, nucleus, and intermediate membrane fractions. The procedure is similar to that described by Caspar et al.15 In each experiment, 0.33 mg of membranes were centrifuged at 150,000 g for 2 hr in a BEEM polyethylene capsule (Polysciences, Inc., Warrington, PA), which was mounted in a Teflon adapter that fits at the bottom of a Beckman (Chicago, IL) SW-41 centrifuge tube. The pellets were removed from the BEEM capsule and allowed to slowly dehydrate in a desiccator maintained at 93% relative humidity with a saturated solution of zinc sulfate. After 24 hr incubation, the specimen was sealed in an x-ray capillary tube and mounted in the x-ray camera in a hollow brass block through which chilled water was circulated to keep the specimen at 6°C. A point collimation x-ray camera was used with a flat plate film cassette loaded with three sheets of Kodak DEF X-ray Film. Exposure times were usually 10 to 12 hr, with a specimen-to-film distance of 10 cm.

Electron Microscopy

Thin sections were prepared with the glutaraldehyde-tannic acid procedure described previously.15 Freeze-fracture replicas were prepared as described in Costello et al.29 Immediately after the membrane isolation was complete, small aliquots of the membrane suspension (3 mg/ml) were sandwiched between copper sheets, plunged into liquid propane and transferred to liquid nitrogen for storage. Some isolated membrane fractions were deposited on Alcian-blue-treated glass coverslips, sandwiched with a copper sheet and plunged into propane as described previously.30 From each of three isolation experiments, one intact bovine lens was set aside for a separate dissection. Small fragments were dissected from the anterior outer cortex and the anterior side of the embryonic lens nucleus. The fragments were first suspended in a drop of aqueous humor, then frozen in propane.30 Samples were fractured and replicated in either a Balzers (Hudson, NH) 360 or a Reichert-Jung (Chicago, IL) Cryofract 190 at a sample temperature of -150°C or lower. Replicas were made with platinum/carbon or, where noted, with tantalum using a new electron gun.31 Micrographs were taken with either a Philips (Mahwah, NJ) 301 or 420 electron microscope. Freeze-fracture images are mounted so that the direction of metal deposition is from the bottom of the figure.

For all regions where large membrane fractures were observed of the cortex from the intact lens, areas of gap junctions and nonjunctional membranes were measured with a planimeter (Numonics, Lansdale, PA, model 1224); areas of square arrays in the intact cortex membrane fractures were obtained from measurements of their length and breadth. In thin sections of the nuclear fraction, the lengths of identifiable junctions were measured with a planimeter.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

The same fractions of membranes isolated as described above from lens cortex, intermediate region, and nucleus were analyzed by SDS-PAGE and Western blot. The fractions were solubilized in sample buffer and separated on 12.5% SDS-PAGE according to previously published methods.32 Some gels were stained with Coomassie blue, whereas other gels were used for Western blot analysis. In the latter case, each gel, containing two identical sets of samples and standards, was divided in half. One half was stained according to the silver method of Oakley et al.33 whereas the other half was electrophotographically transferred to nitrocellulose according to the method of Towbin et al.14 for reaction with MP26 antibodies of Bok et al.35 MP26 and its higher molecular weight aggregates and cleavage products were visualized by horseradish peroxidase immunodetection according to instructions in the kit provided by Bio-Rad (Richmond, CA). The concentration of affinity-purified rabbit anti-bovine MP26 IgG used on the Western blots was 0.14 µg/ml.

Results

Figure 1 shows x-ray diffraction patterns from both cortical and nuclear membranes. The two specimens were prepared and diffraction patterns were recorded using the same experimental procedure. In both cases
The membranes were pelleted and oriented so that the planes of the membranes were horizontal. The pattern from the nuclear membranes (Fig. 1B) contains several sharp reflections (arrows) on the equator (horizontal axis) of the x-ray film, at Bragg spacings of 6.6, 3.3, 1.84, 1.57, 1.11 and 0.95 nm. These spacings index on a square lattice of 6.6 nm, and arise from a crystalline array of protein subunits in the plane of the membranes.15 Note that no such sharp reflections can be observed in the pattern from the cortical membranes (Fig. 1A). Similar results have been obtained in six separate experiments with cortical and nuclear membranes—two sets of diffraction patterns from each of three different membrane preparations. In each of these six experiments the diffraction pattern from membranes isolated from the nucleus contain the sharp equatorial reflections from the 6.6 nm square lattice, whereas these reflections are not recorded from membranes isolated from the cortex. X-ray diffraction patterns recorded from the “intermediate” fraction (data not shown) also contain sharp equatorial reflections from the 6.6 nm square lattice, although these reflections are usually not as intense as those from the nuclear membranes.

Scattering from the partial stacking of membranes and membrane junctions is observed on the meridian (vertical axis) of the x-ray film. For the cortex a sharp meridional reflection is observed at 5.4 nm (inset to Fig. 1A), and for the nucleus a broad meridional scattering band is observed which has an intensity maximum centered at 4.7 nm (inset to Fig. 1B). The amount of arcing of the meridional diffraction is nearly the same in the insets to Fig. 1A and 1B, indicating that the degree of orientation of the cortical and nuclear membranes is similar. In some patterns from the nucleus additional sharp meridional reflections are observed at 3.4 nm and 1.7 nm (the latter reflection is visible in Fig. 1B). These reflections are due to a partial phase separation of cholesterol. These cholesterol reflections can be eliminated, without affecting the equatorial 6.6 nm lattice, by adding 1% Triton before pelleting the membranes.20

Figure 2 shows electron micrographs of thin sections of membranes isolated from the cortex (Fig. 2A,
B) and nucleus (Fig. 2C, D). In both cortical and nuclear preparations, we observe single membranes (open arrows, Fig. 2A–C), and membrane junctions—pairs of closely apposed membranes (closed arrows, Fig. 2A–D). The most obvious difference in the two preparations is that the cortical preparation consists mostly of closed loops, circular profiles and occasional flat sheets, whereas the nuclear preparation contains wavy or undulating membrane profiles. In these undulating membranes, the distance between crests is usually about 0.2 to 0.5 μm and the amplitude is variable from about 40 nm to 140 nm (Fig. 2C, D). We have never observed the undulating membrane profiles in the cortex fraction. In higher magnification views, other differences can be detected between cortical (Fig. 2B) and nuclear (Fig. 2D) preparations. Many of the junctions from the cortex, particularly the flat sheets, have a quite uniform thickness of about 17 nm. In contrast, the junctions from the nucleus are more variable in thickness along their length. In some regions the junctions have a thickness of about 17 nm (large solid arrow, Fig. 2D), whereas in neighboring regions, junctions with a thickness of 13–14 nm can be found (small solid arrow, Fig. 2D). Even thinner profiles are found where the junctions have undulations; in these regions the junction thickness is only about 11 to 12 nm (open arrow, Fig. 2D). In these 11–12 nm junctions, one membrane of the pair is often significantly thinner than the other. The thinner membrane of the pair is always the membrane on the concave side of the undulation (open arrows, Fig. 2D), as noted previously. Another characteristic feature of the nuclear preparation is that the membranes of the junction often separate for short distances (long arrows, Fig. 2C) before reforming the normal pentalamellar appearance of the junction. It should be noted that in both cortex and nucleus there are regions of straight junctions (small solid arrows, Fig. 2B, D) that are symmetric, but narrower than typical gap junctions. In the thin sections of the nuclear fraction (Fig. 2C), symmetrical 16–18 nm wide pentalamellar profiles represent less than 3% of the identifiable membrane profiles, whereas undulating membranes represent about 70% of the membrane profiles.

The asymmetry of the thin junctional profiles in the undulating membranes is readily documented in microdensitometer traces of high magnification images (Fig. 3). The traces in Figure 3G compare the thick symmetric junctions (Fig. 3A, numbers 1 and 2) to thin asymmetric junctions (Fig. 3A, B, numbers 3–5). The images in Figure 3 also reinforce the observation noted previously that the thicker membrane of an undulating membrane pair is always on the convex side of the curve. These membrane pairs in wavy junctions retain their asymmetric appearance when tilted in the electron microscope (data not shown). As one moves along either membrane of the pair, the transition from thick to thin membrane can be gradual (eg, see Fig. 3A, F) or abrupt (arrowheads, Fig. 3C, E), and usually occurs where there is a change in curvature (Fig. 3B–E).

Freeze-fracture results for the cortex and nuclear fractions are shown in Figure 4. A low power view of the cortical membranes (Fig. 4A) shows vesicles of various sizes, with smaller vesicles often enclosed within larger ones. The distribution of particles on membrane fracture surfaces is variable. Often particles form small clusters (arrowheads, Fig. 4A). At higher magnification, in Figure 4B, several types of particle clusters are presented. Dense, irregular clusters of 8–9 nm diameter intramembrane particles in the upper vesicle (arrowheads, Fig. 4B) are probably gap junctions, even though the step to the second membrane is not observed. The cluster of particles in the vesicle to the lower left (open arrow, Fig. 4B) is clearly a gap junction because the fracture step to the apposing EF pitted membrane (thin arrow, Fig. 4B) is present. Small clusters of orthogonally arranged, 8–9 nm intramembrane particles are also present in the cortex fraction (arrows on lower right vesicle in Fig. 4B) and these may represent initial stages of square array formation (see below). In preparations of nuclear membranes, fractures through undulating junctions are observed (Fig. 4C, D). One cluster of intramembrane particles which might be a gap junction is indicated in Figure 4C (open arrow). The edge of one square array is seen in Figure 4D (arrow and inset). As described in the Discussion section, Lo and Harding and Costello et al have explained the paucity of square arrays and predominance of smooth membrane fracture faces in terms of an unusual arrangement of protein molecules in the apposing membranes of the undulating junctions. However, when these same nuclear membranes are anchored to a flat glass support prior to freezing, freeze-fracture replicas reveal large areas of crystalline square arrays with a unit cell size of 6.6 nm; two crystalline areas in Figure 4E contain about 500–700 unit cells each.

Figure 5 shows freeze-fracture images from intact bovine lenses. Membranes from intact cortex contain extensive regions of intramembrane particle clusters on PF fracture faces and well defined steps to the EF pitted membranes that characterize gap junctions (Fig. 5A). Small patches of square arrays (Fig. 5, arrows) are present in the same fracture plane. The gap junctions are characterized by randomly packed arrays of large (9 nm) intramembrane particles, whereas the square patches contain crystalline arrays of a 6.6 nm repeating unit. In preparations from the
intact nucleus (Fig. 5B), the cell membranes are usually undulating, and contain more extensive regions of square arrays.

The relative amounts of gap junctions and square arrays have been quantitated from replicas of intact cortex, such as shown in Figure 6. Dense irregular clusters of intramembrane particles separated from pitted membranes by a small fracture step are classified as gap junctions. Gap junctions are outlined and square arrays are enclosed by squares. It should be noted that the identification and borders of gap junctions and square arrays were confirmed using complementary replicas. For nine large in-plane fractures, the areas and numbers of intramembrane particles for gap junctions, square arrays and nonjunctonal membranes are given in Table 1. About 12% of the total fractured membrane is occupied by gap junctions, compared to less than 1% for
Fig. 4. Freeze-fracture of isolated membranes. (A) Cortex fraction showing single membranes (solid arrows) and small vesicles (open arrow) inside a larger vesicle. Clusters of particles are indicated (arrowheads) some of which may be gap junctions. (B) Composite of three representative vesicles at higher magnification. Clusters of particles (arrowheads on upper vesicle) are commonly seen. Sometimes well defined gap junctions (open arrow on lower left vesicle) are observed which display the characteristic step between pitted and particulated surfaces. The thick solid arrow is a step from ice to one membrane and thin arrow is a step across the extracellular space gap. Small patches of orthogonally arranged particles (arrows on lower right vesicle) are seen occasionally. (C) Nuclear fraction showing an undulating membrane pair. (D) Undulating nuclear membrane pair fractured edge-on and in-plane. One small patch of square array is marked (arrow and inset at ×150,000). (E) Nuclear membranes flattened on glass before fracturing often show large patches of square array (arrows along orthogonal axes). A, B and E are tantalum replicas prepared with the Cryofract 190. A, C and D ×55,000; B, ×155,000; E, ×145,000.
square arrays. Of the total number of intramembrane particles observed, about 32% are in gap junctions, 2% in square arrays and about 66% in nonjunctional membranes. Equivalent large fracture surfaces in isolated or intact nuclear membranes are difficult to find; thus, it is not possible to quantitate accurately the relative amounts of intramembrane particles in gap junctions and square arrays in nuclear membranes. However, in fractures that are observed, square arrays predominate and very few gap junctions can be found. From published freeze-fracture images of undulating membranes (eg, Figs. 2D-G in ref. 19 and Figs. 7, 8 in ref. 20), we estimate that square arrays cover more than 30% and gap junctions cover less than 2% of the membrane surface area.

Table 1. Membrane specializations of intact bovine lens cortex

<table>
<thead>
<tr>
<th></th>
<th>Number measured</th>
<th>Total area, ( \mu m^2 )</th>
<th>% Total area</th>
<th>Average size, ( \mu m^2 )</th>
<th>Approx. IMP* density, IMPs/( \mu m^2 )</th>
<th>Approx. total IMPs</th>
<th>Approx. % IMPs</th>
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<tbody>
<tr>
<td>GJ†</td>
<td>159</td>
<td>7.6</td>
<td>12.4</td>
<td>0.05</td>
<td>5000</td>
<td>38,000</td>
<td>32</td>
</tr>
<tr>
<td>SA‡</td>
<td>72</td>
<td>0.1</td>
<td>0.2</td>
<td>0.002</td>
<td>20,000</td>
<td>2000</td>
<td>2</td>
</tr>
<tr>
<td>Nonjunction</td>
<td>9</td>
<td>53.6</td>
<td>87.4</td>
<td>6.8</td>
<td>1500</td>
<td>80,400</td>
<td>66</td>
</tr>
</tbody>
</table>

* IMP = intramembrane particle.  
† GJ = gap junctions.  
‡ SA = square arrays.

SDS-PAGE and Western blot gels with anti-MP26 used to analyze cortical, intermediate and nuclear membranes are shown in Figure 7. In Coomassie blue-stained gels (Fig. 7A), the major band from all three regions of the lens has an apparent molecular weight of 26 kD (large arrow). Lower molecular bands are observed at about 22–23 kD, 21 kD, 17 kD and 16 kD (small arrows). In agreement with the results of Kistler and Bullivant,23 bands corresponding to MP70 (arrowhead) and MP64 are observed in fractions from the cortex, but not from the nucleus. In all of our gels from the cortical fractions, MP70 stained more intensely than MP64.23,26 A weak band at 24 kD, which is visible in Figure 7A lanes 3, 5 and 7, is more easily detected in the silver-stained gels in
Fig. 6. Freeze-fracture replica of intact cortex. Numerous gap junctions are circled and square arrays are enclosed by squares. Inset shows higher magnification view of region containing two square arrays (arrows) and a gap junction-like cluster of particles (circled). X55,000; inset, X110,000.

Figure 7B. In silver-stained gels (Fig. 7B, lanes 2, 3 and 4), the bands at 26 kD, 24 kD, 22–23 kD, 21 kD, 17 kD and 16 kD are present, but MP70 and MP64 are not observed, probably due to the relatively small amount of protein (300 ng) applied to these gels. A band at 25.5 kD appears as a shoulder on the 26 kD band. For all three regions of the lens, the 26 kD band is labelled with anti-MP26 (lanes 5–7); therefore this band corresponds to the major intrinsic membrane protein (MP26). The bands at 25.5 kD, 24 kD and 16 kD, which are most prominent in the nucleus, are also labelled with anti-MP26 (lanes 5–7, arrows on
GAP AND SQUARE ARRAY JUNCTIONS IN THE LENS / Cosello et al

Fig. 7. (A) Coomassie blue-stained SDS-PAGE of membrane fractions isolated from the cortical (lanes 2 and 3), intermediate (lanes 4 and 5), and nuclear (lanes 6 and 7) regions of the lens. Lanes 2, 4, and 6 were loaded with 3 μg of protein, and lanes 3, 5, and 7 were loaded with 5 μg of protein. Molecular weight standards are shown in lane 1. The arrowhead and large arrow indicate bands at 70 kD and 26 kD, respectively. The small arrows indicate bands at 22-23 kD, 21 kD, 17 kD, and 16 kD, respectively. (B) Silver-stained SDS-PAGE (lanes 1-4) and Western blot (lanes 5-7) of lens cortical (C, lanes 2 and 5), intermediate (I, lanes 3 and 6) and nuclear (N, lanes 4 and 7) membranes. Molecular weight standards are shown in lane 1. Silver-stained lanes were loaded with 300 ng each of dissolved membrane protein and electrophoretically transferred lanes were loaded with 600 ng. The predominant intrinsic membrane-derived protein in all lens regions is MP26 (large solid arrow). The major portion of the band at 22-23 kD and the entire band at 21 kD (open arrow) are α-crystallins as determined by Western blot analysis (data not shown). Fine arrows on the left indicate band at 17 kD, a recently identified membrane protein, and at 16 kD. Staining with anti-MP26 antibody (lanes 5–7) shows three cleavage products of MP26 at approximately 25.5 kD, 24 kD and 16 kD (open arrows on right). The broad band at 50 kD (arrowhead) probably represents multimers of the 24–26 kD peptides.

Discussion

The x-ray diffraction and electron microscopic results indicate that the cortex and nucleus of the bovine lens are quite different, both in terms of membrane morphology and the distribution of membrane specializations. Sharp equatorial x-ray reflections characteristic of a square array of protein molecules in the plane of the membrane are invariably observed in preparations of nuclear membranes, but are not found in corresponding preparations of cortical membranes from the same lenses. In either of these x-ray patterns (Fig. 1A or B), small crystalline arrays of proteins (containing only a few proteins) or large noncrystalline aggregates of proteins would not give detectable sharp reflections. The sharp equatorial reflections in Figure 1B are produced by large crystalline arrays of proteins. Thus, the x-ray results indicate that the large square arrays are found primarily in the nucleus of the lens. The freeze-fracture results are consistent with this. That is, large square arrays have been observed in isolated or intact nuclear membranes, but not in isolated or intact outer cortical membranes. Although there are large regions of membrane junctions in the cortex, these consist primarily of gap junctions (thick symmetric membrane pairs) containing randomly packed intramembrane particles. Square arrays can be observed in the fracture planes of intact cortical membranes, but they are small in number and size compared to the normal gap junctions (Figs. 5, 6 and Table 1). It should be noted that the amount of gap junction in these intact bovine membranes is comparable to the amount in frog, 12%, and rat, 32%. Similar observations have
been made in freeze-fracture images of isolated cortical membranes (Fig. 4A, B), but, since large in-plane fractures are rarely seen, the relative abundance of junctional types is difficult to quantitate. In freeze-fracture images of membranes isolated from the nucleus, gap junctions are seen infrequently (see Fig. 4C), whereas square arrays are often observed, particularly in membranes flattened on glass before fracturing (Fig. 4E).

It should be noted that Brown et al. have reported that the distribution of gap junctions varies along the length of intermediate cortical fibers with the highest density for frog and rat fibers appearing near the equator and the lowest density near the poles. The distribution of junctions cannot be determined in our isolated membrane fractions because concentric shells were examined. The density of gap junctions (about 12%) was determined for intact bovine lenses at one location near to the anterior pole (see Fig. 6 and Table 1). Based on the observations of Brown et al., the density of gap junctions would be expected to be higher at the equator but the key issue of the relative proportions of gap and square array junctions along the length of fibers at different depths will depend on more detailed quantitative measurements which are in progress.

The isolated cortical membranes form circular vesicles or flat sheets (Fig. 2A, B, 4A, B), the walls of which often contain membrane junctions with an overall thickness of about 17 nm. These junctions are symmetrical when observed in cross-section (Fig. 2B, large solid arrow), and in this regard resemble gap junctions isolated from other tissues. In contrast, the nuclear membranes in general do not form circular vesicles, but rather form large wavy or undulating sheets (Figs. 2C, 3C, D). In the regions of the undulations, the junctions have an unusual appearance, described in detail by Lo and Harding and Costello et al. Specifically, Costello et al. noted that in thin sections these junctions are relatively thin and, furthermore, the membrane on the concave side of the junction is usually considerably thinner than the membrane on the convex side (Fig. 2D, open arrows, and Fig. 3). Freeze-fracture experiments show that the thin membrane on the concave side of the junction is nearly devoid of intramembrane particles, whereas the thicker membrane on the convex side of the junction contains the crystalline square array of protein molecules. This unusual distribution of protein in these undulating junctions makes the square arrays very difficult to find in replicas from typical freeze-fracture experiments, even though the x-ray experiments show that large square arrays must be abundant. That is, since the fracture plane usually goes down the center of the membrane that is devoid of intramembrane particles, the apposing membrane, which contains the square array, is not fractured and thus not observed. However, when the undulating membranes from the nucleus are made to adhere to a glass substrate, the fracture plane often proceeds along the surface of crystalline regions, so that large areas of square arrays can be observed (Fig. 4E).

In addition to the thick symmetric and thin asymmetric membrane pairs, there is a third type of junction, the 13–14 nm symmetric junction in Figure 2B and D. Because this junction is thinner and can display variable staining of the central band, it may not be a gap junction, although it can easily be mistaken for a gap junction. The thin symmetric junction is often seen among wavy membranes at points of transition in curvature but its frequency and distribution have not been documented. In addition, the composition and organization of protein (whether random or in a crystalline array) have not been characterized.

The SDS-PAGE and antibody labeling with anti-MP26 indicate that MP26 is a major membrane component in both the cortex and nucleus (Fig. 7). Similar gels and Western blots were obtained from sheep lens membranes by Kistler and Bullivant and Kistler et al. In the case of the nucleus, most of the intramembrane particles in freeze-fracture images are in square arrays. This implies that MP26 (and possibly its breakdown products) forms the square arrays.

The cortex is more complicated to analyze since the structural data (Table 1) indicate that the intramembrane particles are divided primarily between gap junctions and nonjunctional membranes, with an appreciable fraction of intramembrane particles in both. Thus from these data alone it is not possible to be certain whether MP26 or another protein forms the gap junctions. Previously published antibody labeling studies provide further information, but at present do not unambiguously answer the question of whether MP26 is part of the gap junction. Bok et al. and FitzGerald et al. reported that anti-MP26 labeled both junctional and nonjunctional membranes equally, implying that MP26 has the potential to form membrane junctions from a pool of MP26 in the membranes. Using different antibodies, Sas et al. also observed labeling of junctional and nonjunctional membranes. Moreover, they concluded that both thick (17 nm wide) and thin (12 nm wide) junctions were labeled with anti-MP26. However, using affinity-purified antibodies to MP26, Paul and Goodenough found labeling only in thin junctions and nonjunctional regions, and not at gap junctions. In addition, a 70 kD protein (MP70) has recently been localized at gap junctions, and thus MP70 is a component of gap junctions. Since it appears to be currently unresolved whether MP26 is also part of...
gap junctions, the functional role of this protein is uncertain. If MP70 is the only component of gap junctions, then MP26 does not participate in cell-to-cell communication. Alternatively, if MP26 is a part of gap junctions and does participate in intercellular communication, it would imply that MP26 contributes to the formation of two distinct membrane specializations—gap junctions and square arrays. We note that if both MP70 and MP26 compose gap junctions, then in antibody labeling experiments, the labeling of MP26 might be blocked by MP70.

There is no direct evidence to indicate why MP26 forms large square arrays in the nucleus but not in the cortex. There are, however, several possible explanations. For example, as part of the normal aging process MP26 might be slightly modified so that its packing properties are affected. The modifications may be proteolytic cleavage to produce products as seen in Figure 7 or by smaller cleavage products not detectable by SDS-PAGE. Other modifications, such as phosphorylation, are also possible, as enumerated by Takemoto et al. Alternatively, the lipid composition in the nucleus, which has higher protein-to-lipid and cholesterol-to-phospholipid ratios than the cortex, might affect the packing of this intrinsic membrane protein. There are many examples of membrane proteins assuming different packing arrangements as a function of their lipid environments, including bacteriorhodopsin, cytochrome c oxidase and mitochondrial pore protein. The last example is interesting because it was demonstrated that treatment of membranes with phospholipase A2, which removed phospholipid from membranes, induced the crystallization of the pore protein. A third possibility is that MP70, which has been shown to be localized at the gap junction, and is more abundant in the cortex than in the nucleus (ref. 23 and Fig. 7A), might interfere with the formation of square arrays of MP26 in the cortex.

The question arises as to whether gap and square array junctions have distinct functions. It seems very likely that the 16–18 nm thick symmetric junctions, which resemble gap junctions from other tissues, are involved in intercellular communication. However, in the undulating junctions many of the protein molecules do not seem to be arranged in a manner suitable for intercellular communication. In certain regions square arrays in one membrane oppose membranes essentially devoid of intramembrane particles. As noted previously, when membranes containing square arrays are adjacent, the lattices are observed not to be aligned. Moreover, in the cortex, square arrays can be found in areas where the extracellular space between apposing membranes is quite wide. Thus, in these three instances, the square array does not seem to be involved in cell-to-cell communication and, in these circumstances, the function of the square arrays is not known. Two possible roles for the square arrays can be hypothesized. First, they might simply be storage areas or “graveyards” for MP26 proteins that are either not needed for communicating junctions or have been modified by the aging process so that they no longer function in intercellular communication. Second, the square arrays seem to be an integral part of the tongue-and-groove junctions, and might serve to hold adjacent fiber cells together. The uniform packing of fiber cells is thought to be important in maintaining the transparency of the lens. As noted before, the characteristic undulations of the tongue-and-groove junction are maintained in the isolated junctions (Fig. 2C, D, 3C, D). This means that this curvature is caused by the intrinsic proteins (primarily MP26) and lipids of the junction, and not by cytoskeletal supporting proteins, which have been removed along with other cytoplasmic proteins with urea washing during the isolation procedure. Strong electrostatic forces may hold the apposing undulating membranes in close contact. Models of secondary structure of MP26 proposed by Gorin et al. and Revel and Yancy, based on the cDNA-derived amino acid sequence, suggest that MP26 has an excess positive charge on the extracellular surface. The crystalline packing of MP26 would produce a highly charged positive surface and could attract negatively charged lipids (or perhaps extrinsic proteins) in the apposing membrane, which is nearly devoid of intramembrane particles.

Key words: lens, square arrays, gap junctions, fiber cells, membranes, x-ray diffraction, electron microscopy

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Note added in proof: Since this work was submitted, Zampighi, Hall, Ehring and Simon (J Cell Biol, 1989, in press) have demonstrated using immunolocalization that MP26 (denoted by them as MIP) is found in single membranes, thin junctions (11–13 nm thick) and wavy junctions. MP70 was localized to 16–18 nm thick junctions exclusively; antibodies to MP26 were sometimes located within patches of MP70 labels. Importantly, MP26 antibodies were concentrated on the cytoplasmic convex surface in thin sections of wavy junctions and at square arrays in fracture-label images; moreover, MP26 antibodies were distributed asymmetrically across the extracellular space in wavy junctions. These findings provide evi-
dence that MP26 is the protein that forms the square arrays in the lens and that in wavy junctions membranes containing MP26 are paired across the extracellular space with membranes which are nearly devoid of MP26. These authors also propose that, in addition to the possible electrostatic attraction between arrays of MP26 and apposing bilayers, single membrane channels formed by MP26 regulate the volume of the extracellular space.

References


