Aging of Lens Fibers

Mapping Membrane Proteins With Monoclonal Antibodies

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Lenses of sheep contain a variety of membrane proteins that are associated with the aging process. These proteins have been studied using monoclonal antibodies and other methods. The antibodies have been used to map the membrane proteins and to study their spatial distribution in the lens. The results have implications for understanding the aging process in the lens and for cataract research.

Materials and Methods

Membrane Isolation

Lenses were extracted from the eyes of sheep within minutes of death and kept on ice. Lens nuclei became opaque upon cooling, and the transparent, cortical lens portion was peeled from the more compact nucleus. Cortical and nuclear tissues were frozen and stored separately at −80°C. In general, lenses used for membrane preparations and cryosectioning were from less than 1-yr-old sheep. The experiment summarized in Table 1 is based on the comparison of lenses less than 1 yr old with those over 5 yr of age.

For the isolation of crude plasma membranes, cortical or nuclear tissue from 100 lenses was homogenized (Virtis, 90 sec, full speed) in 200 ml 5 mM Tris pH 7.772

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8.0, 1 mM EDTA (= buffer A) with the addition of 5 mM β-mercaptoethanol to prevent oxidation of lens proteins. The membranes were washed repeatedly in A by pelleting at 8,000 rpm in a Sorvall GSA rotor (Sorvall; Wilmington, DE).

Further purification of lens plasma membranes was achieved by extracting the membrane pellets with 4 M urea in B (= 5 mM Tris pH 9.5, 1 mM EDTA) followed by two extractions with 7 M urea in B.10,11,18-20 After centrifugation for 60 min at 24,000 rpm in a SW27 Beckman rotor (Beckman Instruments; Palo Alto, CA) the pellet was resuspended in 4 ml A, and to this, 12 ml 67% sucrose in A were added. Eight ml of this mixture were underlayered in each of two sucrose density gradients with 45%, 25%, and 8% steps. The gradients were spun for 60 min at 24,000 rpm in a SW27 rotor, and lens membranes collected at the 25/45% interface.

The extracted membranes were washed free of sucrose or nucleus were adsorbed 50 μl/well to polystyrene microtiter dishes (Immunoplate IIF Nunc) at 0.2 mg/ml in PBS overnight at 4°C. Unreacted binding sites in the wells were blocked with 10% calf serum, 2% BSA in PBS. Fifty μl aliquots of hybridoma supernatants were then added to the immobilized membranes, and the plates incubated at 37°C for 1 hr. Unbound antibodies were removed by several rinses with PBS. Antibodies bound to membranes were labeled with 50 μl/well 0.5 μCi/ml antimouse Ig 125I(Fab')2 (Amersham; Arlington Heights, IL) in PBS for 1 hr at 37°C. The plates were washed with PBS several times, dried, and exposed at -80°C onto Cronex X ray film (Dupont; Wilmington, DE) using intensifying screens.26 Alternatively, antigen–antibody complexes were solubilized in 200 μl/well 2 M NaOH, and radioactivity was counted with a gamma-counter.

SDS-PAGE for staining or immunoblotting was performed according to established methods.27 For each sample well, 90-μg lens membrane proteins were solubilized in 50-μl sample buffer at room temperature for 15 min immediately before loading the gels. Protein bands were either stained with Coomassie blue or transferred to nitrocellulose (BA85, Schleicher and Schuell) by diffusion according to Bowen et al.28 Alternatively, 1.5-mg lens membrane proteins were separated by SDS-PAGE using a 20-cm wide sample slot and transferred to nitrocellulose sheets. Five-millimeter wide strips thereof were used to test individual hybridoma supernatants for antibody specificity. Reaction of nitrocellulose bound antigen with antibodies and secondary antibody labeling with antimouse Ig 125I(Fab')2 was carried out according to Towbin et al.29 and Renart et al.30 with the modifications described in Kistler et al.31 Antigens were identified by superposition of autoradiographs of immunoblots with their complementary filters stained with amido black (two identical replicas of a gel are obtained by diffusion transfer).

Only hybridomas secreting antibodies that recognized membrane antigens in both the native, membrane-bound conformation and the denatured form were maintained for further analysis. From four fusions we isolated 21 hybridoma lines, which produced lens membrane protein specific antibodies. Antibodies from this collection recognized 11 different protein bands on immunoblots. Five hybridomas secreting antibodies against seven lens membrane proteins with apparent molecular weights between 50,000 and 115,000 have been used for this study.

**Immunofluorescence Microscopy**

Antigens were localized in lens tissue sections using immunofluorescence microscopy. Sheep or mouse lens cryosections (approx. 15 μm thick) were dried on coverslips and kept at room temperature until used. The
Excess antibodies were removed by washing in PBS and sections mounted in 50% glycerol in PBS and 10 mM p-phenylenediamine. Specimens were viewed with a Zeiss fluorescence microscope equipped with a Plan Neofluor 25/0.8 objective, and micrographs recorded on Kodak (Rochester, NY) Tri X Pan 400 film.

Results

Identification of Lens Fiber Membrane Proteins

Treatment of lens fiber homogenates with urea has been widely used to purify plasma membranes. Electron microscopy shows that urea extracts the fibrous material adhering to crude preparations of membranes. Proteins which are insoluble in 7 M urea, are thus likely to be membrane-bound (integral or peripheral), although precipitation of a cytoplasmic protein cannot be excluded.

SDS-PAGE reveals several urea soluble fiber proteins. For example, polypeptides with 50,000 and 115,000 dalton molecular weight are recognized by antibodies derived from hybridomas 18/19-1-A3-E2 and 18/19-3-G7, respectively, in a crude preparation of membranes, but little or none is detected on immunoblots of urea extracted cortical membranes. These proteins appear to be highly antigenic in the mouse, when injected as minor contaminants of the urea insoluble fraction. In contrast, the polypeptides with apparent molecular weights 18,000, 26,000, 34,000/36,000, 64,000, 70,000, 140,000 and 200,000 are urea insoluble (Figs. 1 b, c). MIP (apparent molecular weight 26,000) and MP34/36 have been identified as membrane proteins and have been extensively studied (reviewed in 35). Both the 70,000 and 64,000 dalton proteins are recognized by antibodies secreted by hybridoma 6-4-B2-C6 (Fig. 2d). It seems likely that the 64,000 dalton protein is a limited proteolysis product of the 70,000 dalton polypeptide (unpublished data).

Another group of proteins includes those with apparent molecular weight 57,000, 82,000, and 100,000, which are recognized by antibodies of hybridomas 18/19-3-G7 (Fig. 2c), 18/19-4-F7 (Fig. 2e), and 18/19-2-C4-A1 (Fig. 2f), respectively. These proteins are at least partially urea-insoluble.

Using immunofluorescence microscopy we have localized these antigens in cryosections of sheep lens cortex. Anti 50,000 antibodies labeled membranes and cytoplasm of cortical fibers, consistent with this protein being a component of the urea soluble lens matrix (Fig. 3a). Antibodies secreted by hybridoma 18/19-3-G7, directed against both the urea-soluble 115,000 dalton polypeptide and partially urea-resistant 57,000 dalton protein labeled predominantly fiber membranes with occasional weak fluorescence in the cytoplasm (Fig. 3b).
Fig. 2. Immunoblotting of crude and urea extracted cortical membranes from less than 1-year-old sheep lens (gel lanes as in Fig. 1b, c) with monoclonal antibodies and polyclonal anti-MIP; a, anti-MIP; b, anti-50,000; c, anti-MP57/anti-115,000; d, anti-MP70; e, anti-MP82; f, anti-MP100.

3b), the latter most likely due to the 115,000 molecular weight component. The polypeptides with 82,000 and 100,000 apparent molecular weight were localized in or at the surface of the plasma membrane (Figs. 3 c, f) in agreement with their presence in the urea insoluble, membrane-rich fraction. The 70,000 dalton protein was also found in the membranes, however, in contrast to the uniform membrane labeling from above, the staining intensity was stronger on the broad sides of the cortical fibers and weaker at the corners and on the narrow sides (Fig. 3c). Viewing the fiber broad sides face-on, fluorescent macular patches were revealed (Fig. 3d), demonstrating the partitioning of this protein into discrete membrane domains. Using immunofluorescence and immunoelectron microscopy we have recently identified these membrane regions as lens fiber junctions, most likely gap junctions.17

In summary, the 57,000, 70,000, 82,000, and 100,000 dalton proteins are contained in the urea insoluble, membrane-rich fraction. They are found in fiber membranes by immunofluorescence microscopy, and we will refer to them as membrane proteins MP57, MP70, MP82, and MP100 (see Discussion).

Age Dependent Protein Distribution

Age-related changes in membrane protein distribution have been studied by comparison of cortical and nuclear fibers of less than 1-yr-old-lenses and also by comparing less than 1-yr-old lenses with those older than 5 yr. Firstly, for the less than 1-year-old lenses, the comparison of stained gel lanes loaded with the same total amount of protein from urea extracted, cortical, or nuclear fiber membranes, reveals major differences for the proteins with apparent molecular weights above 50,000 (Figs. 1 c, d). Several cortical membrane proteins are reduced or absent in nuclear fiber membranes. Most strikingly, the junctional domain specific MP70 is a major component of cortical plasma membranes but present only in minor amounts in the lens nucleus. Other proteins, such as MP57 and most lower molecular weight polypeptides, are present in similar quantities in both lens regions. In line with this, immunoblots of gel lanes such as Figs. 1c, d show MP57 with a uniform distribution throughout the lens (Fig. 4). However, MP70 (and MP64), MP82, and MP100 give much weaker or no signals on immunoblots of nuclear fiber membranes. As no aggregated material at the top of the gels could be detected by staining, nor do these proteins appear at different positions on immunoblots, these antigens must have been cleaved to smaller fragments by age-associated proteolysis.

Anti-MP70 antibodies cross-react with a homologous protein in mouse, and the tissue distribution of this
component could thus be directly visualized by immunofluorescence microscopy on mouse lens cryosections. A sharp transition in fluorescence pattern occurred between the outer and deep cortex; the punctate pattern associated with the large intercellular junctions in the peripheral region \(^{17}\) converted into a generally more homogenous and weaker fluorescent membrane staining in the deeper cortex (Fig. 5). This change reveals a gradual loss of MP70 deeper in the lens consistent with the immunoblotting analysis.

We have compared the antigen-distribution in lenses of less than 1-year-old sheep with that in lenses of animals older than 5 yr using radio-immunoassays in microtiter dishes. Equal amounts of total membrane pro-
tein were used in each well, and all membrane fractions contained similar proportions of MIP (Table 1). All the antigens were degraded upon aging with respect to constant levels of MIP and could be grouped in three distinct classes: (1) MP82 was abundant only in cortical fibers of young animals. MP82 was rare or absent in the nucleus and throughout the more than 5-yr-old lens. (2) MP70 and MP100 were more abundant in the lens cortex than in the nucleus independent of the overall age of the lens. (3) The amounts of MP57 were similar to young cortex and nucleus but were strongly reduced in both regions in older lenses. Thus some proteins are only present during a short period of early fiber development, while others are present over a longer time.

Discussion

Fiber membrane proteins have been identified using two criteria: urea insolubility and membrane immunofluorescence staining. In one case, MP70 has previously been localized in fiber junctional membranes by immuno-electron microscopy. MP57, MP82, and MP100 are here referred to as membrane proteins with the following restriction: resolution of fluorescence microscopy is not sufficient to distinguish between protein localization strictly in the plasma membrane or merely in its vicinity. Therefore, we have used the term “membrane proteins” with an operational meaning, namely that these proteins are components in the urea insoluble, membrane-rich fraction and are localized in or near fiber membranes by fluorescence microscopy.

Loss of water- or urea-insoluble protein associated with aging of lens fibers has been reported by several groups. The following post-translational protein modifications have been linked with the aging of lens fibers: protein aggregation, modification of selected amino acid residues, protein conformational changes and proteolysis (reviewed in ref 2). Using radioimmunoassay of purified lens membranes and immunofluorescence microscopy of lens cryosections alone, these protein changes could not be distinguished from each other as they can all lead to the loss of antigenic determinants. SDS-PAGE of membrane proteins under reducing conditions is more sensitive; the loss of protein of nuclear fiber membranes cannot be due to protein aggregation via disulfide bonds as those aggregates are soluble in sample buffer and monomers are resolved. Protein aggregates rendered insoluble by covalent, nondisulfide bonds and thus remaining at the top of the gels (and immunoblots), have never been observed. Protein conformational changes and modification of amino acid residues (eg, deamidation) would hardly affect the apparent molecular weights of the denatured membrane proteins separated by SDS-PAGE. Therefore, proteolysis accounts best for the observed loss of membrane proteins in nuclear fibers and becomes particularly evident because of the absence of protein synthesis in this lens region.

Several classes of proteases have been found in lens tissue (reviewed in ref 20), including leucine-a-minopeptidase, neutral protease, and trypsin-like enzymes. Prominent among lens proteins that become cleaved in older fibers are the crystallins, vimentin, and MIP. However, proteolysis of lens protein appears to be only a secondary age related phenomenon. Primary events must be the age-dependent activation of proteolytic enzymes, age-dependent inactivation of protease inhibitors and/or protein conformational changes that make cleavage sites more accessible for proteases. In fact, an inhibitor for trypsin-like protease has been identified in the lens cortical region but was found absent or inactive deeper in the lens. This correlates well with an increased protease activity in the lens nucleus. Alternatively, conformational changes...
rendering lens proteins more susceptible to proteolytic degradation may be brought about by amino acid modification such as deamidation of asparagine and glutamine residues.\(^2\) It has been suggested in a more general discussion of aging mechanisms that controlled deamidation may determine the half-lives of individual protein species via conformational change and increased susceptibility to proteolysis.\(^3\)

Degradation of higher molecular weight membrane proteins similar to the age-related proteolysis in the lens nucleus occurred upon prolonged incubation of total cortical fiber homogenate at 37°C and in the presence of divalent cations (data not shown). However, we consider it very unlikely that the differences in protein composition between cortical and nuclear membranes are merely isolation artifacts. (1) Procedures for membrane purification from cortex and nucleus were always carried out in parallel and with all precautions taken to prevent endogenous proteolysis. (2) At least for MP70, the immunofluorescence-derived protein distribution in lens tissue sections is in agreement with that concluded from SDS-PAGE and radioimmunoassays of isolated membranes. (3) In gen-

Table 1. Protein composition of urea extracted plasma membranes from the cortical and nuclear region of sheep lenses less than 1 yr old (C\(_1\), N\(_1\)) and more than 5 yr old (C\(_5\), N\(_5\))

<table>
<thead>
<tr>
<th></th>
<th>MIP</th>
<th>57K</th>
<th>70K</th>
<th>82K</th>
<th>100K</th>
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<td>261</td>
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<tr>
<td>N(_1)</td>
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<td>101</td>
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<td>277</td>
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<tr>
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<td>3436</td>
<td>83</td>
<td>82</td>
<td>55</td>
<td>141</td>
</tr>
</tbody>
</table>

The values are counts per minute for \(^{35}\)S in a microtiter radioimmunoassay with equal total protein in each well. The age-related loss of other membrane antigens should be compared to that of MIP, which varies only by ±10% between membrane preparations. (Data are from one experiment; three independent experiments showed the same antigen degradation patterns.)
eral, MIP is very susceptible to proteolysis to 22,000 daltons in vitro.42-44 However, only very little of the degraded protein has been detected on gels of isolated lens membranes from approximately 5-year-old sheep. We therefore consider proteolysis a controlled physiological event of lens fiber aging.

Although proteolysis causes the decrease of all membrane antigens analysed in this study, we consider it significant that each protein follows a specific degradation pattern. Within the limited resolution of our radioimmunoassay MP82 has only been detected in cortical fibers of young animals. Its distribution and age-related degradation pattern is most compatible with specific functions during lens fibers differentiation. This process occurs exclusively in the outer cortical region, which appears more extensive in younger sheep lenses than in older ones. After fiber differentiation and maturation is completed somewhat deeper in the lens cortex, MP82 is degraded. MP70 ages differently, as peak amounts of MP70 in cortical membranes independent of the overall lens age. MP57 appears to be dependent primarily on the overall age of the lens and is homogeneously distributed throughout the organ. Although our gel calibration assigns MP57 the same molecular weight as that published for lens vimentin, the two proteins are distinct as vimentin is largely found degraded in the lens nucleus.37 The function of MP57 is presently under investigation.

There seems to be no sharp division between lens aging and senile cataract formation. Similar protein modifications have been observed in normally aged and cataractous lenses.34 At the membrane level, we could imagine the age-related loss of membrane proteins to result eventually in severe membrane dysfunctions. For example, an accelerated loss of junctional MP70 might reduce the membrane permeability for metabolites and ions thus preventing homeostatic control of deeper lens regions. Membrane protein specific monoclonal antibodies will therefore be useful reagents to study the protein changes in cataractous lenses and to gain further insight into the molecular mechanisms leading to lens cataract.

Key words: aging, membrane proteins, lens fiber junctions, monoclonal antibodies, proteolysis

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References