sults on this abnormal eye are consistent, rather than inconsistent, with the results obtained in other subjects with normal eyes.

In summary, counter to previous occlusion reports, we find that in both normal and abnormal eyes 6.5 days of total occlusion is not sufficient to disrupt the Stiles-Crawford effect. Our results suggest that either 6.5 days total occlusion is an insufficient time interval for inferred receptor malalignment to occur or that total occlusion is not a sufficient condition to induce a change in inferred receptor alignment. This study combined with existing information (eg, receptor alignment is present at birth,1 SCF peak location recovers from pathological stress and can be induced to shift by displacing the pupillary aperture5,6) suggests that receptor alignment depends upon genetically determined factors and is maintained postnatally by a positive phototropic mechanism that is not disrupted by prolonged (6.5 days) occlusion.

Key words: visual function, phototropism, Stiles-Crawford function, Stiles-Crawford effect, occlusion

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Covalent Changes in MIP26K During Aging of the Human Lens Membrane

Larry Takemoro,* Makoto Takehana,* and Joe Horwitz†

Rabbit antisera have been made to the C-terminal octapeptide of bovine MIP26K (residues 256–263) and to an octapeptide near the C-terminus (residues 252–259). Use of these antisera in Western blot analysis of membrane from young vs old human lenses, and from cortex vs nucleus of old human lenses, suggest age-related covalent changes in the C-terminus of MIP26K. These age-related changes in anti-256–263 and anti-252–259 sera can be mimicked by treatment of lens membrane with carboxypeptidase Y, suggesting the loss of 1–8 amino acid residues from the C-terminus of the 26,000 dalton form of MIP26K during aging of the normal human lens. Invest Ophthalmol Vis Sci 27:443–446, 1986
studies have demonstrated an age-related conversion during aging and/or cataractogenesis. Previous role in the physiological functioning of this polypeptide occurs at the C-terminus of MIP26K.6

has been suggested that at least part of this cleavage 24,000 daltons. Using C-terminal specific proteases, it of MIP26K to a component of approximately 22,000-plexes from the lens fiber cell membrane, 1 MIP26K has been implicated as the polypeptide responsible for

As the major component of gap junctional complexes from the lens fiber cell membrane, 1 MIP26K has been implicated as the polypeptide responsible for lens cell communication via the formation of intercellular channels. Recently, this hypothesis has been supported by the demonstration that MIP26K reconstituted into synthetic liposomes can mediate the passage of small molecular components across the lipid bilayer.2,3 Furthermore, antiserum to the C-terminal region of MIP26K can inhibit this passage,3 suggesting that the C-terminal region may be especially important in the control of intercellular communication in the intact gap junctional complex.

It is therefore possible that age-related covalent modifications of MIP26K in the human lens may play a role in the physiological functioning of this polypeptide during aging and/or cataractogenesis. Previous studies have demonstrated an age-related conversion of MIP26K to a component of approximately 22,000–24,000 daltons. Using C-terminal specific proteases, it has been suggested that at least part of this cleavage occurs at the C-terminus of MIP26K.6

To test this hypothesis, we have synthesized two octapeptides corresponding to the C-terminal region of bovine MIP26K. Use of antisera to these peptides confirm the presence of age-related proteolysis in human MIP26K, and strongly suggest that the 26,000 dalton form of MIP26K found in the aged human lens has lost 1–8 amino acid residues during the aging process.

Materials and Methods. Normal human lenses, obtained from The National Diabetes Research Interchange, were stored at –85 degrees C. Lens fiber membrane from whole lenses was prepared as previously described,7 with the exception that the urea treatment step was omitted. In some cases, lens cortex was first removed from lens nucleus by repeatedly washing the intact lens with 0.1 M Tris-HCl (pH 7.4) from a Pasteur pipet. Under these conditions, the approximately 20% of outer fiber cell mass removed was defined as cortex. The cortical cells that were removed and the remaining nuclear cells in the fiber cell mass were then used in the preparation of lens membrane.7

For carboxypeptidase treatment, 18 µg of lens membrane in a total volume of 68 µl of 0.01 M sodium phosphate (pH = 7.5) was treated with 1.2 µg of carboxypeptidase Y (Sigma, C3888 Sigma Chemical; St. Louis, MO) that had been pre-incubated for 10 min at 37 degrees with an equal amount of the endopeptidase inhibitor Pepstatin A (Sigma, P4265). After incubation for 24 hr at 37 degrees, an equal volume of sample buffer containing SDS was added, and the solution was immediately run on an SDS-polyacrylamide gel.

Determination of protein, SDS-polyacrylamide gel electrophoresis, and Western blot analysis using rabbit immunoglobulin and radioiodinated protein A has been described previously.8 Polyacrylamide gels and autoradiographs were scanned with a Bio-Rad model 1650 densitometer (Biorad Laboratories; Richmond, CA) connected to a Shimadzu model C-R3A recorder/integrator (Kyoto, Japan).

Solid-state synthesis of peptides and production of antisera to these peptides followed the protocol of Gooden et al.3 Based upon the sequence of cloned cDNA to bovine MIP26K,8 two octapeptides were synthesized. The sequence of the C-terminal octapeptide (residues 256–263) was H₂N-val-glu-leu-lys-thr-gln-ala-leu-COOH, and the sequence of an octapeptide (residues 252–259) near the C-terminus was H₂N-thr-glu-pro-val-glut lys-COOH.

Results. Figure 1 demonstrates the specificity of the antisera made against synthetic peptides from the C-terminal region of bovine MIP26K. Antiserum against the C-terminal octapeptide of bovine (anti-256–263) and antiserum against an octapeptide near the C-terminus (anti-252–259) both recognize human MIP26K, but not the 23K degradation product. Since the cloned sequence of bovine MIP26K has 263 residues, these
Table 1. Antisera binding to 26K band

<table>
<thead>
<tr>
<th>Membrane sample</th>
<th>Anti-256 serum*</th>
<th>Anti-252 serum*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young lens†</td>
<td>6,241 ± 122</td>
<td>12,648 ± 885</td>
</tr>
<tr>
<td>Old lens†</td>
<td>4,522 ± 389</td>
<td>17,076 ± 850</td>
</tr>
<tr>
<td>Ratio Young/Old</td>
<td>1.39 ± 0.13</td>
<td>0.74 ± 0.08</td>
</tr>
<tr>
<td>Lens cortex† (65 yr)</td>
<td>6,630 ± 854</td>
<td>14,222 ± 1,168</td>
</tr>
<tr>
<td>Lens nucleus† (65 yr)</td>
<td>3,748 ± 528</td>
<td>20,976 ± 1,816</td>
</tr>
<tr>
<td>Ratio cortex/Nucleus</td>
<td>1.77 ± 0.07</td>
<td>0.68 ± 0.07</td>
</tr>
</tbody>
</table>

* C.p.m. of 125I-protein A binding to the 26K band after Western blot analysis.
† Four young lenses (2 day, 2 day, 6 wk, 9 yr) and four old lenses (73 yr, 73 yr, 77 yr, 77 yr) were used for preparation of lens membrane.
‡ Four old lenses (65 yr, 65 yr, 65 yr, 65 yr) were used for preparation of lens membrane.

The results demonstrate that at least part of the age-related cleavage of 26K to 23K must involve the C-terminus. In addition, both the anti-256 and anti-252 sera recognize lower molecular weight components of 20,000 and 15,000 daltons. If these components are degradation products of MIP26K, then most, if not all, of the cleavage must have occurred from the N-terminal end of MIP26K.

Since at least some of the age-related cleavage of the 26,000 dalton component, to the 23,000 dalton component occurs from the C-terminus, it was possible that intermediates of this proteolysis might be present in older human lenses. To investigate this possibility, membrane from old vs young human lenses was subjected to SDS-polyacrylamide gel electrophoresis, followed by electrophoretic transfer of the polypeptide bands to nitrocellulose where they were probed with the anti-256 and anti-252 sera and radioiodinated protein A. The 26,000 dalton band was then cut out and counted in order to quantitate the amount of antisera binding to this component in young vs old lenses. In an identical manner, membrane from the cortex versus nucleus of old human lenses was subjected to this quantitative Western blot procedure.

Table 1 shows that more anti-256 serum binds to the 26,000 dalton band from younger as opposed to older lenses (6241 from young vs 4522 from old). In contrast, more anti-252 serum binds to the 26,000 dalton band from older, as opposed, to younger lenses (17,076 from old vs 12,648 from young). In an identical manner, more anti-256 serum binds to membrane from the younger cortical cells (6630 from cortex vs 3748 from nucleus), and more anti-252 serum binds to membrane from the older nuclear cells (20,976 from nucleus versus 14,222 from cortex). The results of these experiments can be summarized by stating that during aging of human lens fiber cells the binding of anti-256 serum decreases, whereas the binding of anti-252 serum increases.

Since only the 26,000 dalton band was cut out and counted from the Western blot, these results definitively demonstrate the presence of some type of age-related covalent modification of MIP26K that does not significantly change the apparent molecular weight of this component as determined by SDS-gel electrophoresis. Because the 26,000 dalton component is degraded from the C-terminus to a 23,000 dalton component during aging, the most likely mechanism of this covalent modification would be C-terminal clipping of only a few residues that would not be seen by conventional SDS-gel electrophoresis.

To test this hypothesis, membrane from young human lenses was treated with carboxypeptidase Y containing the endopeptidase inhibitor pepstatin. Under the conditions used in this study, all of the proteolysis occurs at the C-terminal end of the molecule. After carboxypeptidase treatment, the membrane preparations were subjected to Western blot analysis and probed with the anti-256 and anti-252 sera. No difference in the apparent molecular weight of the 26,000 dalton band was seen after carboxypeptidase treatments (results not shown).

Quantitation of antisera binding to the 26,000 dalton band reveals the same general trend seen with membrane from young versus old lens fiber cells (Table 2). Anti-256 binds better to untreated membrane (3188 for control vs 1775 for carboxypeptidase Y treated), in contrast with anti-252 which binds better to treated membrane (9983 for carboxypeptidase Y treated vs 7192 for control).

Discussion. Western blot analysis using antisera directed against C-terminal peptides has demonstrated

Table 2. Antisera binding to 26K band after treatment of lens membrane with carboxypeptidase Y

<table>
<thead>
<tr>
<th>Membrane sample</th>
<th>Anti-256 serum*</th>
<th>Anti-252 serum*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young lens†</td>
<td>3,188 ± 72</td>
<td>7,192 ± 188</td>
</tr>
<tr>
<td>Young Lens + Carboxypeptidase Y</td>
<td>1,775 ± 154</td>
<td>9,983 ± 257</td>
</tr>
<tr>
<td>Ratio Young Lens + Carboxypeptidase Y</td>
<td>1.81 ± 0.13</td>
<td>0.72 ± 0.008</td>
</tr>
</tbody>
</table>

* C.p.m. of 125I-protein A binding to the 26K band after Western blot analysis.
† Four young lenses (2 day, 2 day, 6 wk, 9 yr) were used for preparation of lens membrane.
quantitative differences in the amount of antisera binding to the 26,000 dalton band from membrane of young vs old lens fiber cells. Since binding only to 26,000 band was measured, this difference could not be due to differences in other membrane components such as lipids, crystallins, et cetera. Rather, the results demonstrate covalent changes in the MIP26K polypeptide sequence occurring during aging of the normal human lens.

The chemical nature of these covalent changes may include deamidination, phosphorylation, racemization, oxidation, intermolecular crosslinking, glycosylation, and proteolysis. Since direct chemical determination of the C-terminus of MIP26K from old lenses would result in considerable heterogeneity, it is not possible to state with complete certainty that the C-terminus changes during aging. Nonetheless, since the anti-256 and anti-252 sera are directed against sequences near the C-terminus, the most probable covalent change would be clipping of the C-terminus, resulting in decreased binding of the C-terminus antiserum (anti-256) during the aging process. Why the binding of anti-252–259 serum increases during the aging process is not known, but it is possible that clipping of a few residues from the C-terminus may make the 252–259 sequence of MIP26K more accessible to antibody binding. Since the C-terminal antiserum (256–263) is directed to an octapeptide, the number of amino acid residues cleaved would be ≤8. Such a difference would probably be undetectable by SDS-polyacrylamide gel electrophoresis.

This possibility of C-terminal clipping has been supported by in vitro proteolysis with carboxypeptidase Y. As was the case with MIP26K from young vs old fiber cell membranes, treatment with carboxypeptidase Y does not change the apparent molecular weight using SDS-polyacrylamide gel electrophoresis, but does result in decreased binding of anti-256 serum and increased binding of anti-252 serum.

Based upon these findings, we propose that during aging of lens fiber cells the MIP26K molecule is clipped on the C-terminal side. Similar degradation from the C-terminal end has already been reported in the lens during in vivo aging of the αA2 and αA1 crystallin chains. Some (if not all) of this clipped MIP26K polypeptide may then be degraded to the 23K component. It is intriguing to note that we and other investigators do not see the presence of intermediate molecular weight products between the 26K and 23K components. Perhaps clipping of the C-terminus is a necessary prerequisite for cleavage to the lower molecular weight 23K component.

Because of the possible importance of the C-terminal region of MIP26K in coupling of lens fiber cells, any covalent and/or structural changes in this region may have profound effects upon the in vivo functional properties of lens gap junctions. In this regard, this report has demonstrated that antisera to this C-terminal region can act as very specific probes of changes involving a very small yet known region of the MIP26K sequence. These changes can be characterized from relatively small amounts (1–4 μg) of membrane, permitting the study of individual human lens and even microdissected sections from the same lens.

Key words: MIP26K, human lens membrane, aging

From the Division of Biology, Kansas State University, Manhattan, Kansas and The Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles, California. Supported by grants from the National Institutes of Health to L.T. & J.H. Submitted for publication: July 16, 1985. Reprint requests: Larry J. Takemoto, Division of Biology, Ackert Hall, Kansas State University, Manhattan, KS 66506.

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