Main Intrinsic Polypeptide Proteolysis and Fiber Cell Membrane Domains

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The main intrinsic polypeptide (MIP) of ocular lens fiber cells, a putative gap junctional polypeptide, has been shown to undergo a physiologic proteolytic reduction in relative molecular weight with age. Electron microscopic studies of isolated lens fiber cell membranes have revealed the existence of two distinct classes of gap junction-like membrane–membrane interactions, which differ from each other in profile thickness by about 4 nm (“thick” and “thin” fiber cell junctions). The authors report that both classes of membrane–membrane interaction are seen in situ as well. Using exogenous and endogenous proteases to mimic the molecular “aging” of the MIP, we have explored the hypothesis that one junctional class is derived from the other as the MIP is proteolytically degraded. From the results presented the authors conclude that (1) neither limited or exhaustive proteolytic degradation of the MIP substantially alters the relative percentages of the three identifiable fiber cell membrane domains (thick junctions, thin junctions, and unit membranes); (2) thick fiber cell junctions, because they possess a gap between the membrane outer leaflets, are, by definition, gap junctions; (3) thin junctions are not artifacts of tannic acid fixation; and (4) unit membranes are capable of associating in a manner that mimics thin junctions.

Biochemical studies of fiber cell membranes reveal that they are dominated by a single integral membrane protein, the main intrinsic polypeptide (MIP), with a molecular weight of slightly greater than 28 kilodaltons.1 Several lines of circumstantial evidence exist that implicate the MIP as the structural protein of the fiber cell junction,1–7 and a substantial body of work has appeared based on this assumption. However, the role of the MIP as a junctional polypeptide has not been unequivocally established, and, indeed, other possible junctional proteins have been identified.2,8–11

Comparisons of old and young human lenses, as well as the comparison of older and younger regions from a single lens have revealed that the MIP undergoes a slow physiologic reduction in relative molecular weight (MWr) with aging, from 28 kilodaltons to approximately 20–22 kilodaltons, a degradation with a half-life of about 40 yr.12–15

Early studies of isolated fiber cell membranes revealed the presence of two membrane domains: gap junctions and unit (or nonjunctional) membranes.4,16,17 More recent studies have included tannic acid in the fixation process and have resulted in the delineation of three membrane domains in vitro: unit membranes and two classes of pentalaminar profiles (both of which are gap junction-like in profile, neither of which has been reported to have a “gap,” but which differ from each other in profile thickness by about 4 nm).10,18 These two junctional classes have been referred to provisionally as “thick” and “thin” junctions. The authors report that both thick and thin junctions are seen in situ as well. Lo et al19 also have described both junctional classes in situ, and report that thin junctions are more prevalent in the nuclear region of the lens. This distribution of thick and thin junctions parallels the distribution described for the intact and proteolysed MIP, respectively, raising the possibility that thin junctions are generated in the aging process.

The work of Phillipson et al,20 which employed the low-molecular-weight tracer lanthanum hydroxide, has established clearly that a gap does indeed exist between the outer leaflets of the fiber cell gap junction. However, the morphologic demonstration of the gap in the fiber cell gap junction has proved difficult, particularly in vitro, raising the questions: which of the two pentalaminar profiles is the fiber cell gap junction, and what is the nature of the remaining structure?

Using exogenous proteases, the authors have tested the hypothesis that the proteolytic degradation of the MIP from 28 kilodaltons to about 19 kilodaltons results in the conversion of one junctional class to another. The authors also present evidence that (1) identifies...
the thick junction as a gap junction; (2) indicates that thin junctions are not simply an artifact of tannic acid preservation; (3) demonstrated that unit membranes can associate in a manner that mimics the thin junction; and (4) both thick and thin "junctions" are present in situ.

Materials and Methods

Preparation of Bovine and Human Lens Fiber Cell Membranes

Bovine lenses were removed from fresh slaughterhouse eyes, mechanically decapsulated, and homogenized in 50 mM tris, 1 mM diisopropyl fluorophosphate, 1 mM EDTA (pH, 9.5) at 0–4°C, using a Kinematica (Brinkmann Instruments, Westbury, NY) tissue homogenizer at setting 5, for 30 sec. The homogenate from 10 lenses was diluted to 240 ml and centrifuged at 50,000 \( \times g \) for 60 min. The pellet was resuspended in 8 M urea, homogenized as before, diluted to 1:2.6, and centrifuged as stated for 120 min. This pellet was resuspended in 50 mM tris, 0.9% NaCl (pH, 7.0) and centrifuged as stated for 30 min. The final pellet was resuspended in 10 ml of 50 mM tris, 0.9% NaCl (pH, 7.0).

Proteolysis of Fiber Cell Membranes

Aliquots of freshly prepared fiber cell membranes, at approximately 10 \( \mu \)g/\( \mu \)l protein, were diluted 1:1 with (1) trypsin, 4 mg/ml; (2) pepsin 2 mg/ml; (3) staph aurease protease, 1 mg/ml; or (4) no enzyme. All proteases were in tris-saline (pH, 7.0) with 0.02% sodium azide. Incubations with enzymes were performed at 37°C for 10 hr, after which the membranes were washed free of enzymes by 10-fold dilution with 0–4°C tris-saline and centrifugation. Incubations without enzymes were performed at 37°C for 3 or 24 hr. Membranes were washed three times by centrifugation, then prepared for electron microscopy or sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Control membranes were processed immediately after isolation, at 0–4°C.

Electron Microscopy

Samples of membranes containing approximately 100 \( \mu \)g of protein were pelleted in flat-bottomed BEEM "bottle neck capsules" (Ted Pella, Inc; Tustin, CA), and fixed with 2% glutaraldehyde in 50 mM cacodylate buffer. The absence of available amino groups in the trypsinized membranes necessitated the inclusion of 2% bovine serum albumin (BSA) to facilitate the cross-linking of the pellet for subsequent processing. Trypsinized membranes with and without the addition of BSA were compared to evaluate the impact of the addition of the BSA. Pellets were cut from the capsules and suspended in 1% glutaraldehyde, 1% tannic acid in 50 mM cacodylate for 1 hr, at room temperature. One pellet from each paradigm was processed in parallel without tannic acid. Pellets were washed twice with cacodylate buffer and postfixed in 1% aqueous osmium tetroxide. After 30 min the pellets were water washed and stained en bloc with 4% aqueous uranyl acetate. Samples were subsequently dehydrated in ethanol, then propylene oxide, and embedded in Poly Bed 812 (Polysciences, Inc., Warrington, PA). Thin sections were cut and examined without further staining.

Quantitation of the relative percentages of fiber cell membrane domains was performed using a Zeiss MOP 3. Three membrane domains were defined: thick junctions, thin junctions, and unit membranes. Quantitation was performed on nonproteolysed membranes and trypsinized membranes, using 144 micrographs from each category at a final magnification of \( \times 60,000 \). Only membrane domains that could be positively identified as thick junctions, thin junctions, or unit membranes were included. Pellets from two independent isolations were used, with micrographs taken at random through the thickness of the pellet (approximately 0.1–0.4 mm).

To assess the degree and extent of the proteolysis, aliquots from each paradigm were solubilized at room temperature in 2% SDS and 1% \( \beta \)-mecaptoethanol, and frozen until use. Resolution was performed on 12.5% SDS–PAGE, according to the procedure of Laemmli, and stained with Coomassie blue.

Identification of the protein bands as MIP or MIP derivatives was performed using western blotting of nitrocellulose replicas and immunoprobings of nitrocellulose replicas with antiseria to lens crystallins was used to determine the extent of crystallin contamination (not shown).

Results

Membrane Purification

Figure 1 is representative of membranes isolated in this manner. By morphologic criteria the fraction is relatively clean and comparable with that achieved with multiple chaotrope extractions, and/or flotation on sucrose gradients. The virtual absence of intracellular or organellar membranes in the decapsulated lens assures that the membranes harvested are of plasmalemmal origin. Visible nonmembranous contaminants, commonly seen in the form of amorphous and ill-defined densities, were largely extracted as well. Occasional closed membrane vesicles containing what is presumed to be trapped crystallins were found infrequently.

Biochemical analysis supports the conclusion that the membranes resulting from this isolation were substantially free of nonmembrane components. MIP is...
Fig. 1. Electron micrograph of bovine lens fiber cell membranes, purified as described in the text. A portion of this sample, when analyzed by SDS-PAGE (Fig. 2, lane 7), indicates that the main intrinsic polypeptide is present almost entirely in the undegraded form (MW = 27.1 Kd). The overall textural differences that exist between thick (gap) junctions (some of which are indicated by arrows), and other membrane domains is evident. Gap junctions are generally denser, thicker, and have a smoother, less convoluted profile (×22,000).

considered a marker for fiber cell membranes, appearing only in the plasma membrane of lens fiber cells.23-25 The crystallins, which are the dominant fiber cell protein, are not integral membrane proteins.16 Thus, the ratio of MIP to nonmembrane proteins (essentially crystallins) is a measure of the effectiveness of the extraction process. SDS-PAGE analysis of the resulting undegraded membranes indicates that about 95% of
Fig. 2. SDS polyaerylamide gel (12.5%), stained with Coomassie blue. Lanes 1, 8: Standards at arrowheads, from top to bottom—bovine albumin, 66 kilodalton; ovalbumin, 45 kilodalton; carbonic anhydrase, 29 kilodalton; trypsinogen, 24 kilodalton; trypsin inhibitor, 20.1 kilodalton; α-lactalbumin, 14.2 kilodalton. Lanes 2–7: Bovine fiber cell membranes, approximately 15 μg total protein each, after exposure to trypsin, 2 mg/ml, 10 hr, 37°C, relative molecular weight (Mr) = 19.8 kilodaltons (lane 2); pepsin, 1 mg/ml, 10 hr, 37°C, Mr = 21.9 kilodaltons (lane 3); Staph aurease V8, 0.5 mg/ml, 10 hr, 37°C, Mr = 21.7 kilodaltons (lane 4); no enzyme, 24 hours, 37°C, Mr = 23.5 kilodaltons (lane 5); no enzyme, 3 hr, 37°C, Mr = 25 kilodaltons (lane 6); undegraded membranes, solubilized in 2% SDS and 1% mercaptoethanol, immediately after purification as described in the text. Main intrinsic polypeptide is the principal band at Mr = 27.1 kilodaltons (lane 7).

Proteolysis

To facilitate discussion, the relative molecular weights (Mr) of the MIP and its breakdown products have been derived by fitting their relative mobility to a curve generated from the standards. Note that under these conditions the MIP migrates with an Mr = 27.1 kilodaltons in contrast with Mr = 28,200 deduced from its nucleotide sequence. Products generated in the course of this experiment are referred to by the Mr, derived here. Degradation products from other reports are referred to by the Mr, assigned by those investigators.

The relative effects of the different treatments are shown in Figure 2. Incubation of the membrane without the addition of exogenous proteases results in a stepwise reduction in Mr, (lanes 5, 6). However, this reduction does not, under the conditions employed, proceed to the same degree that is achieved by the addition of exogenous proteases. Degradation by staph aurease, pepsin, and trypsin results in peptides with Mr's of 21.7 kilodaltons, 21.9 kilodaltons, and 19.8 kilodaltons, respectively (lanes 4, 3, and 2).

Proteolysis for extended periods, in the presence of excess protease, does not result in a reduction in Mr, beyond the 8–19-kilodalton-level, agreeing with other findings by supporting the belief that this 19.8-kilodalton core is buried within the lipid bilayer and unavailable for degradation.

Electron Microscopy

Isolated membranes. Controls: Controls were defined as membrane samples that did not reveal significant degradation of the MIP, as judged by electrophoretic mobility in SDS-PAGE. Examination of these membranes, both with and without the inclusion of the tannic acid, revealed a number of general features. (1) General preservation of membranes, as judged by their staining density and definition, is better with tannic acid included in the fixation protocol. Unit membranes are more densely stained and easily defined (Fig. 1). Thick and thin junctions are readily apparent (Fig. 3).

the Coomassie blue stainable protein is located at the level expected of the native MIP or MIP-derived multimers and cleavage products. Probing of nitrocellullose replicas of this membrane preparation with anticrystallin antisera shows no trace of crystallin presence, except at high levels of antiseraum or in overloaded gels (not shown).

Fig. 3. Electron micrograph of control membranes. SDS-PAGE of a portion of this sample (Fig. 2, lane 7) suggests that it is undegraded. Both thick (large arrows) and thin (small arrows) junctions are present. Tannic acid included (×240,000).

Fig. 4. Electron micrograph of undegraded membranes as in Figure 3. Arrow indicates the presence of a very narrow gap between the outer leaflets of a thick junction, qualifying the thick junction as a gap junction. No gaps were observed between the outer leaflets of the thin junctions. Tannic acid included (×240,000).

Fig. 5. Undegraded membranes, as in Figure 3, only processed without tannic acid. Both thick (large arrows) and thin (small arrows) junctions can be seen. The narrow gap between the outer leaflets of the two bilayers can be seen at the arrow, g (×240,000).
The transition from thick to thin junction is abrupt. Use of the goniometer stage to examine a given junction from several angles made it evident that the two junctions were not simply the same entity viewed in varying degrees of obliquity. (2) In both tannic acid and nontannic acid fixed membranes, the thicker class of junction can be demonstrated to exhibit a gap between the outer leaflets of the two membranes at the junction (arrow, Fig. 4). (3) Tannic acid is not essential for the discrimination of thick and thin junctional classes. In membrane samples processed without exposure to tannic acid, both thick and thin junctions can be seen. However, the membrane definition that occurs in the absence of tannic acid makes the discrimination more difficult (Fig. 5). (4) Stacks of membranes, several bilayers thick, were commonly found. This finding suggests that fiber cell membranes have the ability, under the conditions employed, to adhere to one another in a manner that—by virtue of profile thickness—resembles the thin junction. Figure 6 shows a thick junction and an assembly of membranes which are either unit membranes, or a mixture of unit membranes and thin junctions. The stack comprises five bilayers, an uneven number, suggesting that at least one unit membrane is involved in this aggregate. Figure 7 shows that a given membrane is capable of folding on itself and presenting a profile that is indistinguishable from a thin junction. (5) In general, unit membranes occurred in smaller fragments and were more irregular in profile. Domains of thick junctions were usually longer and far less convoluted in profile. Domains of thin junctions were the most infrequent, usually occurred in shorter stretches, and were intermediate in the complexity of their profiles. (6) Gap junctions, in both proteolysed and nonproteolysed samples, represent about 13-15% of the membrane profiles present. Because each profile represents the contribution of two cells, this would suggest that gap junctions occupy some 30% of the total membrane area of the bovine lens fiber cell.

Proteolysed membranes: Proteolysed membranes were defined as those samples in which a virtually complete loss of the intact MIP could be demonstrated by SDS-PAGE. In general, regardless of the means that was used to effect the degradation, no obvious differences in the distribution of membrane domains could be seen between the proteolysed and nonproteolysed samples. Figure 8 shows the lower level of background. Derivatives from the serum albumin, which was added to the trypsinized membranes to facilitate their cross-linkage into a pellet. A portion of this pellet was solubilized and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2, lane 2) (×22,000).

Fig. 9. Electron micrograph of trypsinized fiber cell membranes (tannic acid fixed). Both thick and thin membrane-membrane interactions are still present, even after exhaustive proteolysis. Slight changes in the relative percentages of the identifiable domains were observed (Table I), but a quantitative loss of one domain did not parallel the quantitative degradation of the main intrinsic polypeptide from relative molecular weight (MW) = 27.1 kilodaltons to MW = 19.8 kilodaltons (×240,000).

Fig. 10. Electron micrograph of trypsinized fiber cell membranes (tannic acid fixed). Exhaustive proteolysis did not result in detectable changes in the characteristic "gap" (arrow) between the outer leaflets of the plasma membranes (×240,000).
Table 1. Effects of proteolysis on ratios of membrane domains

<table>
<thead>
<tr>
<th>Membrane Domain</th>
<th>µm*</th>
<th>N†</th>
<th>%‡</th>
<th>Average length $\overline{\text{§}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Undegraded membranes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unit membranes</td>
<td>730</td>
<td>1,546</td>
<td>76%</td>
<td>0.409</td>
</tr>
<tr>
<td>Thick junctions</td>
<td>124</td>
<td>262</td>
<td>15%</td>
<td>0.477</td>
</tr>
<tr>
<td>Thin junctions</td>
<td>67</td>
<td>252</td>
<td>08%</td>
<td>0.269</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>921</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trypsinized membranes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unit membranes</td>
<td>620</td>
<td>1,735</td>
<td>82%</td>
<td>0.358</td>
</tr>
<tr>
<td>Thick junctions</td>
<td>92</td>
<td>300</td>
<td>13%</td>
<td>0.315</td>
</tr>
<tr>
<td>Thin junctions</td>
<td>39</td>
<td>202</td>
<td>05%</td>
<td>0.193</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>754</td>
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* µm = total length of measured membrane domain.
† N = number of measurements made in that domain.
‡ % = given domain divided by total measured membranes.
§ Average length = total length of membrane domain/N.

membranes. Figure 8 is a lower-power micrograph of trypsinized membranes. The greater amount of flocculent material that is present in the trypsinized membranes is because of the inclusion of serum albumin to promote their cross-linkage into a pellet during fixation. Membranes processed in the absence of the serum albumin showed similar distributions of membrane domains, but were disinclined to remain in a pellet form and were difficult to process. In all cases, the three membrane domains could be found in proportions that were not noticeably different from those of the control. Proteolysis had no discernible impact on the narrow gap that characterizes the thicker class of junction (Fig. 10). There was clearly no loss of one junctional class or domain that corresponded to the quantitative loss of the intact MIP.

Trypsinization resulted in the severest reduction in MW, of the MIP, and for this reason the authors selected trypsinized membranes for statistical analysis. The total length of each identifiable membrane domain was determined, divided by the number of measurements (N) to establish average length per measurement, and divided by the total measured membrane to establish relative percentage. The results of the quantitation are presented in Table 1.

**Lens:** Electron microscopy of the lens nuclear region reveals the presence of both thick and thin classes of membrane interactions (Fig. 11). Variation in the plane of the section confirms that, as in the isolated membranes, the variation in “junction” thickness does not derive from variations in the plane of the junction.

**Discussion**

**Membrane Purification**

For the purposes of this investigation it was desirable to generate a membrane fraction that was representative of the total fiber cell plasma membrane population. For this reason, the authors avoided commonly used membrane isolation procedures such as gradient centrifugation and detergent extraction that might artifactsubfractionate the membranes, inadvertently enriching one membrane domain or another.

For biochemical reasons it was important to remove the abundant crystallins in a virtually quantitative manner. Many of the crystallin components have...

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Fig. 11. Electron micrographs of both thick (large arrows) and thin (small arrows) junctions in the same field of lens tissue. This region was within 2 mm of the lens center (x255,000).
MWs in the same range as the MIP and its proteolytic derivatives, and failure to extract them would have confused the assessment of proteolytic degradation.

More traditional membrane isolation procedures involve multiple extractions in chaotropes to remove the less soluble crystallins and cytoskeletal elements. These procedures are time consuming, but result in a membrane preparation that is morphologically sound. Russel et al described a procedure for the rapid, single-step extraction of peripheral membrane proteins using an alkaline wash. This latter procedure is extremely effective in “scrubbing” the membranes free of nonintegral membrane proteins but is morphologically harsh. The procedure described here blends the two of these approaches, resulting in a purification that is rapid, biochemically effective, and morphologically gentle.

Proteolysis

MIP has been shown to undergo an age-dependent degradation from 28 kilodaltons to approximately 22 kilodaltons in situ. A number of exogenous proteases have been shown to be capable of approximating or exceeding this physiologic degradation, at least as judged by relative electrophoretic mobility. No protease, however, has been capable of degrading the MIP beyond the 19–20-kilodalton level without the use of detergents or other membrane perturbants. Presumably the 19-kilodalton core of the MIP is buried within the lipid bilayer and not susceptible to proteolytic attack.

Both the amino and carboxy termini of the MIP are exposed to the cytoplasmic surface and are susceptible to the proteolytic degradation. The precise location of the naturally occurring proteolysis, and the amino acid sequence of the resulting core polypeptide, are, as yet, unpublished. Thus, the degree to which exogenous proteolytic degradation mimics the naturally occurring degradation is also unknown. Specifically, the authors were concerned with the possibility that exogenous and naturally occurring degradation might occur at different sites on the MIP, resulting in polypeptides with similar electrophoretic mobilities but significantly different primary sequences. Variations in the primary sequences may well result in different tertiary structure, resulting in observable differences in membrane architecture. For this reason the authors employed several means of degrading the MIP and examined the effects of each.

Membrane Structure

In an effort to study the relationship between MIP degradation and the presence of what appears to be two classes of fiber cell intercellular junctions, the authors have employed both exogenous and endogenous proteases to mimic the naturally occurring degradation of the MIP. The authors have hypothesized that the thin junction found in isolated membranes may be derived from the thick junction through proteolytic degradation of the MIP, and thus may represent the structural counterpart to MIP aging. The authors have found that a variety of proteolytic regimens, including the exhaustive tryptic degradation, are not paralleled by a significant shift in the relative percentages of the three membrane domains. Some shift in the relative ratios was noted, but it clearly did not parallel the virtually quantitative degradation of the MIP. Therefore, the authors conclude that the degradation of the MIP is insufficient, in itself, to alter the ratios of fiber cell membrane domains.

Certain limitations, however, must be considered in the interpretation of the data presented here. (1) Most of the membrane in any given micrograph cannot be positively identified as belonging to any specific class of membrane domain. In order to identify a given stretch of membrane, that membrane must fall within a narrow range of deviation from perpendicular to the optical axis. It is unlikely that the range of this deviation is identical for all three domains. Specifically, it is probably easier to identify unit membranes over a wider range of deviation than either of the two junctional classes, meaning that unit membranes may be over-represented in these figures. Thus the suggestion that gap junctions represent 30% of the fiber cell surface may possibly be a low estimate. (2) In-vitro degradation of the MIP is a rapid event, allowed here to proceed for a maximum of 12 hr, but essentially complete within 30 min. We have shown that quantitative degradation of the MIP is not paralleled by a comparable loss of one membrane domain or another. However, it is conceivable that MIP degradation represents the mechanism by which the thin class of fiber cell junction arises, and that the reorganization of membrane components following the MIP degradation is a relatively slow event, not observed within the time frame of this investigation.

The use of tannic acid as a membrane preservative, and the discrimination of a second class of pentalaminar, gap junction-like membrane domains in vitro, where only one class had been described previously, have been coincident. This has raised the question of whether the second class of junction is an artifact of the isolation procedure or of tannic acid fixation. We demonstrate here that thin junctions are seen in situ as well as in vitro, and that tannic acid is not essential for the visualization of these two classes of junctions. Lo et al demonstrate that thin junctions are found in situ, predominantly in the nuclear region. Together these reports imply that thin junctions are
genuine biologic entities, and not artifacts of either tannic acid or the isolation process. This, in turn, raised the question of which of the two pentalaminar profiles observed in vitro represents the fiber cell gap junction. By definition, the presence of the diagnostic 2 nm is sufficient for the designation of gap junction. However, this gap, although experimentally demonstrated by Phillipson et al., has proved difficult to demonstrate from a morphologic vantage point—possibly because the gap is narrower. This difficulty has been exacerbated by the preservation qualities of the tannic acid, which tends to fill in the extracellular gap with stained material. However, the authors were able to demonstrate the presence of a gap between the outer leaflets of the membranes in the thick junctions by appropriately understaining. Although this does not eliminate the possibility that the thin junction is gap junction related, it does permit the positive identification of the thick junction, by definition, as a gap junction.

Remaining to be resolved is the nature of the thin junction. Whether it represents a genuine intercellular junction, a site of membrane differentiation whose function remains to be established, or the simple adhesion of adjacent membranes is, as yet, unclear. The authors have shown that fiber cell membranes are capable of adhesion in a manner that is thin junction-like in appearance. However, this does not preclude the existence of a genuine intercellular junction of similar morphology. The nature and biologic role of these different domains is made all the more exciting by reports indicating that these domains exhibit different immunoreactivity.

**Key words:** main intrinsic polypeptide, gap junctions, aging, membrane domains, lens

**References**


