Complete Map and Identification of the Phosphorylation Site of Bovine Lens Major Intrinsic Protein

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Purpose. To determine the complete primary structure, including posttranslational modifications, of bovine lens major intrinsic protein (MIP) using a recently developed combination of liquid chromatography and mass spectrometry.

Methods. The MIP was isolated from bovine lenses by sucrose gradient centrifugation and was cleaved with cyanogen bromide (CNBr). A high-performance liquid chromatographic system, developed for hydrophobic protein analysis, was used to separate the cleavage fragments. Matrix-assisted laser desorption ionization and electrospray tandem mass spectrometry were employed to obtain molecular weight and sequence data from bovine MIP CNBr fragments, directly or after subsequent digestion with trypsin.

Results. The complete sequence of bovine MIP was mapped by molecular weight measurements of CNBr fragments, confirming the reported DNA sequence. The C-terminal peptide (177 to 263) was fully sequenced and the major site of phosphorylation was determined to be at serine 235 rather than at the previously reported serine 243. The level of phosphorylation in the native protein was determined to be 25%. No other posttranslational derivatizations were observed with the exception of the previously detected deamidation of asparagine 246.

Conclusions. These results represent the first complete MIP sequence map at the amino acid level and identify the single major phosphorylation site at serine 235. Invest Ophthalmol Vis Sci. 1997;38:2508-2515.

The major protein of the plasma membrane of the lens fiber cells is an integral membrane protein termed major intrinsic protein (MIP), also known as MP26 or MIP26. This protein is a member of a family of integral membrane proteins that have a variety of physiological functions and are found in bacteria, animals, and plants. The proteins in this family are proposed to have the same topology, consisting of six α-helices spanning the membrane, and to function in the transport of small molecules, water in the case of MIP, across the membrane. The best characterized member of this class of proteins is MIP, which exists as a tetrameric complex in the membrane. However, the posttranslational modifications, three-dimensional structure, function, and functional regulation of this protein still are not well defined.

The primary sequence of MIP has been determined by complementary DNA (cDNA) cloning, and two of the major cyanogen bromide fragments have been partially sequenced. The protein in bovine lenses has been reported to be phosphorylated on serines 243 and 245 and has been shown in vitro to be a substrate for several protein kinases. Using phosphorylation with protein kinase A in situ, Ehring et al. have shown that phosphorylation modulates the voltage dependence of reconstructed MIP channels, which suggests that this posttranslational modification may be physiologically significant.

Other posttranslational modifications have also been reported. In vitro, the protein is palmitylated on incubation with palmitic acid, although this modification has not been observed in native material. Deamidation in aged MIP and glycation in MIP from...
Phosphorylation of Major Intrinsic Protein on Serine 235

Yee Kin Ho, University of Illinois, Chicago) were de-

All experiments were performed in accordance with

bodies to the C-terminal of MIP has revealed changes

Western blot analysis using antibodies to the C-terminal of MIP has revealed changes in cataractous mouse lens MIP, and age-related changes were also observed in human MIP. The exact structural identity of these changes remains to be determined. It is clear that alterations of MIP can play a role in cataractogenesis, evidenced by genetic mutations identified in the MIP gene as the cataract Fraser mutation (CaF) and the lens opacity mutation (Lop), both of which lead to congenital cataracts in mice homozygous for either of these mutations.

In that it is an integral membrane protein, MIP is extremely hydrophobic and presents the well known challenges to structural analysis of hydrophobic proteins. No crystallization techniques have been developed for these proteins, and the hydrophobic nature of the transmembrane segments has made generation and solubilization of the products of proteolytic and chemical cleavage difficult. The resistance of MIP to proteolytic cleavage is well documented. In this study, we have combined a recently developed high-performance liquid chromatography (HPLC) method for integral membrane proteins with mass spectrometry to confirm the primary covalent structure of MIP, and we have discovered a major, previously unreported, site of phosphorylation. This methodology will allow further investigation into the three-dimensional structure and modifications of lens membrane proteins in normal and diseased states.

METHODS

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Bovine lens MIP was prepared according to the procedure of Goodenough. Briefly, 10 frozen bovine lenses (Dr. Yee Kin Ho, University of Illinois, Chicago) were de-capsulated and homogenized in 1 mM NaHCO3, 5 mM EDTA, and 10 mM NaF, pH 8 at 4°C. Lens homogenate was then centrifuged at 13,300g at 4°C for 15 minutes. The resulting pellet was washed with Tris buffer solution (5 mM Tris, 1 mM EDTA, and 1 mM CaCl2, at pH 9) and 10 mM NaF, then with 4 M urea in Tris buffer solution, followed by 7 M urea in the same buffer solution. The final wash was followed by a 10,000g centrifugation for 60 minutes.

The urea-washed pellet was suspended in 2 ml Tris buffer solution and 7 ml 67% sucrose, buffered in the same buffer. Tris-buffered solutions of 45%, 41%, and 25% sucrose were placed over the 67% layer and centrifuged for 45 minutes at 10,000g. The 25%:41% interface was collected and washed with de-ionized water (dH2O).

Reduction and alkylation of cysteine residues was accomplished by incubating the pellet with tributylphosphine (1000-M excess over cysteine content) and 4-vinylpyridine (2500-M excess over cysteine content), with subsequent sonication for 1 minute, followed by 5 minutes’ cooling. The sonication procedure was repeated for 60 minutes. Excess reagents were removed by washing with deionized water. Delipidation was carried out on an LH-20 column and MIP was eluted with ethanol–formic acid (70/30, vol/vol). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on a precast 12% acrylamide gel; samples were not heated before loading, to avoid irreversible aggregation.

The MIP (700 μg) was solubilized with trifluoro-acetic acid (TFA) (75%) and a 500-fold molar excess of CNBr over methionine residues was added to cleave the protein chemically. The reaction was carried out for 18 hours at room temperature under nitrogen in the dark. The reaction was terminated by a fivefold dilution in deionized water, followed by vacuum evaporation.

High-performance liquid chromatography was accomplished, using a gradient system designed for hydrophobic membrane protein–peptide analysis (DR Knapp, personal communication, 1996). The dry protein (112 μg) was solubilized for 1 minute by sonication in 5 μl TFA, followed by the addition of 42 μl acetonitrile and then 84 μl isopropanol, with 1 minute of sonication after each addition of solvent. This solution was diluted to 0.05 to 0.07 μg/μl with 4.869 ml deionized water. The entire sample volume (5 ml) was injected onto a 30-mm Brownlee Aquapore C2 column (Bodman, Aston, PA) and eluted with a gradient of acetonitrile in 5% 10 mM (NH4)2CO3 and 95% 10 mM NH4HCO3, pH 8.5 at 33°C, with an enzyme-substrate ratio of 1:10. Endoproteinase Glu-C (Boehringer-Mannheim) digestion was carried out on HPLC fractions by solubilizing the sample in 90% 10 mM NH4HCO3 and 10% acetonitrile, pH 8.5 at 33°C, with an enzyme–substrate ratio of 1:10. Endopeptinase Glu-C (Boehringer-Mannheim) digestion was carried out on HPLC fractions by solubilizing the sample in 95% 10 mM (NH4)2CO3 and 5% acetonitrile, pH 7.9 at 25°C, with an enzyme–substrate ratio of 1:10.

Matrix-assisted laser desorption ionization (MALDI) mass spectra were acquired on a custom MALDI time-of-flight instrument incorporating a nitrogen laser (Laser Science, Newton, MA). Samples were solubilized in 85% acetic acid and mixed 1:3 with α-cyano-4-hydroxycinnamic acid matrix, spotted in 1-μl aliquots, and allowed to air dry. Typically, 30 laser shots were used acquire one mass spectrum. Peptide
external standards were used to calibrate the mass scale, and peptide molecular weights were measured with typical standard deviations of 0.02%. Direct electrospray analysis was carried out on a JEOL (Peabody, MA) HX110/HX110 tandem mass spectrometer or on a Sciex API-III triple quadrupole instrument (Perkin-Elmer-Sciex, Thornhill, Ontario). Dried fractions were solubilized (1 μg/μl) in dH₂O–methanol–acetic acid (47/47/6) and were sprayed at a flow rate of 3 μl/min. Typical peptide molecular weight determinations were made with standard deviations of ±2 daltons (Da) for large CNBr products and ±0.1 Da for smaller tryptic peptides. HPLC tandem mass spectrometry (LC/MS/MS) analysis was accomplished on a Finnigan LCQ instrument (Finnigan MAT, San Jose, CA). A dried mixture of tryptic peptides was resuspended in 75 μl deionized water and injected (2-μl aliquots) onto a C₁₈ column (1 mm in diameter; Michrome Bioresources). The gradient used was 98% solvent A (98% water, 2% acetonitrile, 0.02% TFA, and 0.1% acetic acid) to 67% solvent B (90% acetonitrile, 10% water, 0.02% TFA, and 0.1% acetic acid) for 38 minutes followed by 95% B for 5 minutes. The HPLC effluent (200 μl/min) was directed into the ion-trap mass spectrometer for molecular weight and sequence analysis. Typically, 20 pmol of sample was injected per analysis. Automated MS/MS analysis was carried out in the ion trap while a chromatographic peak, eluted by mass selection of the ion of interest, followed by radio frequency excitation of that ion, causing fragmentation.

RESULTS

To observe the purification of MIP, SDS–PAGE was used, and the results are displayed in Figure 1. Although not completely pure after the final step, the amount of MIP is greatly enhanced in the membrane by this isolation scheme. The sucrose gradient appears to remove some of the high molecular weight membrane species present in the urea-washed pellet.

Because of its hydrophobic nature and putative three-dimensional structure, MIP is somewhat resistant to extensive cleavage by proteases. Therefore, chemical cleavage by CNBr in 75% TFA was used to generate large peptide fragments, which could then be manipulated more easily. The CNBr products were isolated by a recently developed HPLC methodology, specifically designed for hydrophobic membrane protein and peptide analysis (DR Knapp, personal communication). The resulting chromatogram is shown in Figure 2. Mass spectrometric analysis, MALDI or electrospray was carried out on each fraction; the masses corresponding to MIP peptides are reported in Table 1, showing complete coverage of the MIP sequence. Other signals are probably caused by cleavage products from one of the minor contaminating proteins in our preparation.

MALDI mass spectrometry involves mixing a protein or peptide sample solution with a matrix solution and allowing the matrix to crystallize. When dry, sample molecules incorporate into the matrix crystals. A laser, usually one emitting ultraviolet light, is focused on the dried sample in the mass spectrometer to desorb intact sample molecules and ionize them by pro-
TABLE 1. Molecular Weights of Major Intrinsic Protein Cyanogen Bromide Products

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Measured Mass (Da)</th>
<th>Predicted Mass</th>
<th>Elution Time (minutes)</th>
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<td>2–81</td>
<td>8670.4</td>
<td>8668.2*</td>
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<td>82–90</td>
<td>1125.6</td>
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<td>91–176</td>
<td>8988.0</td>
<td>8984.6*</td>
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<tr>
<td>177–263</td>
<td>9512.7</td>
<td>9510.9</td>
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<td>177–263</td>
<td>9558.5</td>
<td>9556.9†</td>
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<tr>
<td>177–263</td>
<td>9637.6</td>
<td>9636.9†</td>
<td>38.4</td>
</tr>
<tr>
<td>184–263</td>
<td>8799.9</td>
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<td>38.4</td>
</tr>
<tr>
<td>184–263</td>
<td>8878.9</td>
<td>8877.1‡</td>
<td>38.4</td>
</tr>
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</table>

* Includes pyridylethylation of cysteine residues.
† Includes oxidation of methionine.
‡ Includes phosphorylation.

Phosphorylation of Major Intrinsic Protein on Serine 235

**Tandem Mass Spectrometry**

Tandem mass spectrometry is used to sequence peptides by generating fragments of a peptide selected on the basis of its molecular weight in the first stage of mass analysis. A second stage of mass analysis is employed to measure fragment ion masses. Because...
peptides fragment predominantly along their amide bonds, differences in fragment ion masses can be assigned to amino acids, and if a series of related ions is present— for example, N-terminal-containing ions—a sequence can be obtained. Typically, complementary N-terminal- and C-terminal-containing fragment ions are present and, according to nomenclature devised by Roepstorff and Fohlman and revised by Biemann, these ions are given the symbols b, n, and y, respectively, where n represents the amide bond that is broken. A significant advantage of using tandem mass spectrometry for peptide sequencing is that N-terminally blocked peptides and posttranslationally modified peptides can be sequenced and their modifications identified.

Sequence information obtained by LC/MS/MS analysis combined with peptide molecular weights for these tryptic peptides provided unambiguous peptide identification and confirmed the entire C-terminal MIP sequence. These data are summarized in Table 2. Note that homoserine at position 183 was identified as a modification that results from incomplete cleavage during CNBr treatment, leaving a homoserine residue in the middle of the tryptic peptide sequence 177 to 187. The peptide molecular weight corresponding to residues 239 to 259 was 1 Da higher, compared with the predicted sequence molecular weight. When sequenced, a shift in predicted fragmentation pattern of one mass unit occurred at residue Asn246. This reflects the deamidation Asn246 to aspartic acid as reported by Takemoto and Emmons.

Two possible phosphopeptide signals are observed in the MALDI spectrum at m/z 2278 and 1212, corresponding to residues 239 to 259 and 229 to 238, respectively. It is interesting to note that we do not observe the unphosphorylated 229 to 238 peptide, presumably because when serine 235 is phosphorylated, tryptic cleavage is blocked, whereas in the unphosphorylated situation, efficient tryptic cleavage can occur. Alternative cleavage with endoproteinase GluC (data not shown) indicated a phosphopeptide including residues 233 to 250; however, this signal does little to assign the specific modified site, because it includes serines 235, 240, 243, and 245. Reverse-phase HPLC–MS of the tryptic peptides revealed a large signal at 1212, which was subsequently sequenced by tandem mass spectrometry. In multiple MIP preparations, the signal at m/z 2278, corresponding to phosphorylated 239 to 259, was present in too low an abundance to sequence in our experiments.

The tandem mass spectrum of the putative phosphopeptide 233 to 238, (M + 2H)²⁺, m/z 606.7 shown in Figure 5 indicates unambiguously that serine 235 is the site of phosphorylation. The most abundant signal at m/z 557.4 corresponds to the doubly charged deamidation Asn246 to aspartic acid as reported by Takemoto and Emmons. Moreover, the tandem mass spectrum of the putative phosphopeptide 229 to 238, (M + 2H)²⁺, m/z 606.7 shown in Figure 5 indicates unambiguously that serine 235 is the site of phosphorylation. The most abundant signal at m/z 557.4 corresponds to the doubly charged deamidation Asn246 to aspartic acid as reported by Takemoto and Emmons.

TABLE 2. Major Intrinsic Protein Sequence Information Obtained by Tandem Mass Spectrometry

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Measured Mass (Da)</th>
<th>Predicted Mass</th>
<th>Sequence Confirmed</th>
</tr>
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<tr>
<td>177–187</td>
<td>1169.6</td>
<td>1169.6</td>
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<td>456.3</td>
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<td>491.2</td>
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<td>188–194</td>
<td>717.4</td>
<td>717.4</td>
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<td>188–195</td>
<td>818.5</td>
<td>818.4</td>
<td>193–195</td>
</tr>
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<td>974.6</td>
<td>190–196</td>
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<td>188–226</td>
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<td>204–206, 209–212</td>
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<td>501.4</td>
<td>501.3</td>
<td>193–196</td>
</tr>
<tr>
<td>206–226</td>
<td>2203.6</td>
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<td>211–223</td>
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<tr>
<td>227–233</td>
<td>817.6</td>
<td>817.5</td>
<td>227–230, 233</td>
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<tr>
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<td>576.4</td>
<td>576.3</td>
<td>229–233</td>
</tr>
<tr>
<td>229–238</td>
<td>1210.8</td>
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<td>2197.4</td>
<td>2197.1t</td>
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<td>1897.2</td>
<td>1896.9t</td>
<td>246, 251–252, 255–259</td>
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<tr>
<td>260–263</td>
<td>431.2</td>
<td>431.2</td>
<td>260–263</td>
</tr>
</tbody>
</table>

* Includes homoserine 183.
† Includes phosphoryserine 235.
‡ Includes deamidation of asparagine 246.
sequence SVSERL (b9); however, the b7 ion (which contains the next residue, S235) has lost H3PO4, resulting in a fragment mass corresponding to dehydroalanine. Complementary information is provided by the y ion series, which shows that the y3 ion is not phosphorylated but the y4 ion that contains S235 loses H3PO4.  MS/MS/MS was carried out to fragment the 557.4 ion generated from the 606.7 molecular ion. The fragmentation pattern (not shown) reveals ions at m/z 854.6 (b8), 927.7 (y8), 967.7 (b9), and 711.4 (y6), which are 18 Da lower than predicted for unmodified 233 to 238 because of loss of H3PO4 from a serine residue. Fragment ions at m/z 187.1 and 260.2 match predicted sequence ions. This sequence information obtained from the tandem mass spectrometry experiments provides unambiguous evidence that S235 is a site of MIP phosphorylation in vivo.

The intensity of the phosphorylated 184 to 263 signal in Figure 2 was approximately 25% of the unphosphorylated signal. Although molecular ion intensities in MALDI or electrospray mass spectra can vary on the basis of ion structure, making quantitation difficult, the signal obtained for phosphorylated 239 to 259 represented from 2 to 10 times less signal relative to the phosphorylated signal obtained for 229 to 238 in several analyses.

In other HPLC fractions, signals corresponding to possible truncated peptides are observed; however, tandem mass spectrometric sequencing of these peptides is currently underway, and the identification of truncation sites will be the subject of a future report.

**DISCUSSION**

Structural elucidation of integral membrane proteins has previously been a difficult enterprise because of hydrophobic considerations. Structural work on lens MIP has focused mainly on the C-terminal, because it is easily generated and isolated from the core integral membrane portion of the protein. Limited success has formerly been achieved on the central-membrane-spanning regions of the protein. Our results show that all regions of purified MIP can be accessed in great detail by mass spectrometric methods, using the cleavage and novel separation methods described.

Chemical cleavage of the intact protein by CNBr generates smaller hydrophobic fragments that can be isolated and further analyzed. A recent advance in the chromatographic separation of hydrophobic peptides allows isolation of each CNBr fragment from the MIP protein. Mass spectrometric analysis of these fragments and subsequent proteolytic fragments mapped the entire sequence of bovine MIP. These data, along with the sequence data obtained, confirmed the DNA reported sequence of Gorin et al. Mass spectrometric analysis also revealed the presence of a phosphorylated C-terminal in two CNBr products, 184 to 263 and 177 to 263. Both of these signals were approximately 25% of the unphosphorylated signal, which is in good agreement with published results of Ehring et al but is larger than the 5% to 10% value initially reported by Lampe and Johnson.

Early identification of bovine MIP as a phosphoprotein localized the site of phosphorylation to a 40-amino acid region on the C-terminal of the protein. Results in more recent biochemical studies indicate that serine 243 is the major site of phosphorylation in vivo and by in vitro incubation with protein kinase A. Evidence for a small amount of phosphorylation on serine 243 or 245 was also obtained in our studies.
A sites are often blocked to trypsin cleavage by phospho-
were observed.

structural examination of the major intrinsic protein
and specifically for the phosphorylation site at serine
235 is located in a highly conserved region (100% ~
known MIP sequences from vertebrates. However, ser-
in Figure 6, serine 243 is not conserved among the
physorylation,24 which is observed in our tryptic maps.

Previous biochemical work did not closely examine
such a modification. Furthermore, excluding possible
modifications on MIP can now be accomplished on
by tryptic mapping. However, evidence of the major
phosphorylation site in vivo was obtained in the tryptic
map of the C-terminal peptides, where the peptide
229 to 238 was observed to be phosphorylated. Tand-

t mass spectrometric analysis unambiguously identi-
ified serine 235 as the predominant phosphorylation
site in our preparation. Although previous reports cite
a protein kinase A consensus sequence of R-X-S
(where X is a neutral amino acid) for phosphorylation
of serine 243,9 this sequence is also present for serine
235. Consistent with our results is that protein kinase
A sites are often blocked to trypsin cleavage by phos-
phorylation,24 which is observed in our tryptic maps.
Previous biochemical work did not closely examine
the region containing serine 235. The approach used
in this study easily lends itself to detailed sequence
analysis of the MIP C-terminal, and the results pre-

ted here bring into question the relevance and validi
try of previously published phosphorylation sites
identified after incubations in vitro.

As shown in the amino acid sequence alignment
in Figure 6, serine 243 is not conserved among the
known MIP sequences from vertebrates. However, ser-
ine 235 is located in a highly conserved region (100% ~
identity) spanning residues 231 to 239, suggesting an
important conserved role for this sequence in general
and specifically for the phosphorylation site at serine
235.

Previous reports also identified fatty acid modifi-
cations to MIP12; however, we found no evidence for
such a modification. Furthermore, excluding possible
truncation, no other posttranslational modifications
were observed.

In summary, recent advances in mass spectrom-
etry and HPLC have allowed us to carry out a detailed
structural examination of the major intrinsic protein
MIP in bovine lenses. Having now established a com-
plete protein map, further studies on posttranslational
modifications on MIP can now be accomplished on
protein from aged and diseased lenses. The identifi-
cation of serine 235 as the major phosphorylation site
brings into question which kinase is responsible for
phosphorylation at this site, and further, which ser-
ine(s) is important in any regulatory function.

Key Words
major intrinsic protein, mass spectrometry, membrane pro-
tein, phosphorylation

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ries Laboratory (Fort Johnson laboratory), and Eric Finley
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spectively; Dr. Jack Wang for operation of the JEOL instru-
ment; and the Medical University of South Carolina,
Charleston, for use of their mass spectrometry facility.

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Phosphorylation of Major Intrinsic Protein on Serine 235


