Posttranslational Modifications of the Bovine Lens Beaded Filament Proteins Filensin and CP49

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PURPOSE. The lens beaded filament proteins filensin and CP49 are phosphorylated proteins that undergo proteolytic degradation with fiber cell age; however, the specific sites of modifications remain largely unknown. The purpose of this study was to identify posttranslational modifications (PTMs) in bovine lens beaded filament proteins.

METHODS. Filensin and CP49 were enriched by urea extraction of lens fiber cell homogenates after the water-soluble fraction was removed. The urea-soluble fraction was separated by SDS-PAGE, and the corresponding filensin and CP49 bands were digested by trypsin, Lys C, or Glu C. The enzymatic digests were analyzed by HPLC mass spectrometry.

RESULTS. The sequences of lens beaded filament proteins were systematically mapped, and putative database sequence errors of filensin were identified. The data also indicated that Met-1 of CP49 was removed and Ser2 was acetylated. Nine phosphorylation sites on filensin and seven phosphorylation sites on CP49 were identified. Filensin was found to be truncated at D431 and L39, and the resulting new N termini were *N*-myristoylated and *N*-acetylated, respectively. Truncation of CP49 occurred at D37. Aspartic acid isomerization to isoaspartic acid occurs at the major truncation sites of filensin (D431) and of CP49 (D37).

CONCLUSIONS. This study identified sites of phosphorylation and truncation in filensin and CP49 and revealed two unusual PTMs: postproteolytic *N*-acetylation and *N*-myristoylation of filensin. The detailed knowledge about these PTMs provides important information for further study of their functional consequences—for example protein redistribution during lens fiber cell differentiation and aging. (*Invest Ophthalmol Vis Sci*. 2010;51:1565–1574) DOI:10.1167/iops.09-4565

The ocular lens is a transparent tissue composed of highly differentiated and elongated lens fiber cells that synthesize lens-specific proteins. Filensin (BFSP1) and CP49 (phakinin, BFSP2) are two lens fiber cell-specific proteins that co-assemble to form a unique intermediate filament structure: the lens beaded filaments (BFs).^{1–4} Primary amino acid sequence analysis shows that filensin and CP49 are homologous to other

members of the intermediate filament protein family.^{2,3,5–7} The structure of filensin consists of a head domain, a rod domain that can be divided into three subdomains (1A, 1B, and 2), and a C-terminal tail domain,⁸ whereas CP49 completely lacks the non- α -helical C-terminal domain.⁵

The lens beaded filament has a 6- to 8-nm filament backbone that is decorated by 12 to 15 nm beads at regular intervals. This structure is found exclusively in the fiber cells of the lens and is believed to play a functional role that is unique to lens fiber cell biology. Studies have shown that knockout of filensin or CP49 each reduces the expression of the other co-assembly partner and causes earlier emergence of opacification.^{9,10} Structural changes in fiber cell shape and plasma membrane organization were also observed in a CP49 knockout model.¹¹ Some forms of hereditary cataracts in humans have been linked to mutations of filensin or CP49.^{12–14} These data suggest that the lens beaded filaments play an important role in the development and maintenance of lens transparency.

Posttranslational modifications increase functional diversity of proteins, adding an ever-increasing complexity to the functions of the cell. In long-lived lens fiber cells, both regulatory and age-related modifications can be found. Both filensin and CP49 are reported to be phosphorylated proteins^{15,16}; however, no phosphorylation sites on filensin and CP49 have been identified. Ser 5 of filensin located in a conserved di-arginine/aromatic residue motif (YRRSY), a consensus site for protein kinase A phosphorylation¹⁴; however, phosphorylation on Ser 5 in vivo has not been reported. Both filensin and CP49 have been shown to be extensively processed during lens fiber cell differentiation.^{17–19} At least two major filensin fragments are formed during proteolytic cleavage—one containing the N-terminal domain and the other containing the C-terminal non- α -helical tail domain¹⁷—and a change in cellular localization of these two fragments during fiber cell differentiation was reported.^{17,20} Both fragments have similar electrophoretic mobilities (51–54 kDa) by SDS-PAGE.^{2,17,19} One of the cleavage sites was estimated to be in the vicinity of residue 453, by measuring the molecular mass of the truncated fragment¹⁸; however, detailed information about the cleavage site(s) is missing.

It is important to map the proteolytic pathway of filensin and CP49 to understand the normal and pathogenic roles of these proteins. In this article, the nearly complete sequences of filensin and CP49 were mapped and sites of truncation and phosphorylation were identified. Two unexpected posttranslational modifications including postproteolytic *N*-acetylation and *N*-myristoylation were characterized. This study represents the first comprehensive examination of posttranslational modifications of lens fiber cell-specific intermediate filament proteins. The newly identified postproteolytic *N*-acetylation and *N*-myristoylation events provide insight into the functional consequence of filensin degradation during lens fiber cell differentiation.

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MATERIALS AND METHODS

Lens Fractionation

Frozen 1-year-old or older bovine lenses (PelFreez Biologicals, Rogers, AR) were decapsulated and dissected into cortex and nucleus before homogenization. Tissue was homogenized in homogenizing buffer (5 mM Tris buffer containing 1 mM EDTA, 1 mM PMSF, and 5 mM β -mercaptoethanol; pH 8.0) and centrifuged at 20,000g for 30 minutes, and the supernatant was discarded. The pellets were washed three times with homogenizing buffer. The remaining pellets are called the water-insoluble fraction (WIF). The WIF was extracted by 8 M urea twice in the homogenizing buffer followed by centrifugation at 20,000g for 30 minutes. The supernatant was pooled and called the urea-soluble fraction (USF). The remaining pellets were washed with 8 M urea three additional times and were collected as the urea-insoluble fraction (UIF). The supernatants contained only low concentrations of proteins and were discarded.

Electrophoresis and In-gel Digestion

The protein concentration in the USF was determined by using the Bradford Assay. Proteins were reduced by adding DTT to the USF (5–7.5 mg/mL), to a concentration of 10 mM, and incubating them at 55°C for 1 hour. The samples were then alkylated by adding iodoacetamide, to a concentration of 55 mM, and were incubated in the dark at room temperature for 45 minutes. After reduction and alkylation, the USF was separated by SDS-PAGE using a 4% to 12% Bis-Tris gel (NuPAGE Novex, Invitrogen, Carlsbad, CA) and MES running buffer (Invitrogen). The gel was then stained with colloidal blue (Invitrogen). The major bands were excised and destained with three consecutive washes with a 50:50 mixture of 50 mM ammonium bicarbonate and acetonitrile for 10 minutes. The gel bands were then dried (SpeedVac; Savant Instruments, Inc., Holbrook, NY). Each sample containing an individual band was rehydrated in 10 to 15 μ L of solution containing 20 ng/ μ L trypsin (Promega, Madison, WI), Lys C (Sigma-Aldrich, St. Louis, MO), or Glu C (Sigma-Aldrich) in 50 mM ammonium bicarbonate buffer for 15 minutes. Thirty microliters of 50 mM ammonium bicarbonate buffer was added to each sample, and the samples were incubated at 37°C for 12 to 14 hours. The peptides were extracted by using 20% ACN/0.1%TFA once, 60%ACN/0.1%TFA twice, and 80%ACN/0.1%TFA once. The extracted samples were pooled and dried (SpeedVac; Savant Instruments, Inc.) and reconstituted in 0.1% formic acid for subsequent analysis.

Liquid Chromatography–MS/MS

Enzymatic digests were separated on a fused silica capillary column (110 mm $\times$ 75 μ m) custom packed with monitor-spherical silica (5- μ m mean particle size; Column Engineering, Ontario, CA). A solvent delivery system (Agilent 1100; Hewlett Packard, Palo Alto, CA) was used for HPLC separation, which was performed with the following gradient: 0 to 10 minutes of 5% ACN (0.1% formic acid), 10 to 45 minutes of 5% to 25% ACN (0.1% formic acid), 45 to 60 minutes of 25% to 52% ACN (0.1% formic acid), and 60 to 61 minutes of 52% to 100% ACN (0.1% formic acid), with a 0.25- μ L/min flow rate. The eluate was directly infused into a mass spectrometer (LCQ DecaXp; ThermoFisher, San Jose, CA) or into an ultrahigh-capacity (HCT) ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with a nanoelectrospray source. The mass spectrometer was operated in data-dependent mode, with the top three most abundant ions in each full mass spectrum being selected for subsequent MS/MS scans by collision-induced dissociation (CID). HCT MS scans were performed in the standard enhanced mode, and all MS/MS scans were performed in electron transfer dissociation (ETD) mode. For ETD analyses, the reactant temperature was set at 65°C, although the ionization energy and emission current were set at 75 eV and 1.7 μ A, respectively. Fluoranthene radical anions were allowed to react with the peptide ions for 180 ms. Dynamic exclusion (repeat count, 2; repeat duration, 0.5 minute; exclusion duration, 0.7 minute for CID; and exclusion after 2 spectra

and release after 1 minute for ETD) was enabled, to allow detection of less-abundant ions. For accurate mass measurement, tryptic or Glu C peptides were separated by a similar HPLC column and elution gradient. The eluate was directly infused into a mass spectrometer (LTQ Orbitrap; ThermoFisher) with a nanoelectrospray source. MS scans were acquired with a resolution of 60,000. Data were acquired on the mass spectrometer in the data-dependent mode, with the top five most abundant ions in each MS scan fragmented in the spectrometer.

Reductive Amination

The water insoluble fraction was washed immediately with sodium acetate buffer (100 mM; pH 5.5) twice. The pellets were suspended in 100 μ L sodium acetate buffer. Ten microliters formaldehyde (37%) and 50 μ L 0.6 M sodium cyanoborohydride were added to the pellets. The samples were incubated at room temperature for 2 hours. The samples were then centrifuged at 20,000g for 20 minutes, and the pellets were washed with water three times followed by 8 M urea extraction. The USF was then isolated, reduced, and alkylated, as described earlier and separated by SDS-PAGE.

Data Analysis

All CID spectra were searched by using the Sequest algorithm (BioWorks 3.3 software package; ThermoFisher) with a custom bovine lens protein SwissProt database (<http://www.expasy.org>; provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland). Peptide identifications were made according to the following criteria: cross-correlations were at least 1.5 for +1-charged ions, 2.0 for +2-charged ions, and 2.5 for +3-charged ions. All ETD spectra were searched via an in-house Mascot server (<http://www.matrixscience.com>; provided in the public domain for use in research by Matrix Science, Boston, MA). Potential posttranslational modifications including acetylation, deamidation, phosphorylation, Met oxidation, and truncation were considered. Dimethylation of the protein N terminus and Lys ϵ -amino groups was considered for the reductive amination experiment. All the identified peptides and posttranslational modifications presented in this article were manually verified. To identify the truncation sites, we subjected raw data to Sequest searches with the partial enzymatic cleavage setting.

RESULTS

Isolation and Characterization of the USF from the Bovine Lenses

The USFs were isolated as described in the Methods section from three regions of the lens, including a 1-mm-thick surface layer, the remaining cortex, and the nucleus. The USFs were then separated by SDS-PAGE; the gel profile is shown in Figure 1. Major protein(s) in each band were assigned according to the peptide identification of the most intense peaks in the base peak chromatogram of the LC-MS/MS analysis. For example, peaks in the base peak chromatogram of the tryptic digest of the 100-kDa band were all identified as filensin; therefore, filensin was assigned as the major protein in the 100-kDa band. By this method, filensin was identified as the major protein in both the 100- and the 53-kDa bands, and CP49 was identified as the major protein in the 45- and 41-kDa bands. The distribution of these assigned proteins is in agreement with other reported results.^{15,20,21} Some filensin and CP49 peptides were also detected in other bands; however, the signal was weak compared with the major protein of the band. The SDS-PAGE profile contains many less-intense bands that may contain additional truncated fragments of filensin or CP49; however, this study was focused on characterization of the major truncated fragments.

FIGURE 1. Identification of gel-separated USF proteins from bovine lens fiber cells. The major proteins in the USFs of bovine lens fiber cells were identified by dissection and processing of tissue and separation of the proteins by SDS-PAGE on a 4% to 12% Bis-Tris gel. The gel was stained with colloidal blue. The major bands were excised and subjected to in-gel tryptic digestion and LC-MS/MS analysis for protein identification. The mass spectrometry-based protein identity is indicated next to the corresponding protein band on the gels. Protein ladder (lane 1; BenchMark; Invitrogen, Carlsbad, CA); OC, USF from the outer cortex (lane 2); IC, USF from the inner cortex (lane 3); and N, USF from the nucleus (lane 4).

Sequence Coverage of Filensin and CP49

The LC-MS/MS (CID) raw files of the 100- and 45-kDa band tryptic digests were searched by Sequest against a custom SwissProt bovine lens database. The protein sequence coverage of filensin in the 100-kDa band and CP49 in the 45-kDa band determined by this approach gave 95% coverage of filensin and 97% coverage of CP49. The sequences of filensin and CP49 that are not detected in tryptic digests contain many closely spaced tryptic cleavage sites resulting in very short peptides that are difficult to confidently detect by mass spectrometry. Some of these sequences were confirmed by Lys C or Glu C digestion. Combining results from multiple digests confirmed 98% of the filensin sequence and 99% of the CP49 sequence. The confirmed sequences of filensin and CP49 are shown in Figure 2. The N termini of filensin and CP49 were not detected in the tryptic digest, because the trypsin digestion of filensin and CP49 yields very short N-terminal peptides (MYR and MSTR, respectively). Lys C or Glu C was then used to digest the proteins. In the 45-kDa band, Lys C peptide corresponding to residues 2–22 of CP49 was detected with acetylated Ser 2. The tandem mass spectrum of the acetylated peptide 2–22 of CP49 can be found in Supplementary Figure S1, <http://www.iovs.org/cgi/content/full/51/3/1565/DC1>. No nonacetylated Ser 2 was detected. Unfortunately, the N terminus of filensin was not detected in the Lys C or Glu C digests.

Two putative filensin sequence errors in the bovine sequence were detected in the MS/MS sequence data. These two sequence errors include G165 to D165 and R207 to E207. By changing from G165 to D165 in filensin, this site becomes conserved between bovine and human. Changing R207 to E207 makes residue 207 a conserved site among human, bovine, rat, and mouse. The tandem mass spectra of the peptides 156–171 and 177–210 of filensin are shown in Supplementary Figures S2 and S3. In addition, the combination of accurate

A	MYRRSYVFT	RKEQYERAE	GRAEPRDLA	BARAAAPNLA	ALQGLGERVA	AHVORARALE
	QRHAVLRRL	DAFQRLDELA	GPEALARHV	EGNRQRARDL	AAERTRLRQ	GABQARALDE
	FRSKYENEC	COLLKEMLE	RINKADEAL	LNNLRQTEA	QFLQDDI SAA	KDRYKNNLE
	ITQYVTILQ	ITQTPQAAA	ITSGMRBEKI	LTEREAAALQ	COLEDGREMI	CLLQAQTEL
	QAQTAALQA	IRDAHECYDD	ETQLYNEQID	TLRKEIEEAE	RSLSRSSYDC	ROLVVVQOTL
	RNELDRYRI	INENGRILSS	AFIETPTTLY	TASHGASLSP	RHGGKDLTRA	VODITAAKPR
	LKGLPPLNR	KKEMVAKDRA	DEILEETILR	GPEDMKPRV	VKEEGESKL	EPGDEEASPP
	TQGAPEPVP	DGGKISKAFE	KLGMHIEKV	KGPKEPPEA	DIYTKGRVYM	VSGDGSFVDP
	GFCVFSVPAK	GVVVSIGDD	SVPPDSGVEP	SPQQPEPPEL	EGQGPQOEKE	DGLKEGGGPP
	EGKGEPEPK	GDSVKEGGP	PEGKDGVEK	EGGPPEGGKD	GVKEEGGPPE	GKGDGVKKEG
	EPPEGKGEGL	KEEGEGLQEK	EDGRPTTTPH	ADKGDENAK	ELKGLQKQD	DQKEEGARGP
	CMVAPGPEG	PSTPRSQGPQ	VILGSGSEHG	ARSGSLRLS	PERKLAYEKV	EVMESEKFS
	TESIQTYEET	AVIVETMIEK	TKANKKKLGE	KGSSSA		
B	STRVVVDA	PAGASSMPL	QRHKASFR	QSPSSLDGLE	ASRTVAVSGI	VTRPRVYVGM
	APSGPTGGLG	ARVTRRALGI	SSVFLQGLRS	SLGATAPAPS	LERDILGAVED	LGGLVVEYMA
	KVHLEKVSQ	ELAAQLRMHL	ESKATRSNW	GALRASWASS	QQVGVAVLE	NARIMLOTEN
	IQAGADDFKE	RYENBQPRK	AAEEBINSLY	KVIDEANSK	MDLESQTESL	KEELGELRS
	YEDDVMLYK	QLAGSELEQL	NVPIGTGLDD	ILETIRTHWE	RIVEKRLQA	GALLQAKQQA
	ELARRAQTE	EKLAAALRVE	LHNTSCQIOS	QAETESLRA	LKRGLENTLH	DAKHWHDEL
	QNLGAVVSR	EALREMRAR	AEQQIQAREH	LISHKCOLQR	DVASYHALID	REBSS

FIGURE 2. The protein sequence coverage map of filensin and CP49. The sequences of filensin (A) and CP49 (B) that were detected by LC-MS/MS analysis through a combination of trypsin, Lys C, and Glu C digestion are *underscored*. The residues of filensin that do not match the database sequence entry are *shaded*.

mass and tandem mass spectra supports the finding that residues 624–625 of filensin are Arg-Pro as reported by Gounari et al.,⁸ instead of the Gln-Ser present in the database. The high-resolution full mass spectra and the tandem mass spectra of the singly phosphorylated and nonphosphorylated peptides 621–637 of filensin are shown in Supplementary Figure S4.

Identification of Phosphorylation Sites in Filensin and CP49

To identify the phosphorylation sites in filensin and CP49, we analyzed enzymatically digested peptide mixtures by LC-MS/MS. We looked for the phosphorylated peptides in CID mode by searching for the characteristic signal of the neutral loss of H_3PO_4 . Neutral loss of H_3PO_4 from phosphorylated peptides typically dominates tandem mass spectra, resulting in insufficient fragmentation in CID mode for identification of the specific site of modification. In addition, an element of uncertainty arises when assigning the site of phosphorylation when there are several potential phosphorylation sites in close proximity. In these cases, the relatively new technology of ETD was used to confirm the sites of phosphorylation. Combining the data from both the CID and the ETD modes, phosphorylation sites were detected on either serine or threonine residues in filensin and CP49. Addition of 80 Da to tyrosine 258 in filensin was detected in some, but not all, samples, which may indicate that either phosphorylation or sulfation of tyrosine occurs. No further confirmation was attempted. Identified phosphorylation sites on filensin and CP49 are summarized in Tables 1 and 2, respectively. For each phosphorylation site identified, a

TABLE 1. Identified Phosphorylated Peptides in Filensin

Phosphorylation Sites	Observed Peptides	Enzyme
S5	5–11	Trypsin
S339*	331–341, 318–341	Trypsin
S418	410–434, 400–434	Trypsin
S511	491–534	Lys C
T627	621–637, 624–640	Trypsin, Lys C
T673*	659–675, 664–675	Trypsin
S700	697–703, 697–709	Trypsin
S753	748–756, 747–756	Lys C
S754	748–756, 747–756	Lys C

* Sites of phosphorylation confirmed by ETD.

TABLE 2. Identified Phosphorylated Peptides in CP49

Phosphorylation Sites	Observed Peptides	Enzyme
S2 or T3 single	2-24	Lys C
S26	23-28	Trypsin
S32*	29-43	Trypsin
S35*	29-43	Trypsin
S32+S34 double	29-43	Trypsin
S32 or S34+S35 double	29-43	Trypsin
T53	44-55	Trypsin
S90	90-103	Trypsin
S100	90-121	Trypsin
S415 or S416 single	401-416	Trypsin

* Sites of phosphorylation confirmed by ETD.

supporting tandem mass spectrum is presented in Supplementary Figures S5 to S20.

Nine phosphorylation sites were identified on bovine filensin. With the exception of S5, all phosphorylation sites identified are located in the tail domain of filensin. S5, S339, and S511 are highly conserved sites among bovine, human, rat, and mouse sequences. Examination human lens proteomics data, acquired previously for other purposes, revealed phosphorylation on S5 and S511 of filensin (data not shown). Six of the nine phosphorylation sites observed on bovine filensin are present in a Ser/Thr-Pro motif, a putative consensus site for a proline-directed kinase.

A total of nine potential phosphorylation sites were identified on bovine CP49. Two singly phosphorylated peptides, 2-24 and 401-416, were detected in CID mode, but the sites of phosphorylation could not be distinguished between S2 or T3 and S415 or S416. Unfortunately, the 401-416 peptide was not detected in ETD mode to help to assign the exact site of phosphorylation, and the Lys C digest that produced the 2-24 peptide was not analyzed in ETD mode. Except for the S415/S416 site, all the other identified phosphorylation sites on CP49 were clustered in the head domain. Among these phosphorylation sites, S26, S32, S34, S35, and S90 are highly conserved in different species (human, bovine and mouse); however, more analyses are needed to confirm phosphorylation at these sites

in other species. Unlike filensin, only S32 and T53 possess the Ser/Thr-Pro motif.

Identification of Truncation Sites in Filensin and CP49

To identify the truncation sites of filensin and CP49, the LC-MS/MS raw data were searched with Sequest for partial enzymatic cleavage (i.e., one end of the peptide results from non-specific cleavage). Many nonspecifically cleaved peptides from both filensin and CP49 were detected. A list of these peptides can be found in Supplementary Table S1, <http://www.iovs.org/cgi/content/full/51/3/1565/DC1>. The relative abundances of these peptides were estimated by calculating the ratio of their peak areas to the areas of the corresponding fully tryptic peptides. The signals for the putative truncated peptides detected in the gel bands were weak where the estimated relative abundance of them was below 1% except for peptides 263-276 and 401-414 of CP49, which reached 3% to 4%. All the peptides listed in Supplementary Table S1 were present in bands corresponding to both the full-length protein and the truncated fragments, and the signals for these peptides were not significantly different between both bands. Therefore, these peptides do not provide information regarding the major truncation sites of filensin and CP49 and they could be due to nonspecific enzymatic cleavage or sample preparation.

In addition to the low-abundance nontryptic peptides, the filensin peptides 410-431 and 400-431 were detected in significant abundance only in the 53-kDa band, not in the 100-kDa band. In addition, fully tryptic peptides 400-434 and 410-434 were detected only in the 100-kDa band, but not in the 53-kDa band. The selected ion chromatograms (SICs) of these peptides are shown in Figure 3. These data indicate that D431 is a major truncation site in filensin. The calculated molecular mass of the N-terminal truncated fragment is 49,209 Da, without considering posttranslational modifications, which is close to the estimated gel molecular mass.

Earlier studies have shown that the 53-kDa band contains two truncated fragments of filensin corresponding to an N-terminal non- α -helical/rod domain and a non- α -helical C-terminal domain.¹⁷ In the present study both filensin C- and N-

FIGURE 3. SICs from LC-MS/MS analysis indicate filensin peptides derived from digestion of (left) 100- and (right) 53-kDa gel bands. SICs of expected filensin tryptic peptides 410-434 and 400-434 (top rows) and the truncated peptides 410-431 and 400-431 (bottom rows) are plotted. *Peaks of interest. Intact tryptic peptides 410-434 and 400-434 were detected in the 100-kDa band, but not in the 53-kDa band. The truncated peptides 410-431 and 400-431 were detected in the 53-kDa band, but not in the 100-kDa band.

FIGURE 4. SICs from LC-MS/MS analysis indicate CP49 peptides derived from digestion of (left) 45- and (right) 41-kDa gel bands after chemical demethylation. The SICs of the expected tryptic peptide 29–43, the truncated dimethylated peptide 38–43, and expected tryptic peptide 44–52 of CP49 are plotted. *Peaks of interest. The signal of full tryptic peptide of 29–43 was much higher in the 45-kDa band than in the 41-kDa band (top row), whereas the signal of N-terminal dimethylated (truncated) 38–43 was much higher in the 41-kDa band than in the 45-kDa band (middle row). The signals of the expected tryptic peptide 44–52 in the 45-kDa band and in the 41-kDa band were similar (bottom row).

terminal peptides were detected in the 53-kDa band. These fragments can be separated by 2D SDS-PAGE, as shown by Sandilands et al.¹⁷ and as confirmed in the present study (data not shown). As mentioned, several nonspecific tryptic peptides in the region 318–341 of filensin were detected, and truncation in this region results in a C-terminal fragment with a molecular mass of ~50 kDa. We tested whether the low abundant nontryptic peptides described earlier were due to nonspecific trypsin cleavage or to *in vivo* truncation by chemically dimethylating the N terminus of the proteins before urea extraction. After searching for the N-terminal dimethylated peptides, we found no N-terminal dimethylated filensin peptides. However, the reductive amination reaction was successfully completed because all the lysine residues in the protein were dimethylated. These results suggest that the low-abundance peptides were not generated by *in vivo* mechanisms.

The nonspecifically cleaved CP49 peptide 38–43 was identified as dimethylated after reductive amination. This peptide was detected in both the 45- and 41-kDa bands; however, the signal of this peptide in the 45-kDa band was very weak compared with the strong signal observed from the 41-kDa band (Fig. 4). The SICs of the fully tryptic CP49 peptides 29–43 and 44–52 are also shown in Figure 4. These data indicate that the 29–43 peptide is dramatically decreased in the 41-kDa band, but the 44–52 peptide did not change significantly. This result suggests that CP49 truncation at D37 forms the 41-kDa fragment. The calculated molecular mass of the remaining 38–415 fragment is 42,068 Da, which is consistent with the estimated gel molecular mass.

Identification of N-terminal Acetylated or Myristoylated Filensin Truncation Products

Both previous reports^{17–19} and our results support the finding that truncation of filensin results in the formation of both C- and N-terminal fragment(s) of ~50 kDa; however, the reductive amination experiment did not allow detection of any dimethylated peptides from filensin. A possible reason for this is that the newly formed N-terminal amino group of C-terminal fragment has been modified. By searching for N-terminal mod-

ified peptides, an N-terminal acetylated peptide of filensin (residues 40–48, AALQGLGER) was detected in the 100-kDa band. Acetylation of A40 of filensin was also confirmed by Glu C digestion of the 100-kDa band. The high-resolution mass measurement results are shown in Table 3, and the tandem mass spectra of these peptides are shown in Figure 5. The presence of the acetylated peptide 40–48 of filensin suggests that filensin is truncated at Leu39 and that the newly formed N terminus is subsequently acetylated *in vivo*. Truncation at L39 results in the loss of 4610 Da, a mass difference that cannot be well resolved from the full-length filensin in the gel; therefore, this fragment was probably cut from the gel along with intact filensin. Analysis of the MS/MS data from the tryptic digest of the 45-kDa band revealed that the N-terminal acetylated 40–48 peptide of filensin was present in the 45-kDa band, probably due to truncation at both L39 and D431.

Acetylated A40 of filensin is not the N-terminal residue of the 53-kDa C-terminal fragment, and the acetylated 40–48 peptide was not detected in the 53-kDa band. The absence of the 410–434 fully tryptic peptide in the 53-kDa band suggests that the C-terminal fragment of filensin results from truncation at D431. Data analysis revealed that the peptide containing residues 432–435 of filensin (GGK) was present in the tryptic digest of the 53-kDa band and that the N terminus of the peptide was modified by a mass of 210 Da. This signal was not detected in the 100-kDa band. The selected ion chromatogram of this peptide is shown in Supplementary Figure S21. The tandem mass spectrum shown in Figure 6, top, confirmed the

TABLE 3. Measured and Predicted Masses of N-Acetylated and N-Myristoylated Filensin Peptides

Peptides	Modification	Predicted [M + H] ⁺ *	Measured [M + H] ⁺ *
40–48: AALQGLGER	N-acetylation	956.516	956.514
432–434: GGK	N-myristoylation	471.354	471.352
432–440: G GKISKAFE	N-myristoylation	1146.714	1146.713

* Monoisotopic masses.

FIGURE 5. Tandem mass spectra of the Glu C peptide acetyl-AALQGLGE (40–47) (*top*) and the tryptic peptide acetyl-AALQGLGER (40–48) (*bottom*) of filensin are plotted. Tandem mass spectra are labeled with the predicted b- and y-ions. All b-ions are shifted in mass by 42 Da from their expected m/z values.

sequence; however, the peptide 432–435 is very short, and it is difficult to assign the sequence with confidence. The 53-kDa band was then digested by endoproteinase Glu C. The tandem mass spectrum of the Glu C peptide 432–440 (GGKISKAFE) is shown in Figure 6, bottom. The tandem mass spectral data confirm the sequence and identify the site of the 210-Da modification as the N-terminal G432 residue. The accurate mass data for both the tryptic 432–434 peptide and the Glu C 432–440 peptide were obtained (Orbitrap mass spectrometer; ThermoFisher), and the results are shown in Table 3.

The accurate mass of these peptides indicates an additional mass of 210.199 Da to the predicted peptide molecular mass. Based on the mass and the significantly increased hydrophobicity of these modified peptides indicated by HPLC elution times, this modification is assigned as addition of myristate to the N-terminal amine. This myristoylated peptide was also detected in the UIF (Supplementary Fig. S22), which indicates that this C-terminal fragment of filensin has strong membrane-binding properties. Of note, a similar myristoylated peptide was detected in a human lens

FIGURE 6. Tandem mass spectra of the tryptic peptide myristoyl-GGK (432–434) (*top*) and the Glu C peptide myristoyl-GGKISKAFE (432–440) (*bottom*) of filensin are plotted. Tandem mass spectra are labeled with the predicted b- and y-ions. All b-ions are shifted in mass by 210 Da from their expected m/z values.

FIGURE 7. ETD MS/MS spectra of filensin peptide 410–434 containing isoaspartic acid residue D431 (*top*) and aspartic acid residue D431 (*bottom*) are labeled with the predicted c- and z-ions. *Boxed labels:* isoaspartic acid diagnostic ions.

sample after truncation at D433 (the homologous site for bovine D431; data not shown).

Isomerization of Aspartic Acid Residues

It is interesting to note that the major sites of truncation for both CP49 and filensin are located at aspartic acid residues. Inspection of the chromatographic data in Figures 3 and 4 reveal multiple peaks for peptides 410–434 of filensin and 29–43 of CP49. The molecular masses of the peptides in the multiplets were same (± 0.5 Da). This result suggests that

isomerization of aspartic acid has occurred. To confirm the isomerization of aspartic acid, the trypsin digests of both the 100- and 45-kDa band were analyzed by HCT with ETD. According to O'Connor et al.,²² electron capture dissociation (ECD), a technique similar to ETD, allows differentiation of isoaspartic acid and aspartic acid residues by using the c+57 and z–57 peaks. The ETD tandem mass spectra of the 410–434 peptide of filensin in distinctly separated peaks are shown in Figure 7 and the tandem mass spectra of the 29–43 peptide of CP49 are shown in Figure 8. In the ETD tandem mass

FIGURE 8. ETD tandem mass spectra of CP49 peptide 29–43. ETD MS/MS spectra of 29–43 of CP49 containing isoaspartic acid residue D39 (*top*) and aspartic acid residue D39 (*bottom*) are labeled with the predicted c-, z-, and y-ions. *Boxed label:* isoaspartic acid diagnostic ion.

spectrum of the early eluting filensin 410–434 peptide, characteristic z4–57 and c21+57 fragments indicate the presence of isoaspartic acid. Although present in low abundance, these fragments were not present in the MS/MS spectrum of the 410–434 peptide that eluted later. Similarly, the z7–57 fragment was present in the MS/MS spectrum of the early eluting 29–43 peptide of CP49, but not in the later fraction. These results indicate that isomerization of aspartic acid occurs at D37 of CP49 and D431 of filensin. Figure 3 also shows that there is only one isomer of D431 in the truncated filensin N-terminal fragment. These results do not allow distinction between D-isoaspartic acid or L-isoaspartic acid.

DISCUSSION

The experimental approach presented herein permitted the analysis of posttranslational modifications of the lens fiber cell beaded filament proteins filensin and CP49, known to be phosphorylated and to undergo truncation with fiber cell differentiation and age.¹⁵ It has been reported that phosphorylation of intermediate filament proteins plays an important role in regulating IF protein dynamics including: protein solubility, localization, turnover, and filament organization.^{23–25} In the present study, bovine filensin and CP49 were found to be extensively phosphorylated on sites S339, T627, and T673 of filensin and on S32 of CP49. Further study is needed to determine the functions of each site of phosphorylation identified in this report.

Six of the nine identified phosphorylation sites on filensin are on serine or threonine residues preceding a proline (Ser/Thr-Pro) and two phosphorylation sites on CP49, S32, and T53, are present in the same motif. NetPhosK²⁶ predicts that five of six phosphorylation sites in the Ser/Thr-Pro motif in bovine filensin are potentially phosphorylated by cdk5, a proline-directed kinase that is present and active in the lens.²⁷ S5 in filensin is present in a typical protein kinase A phosphorylation site (RRS) and phosphorylation on S5 was confirmed in this report.

According to previous reports, phosphorylation sites of intermediate filaments are clustered in the head and/or tail domain, and the central rod domain lacks any characterized *in vivo* phosphorylation sites.²³ Most of the phosphorylation sites of filensin are located in the tail domain, whereas most of phosphorylation sites of CP49 clustered in the head domain. No phosphorylation sites were identified in the central rod domain, with the possible exception of the variably observed Y258 site on filensin.

Previous research determined that filensin is proteolytically processed during fiber cell differentiation and that two major fragments are formed that may derive from the two major subdomains of filensin; the C-terminal non- α -helical tail domain and the N-terminal non- α -helical/rod domain. In this study, the major truncation site of filensin was detected as D431, a residue located in a highly conserved C-terminal motif PEDVP-DGxxISKAF. Truncation at D431 results in the formation of two fragments: one contains the head domain, rod domain, and a small part of the tail domain, and the other contains the remaining tail domain. The calculated molecular mass for the truncated N-terminal fragment is 49,209 Da, without considering posttranslational modifications. Masaki and Quinlan reported a molecular mass of 49,610 Da of the filensin fragment measured by mass spectrometry.¹⁸ The mass difference between these two numbers suggests the existence of posttranslational modifications. The calculated molecular mass of the C-terminal fragment produced from truncation at D431 is 34,012 Da which is not consistent with its 53-kDa position in the gel. Considering the anomalous behavior of intact filensin

during gel electrophoresis (predicted 83-kDa protein runs at 100 kDa), it is reasonable to assume that this anomaly is due to the C terminus of filensin, since the N-terminal fragment did not show a dramatic shift in the gel from its predicted molecular mass. The 17-kDa mass difference observed for full length filensin is consistent with that observed for the C-terminal fragment; however, the cause of this anomalous behavior in gel electrophoretic mobility remains unknown.

Two unexpected posttranslational modifications were detected on filensin fragments. N-terminal acetylation and N-terminal myristoylation occur on newly formed N termini after truncation making this processing a posttranslational event. Typically, both N-myristoylation and N-acetylation are irreversible protein modifications that occur co-translationally after remove of an initial methionine. Postproteolytic acetylation and myristoylation of a newly formed N terminus has been reported in only a few proteins, including proapoptotic BID after cleavage by caspase 8.²⁸ Myristoylation of the newly exposed N terminus of caspase-cleaved proteins was also found in actin, gelsolin, and p21-activated protein kinase 2 (PAK2).^{29,30} Since several of these myristoylated proteins are either cytoskeletal proteins or cytoskeletal-regulated proteins, it has been suggested that posttranslational myristoylation may play a general role in the regulation of cytoskeletal structure during apoptosis.³⁰ The filensin result in the present study provides another example of a cytoskeletal protein with postproteolytic N-myristoylation and suggests a role for filensin during lens fiber cell differentiation, an apoptosis-like event. In addition, the major truncation site of filensin, D431, is in a consensus motif (DXXD) for group II caspases (caspase 2, 3, and 7).³¹ Note that caspase 3-like activity has been detected during lens fiber cell differentiation.³²

The membrane-binding properties of the filensin C-terminal fragment have been examined previously where a change in cellular localization during fiber cell differentiation was observed.^{17,20,33} The C terminus of filensin was found to be primarily located in the cytoplasm of newly formed fiber cells and in the membrane regions of the older fiber cells of the nucleus, whereas the N terminus was found to relocate from the membrane to cytoplasmic regions with the fiber cell differentiation.¹⁷ The reasons that the polar C-terminal fragment of filensin binds strongly to the membrane fractions has remained unknown.¹⁷ Typically, N-myristoylation results in alterations in protein/protein interactions, membrane binding, and targeting.^{28,34–36} Thus, N-myristoylation of G432 of filensin is expected to play an essential role in the relocation of this C-terminal fragment from the cytoplasmic region to the membrane. In addition, a cluster of basic residues is positioned adjacent to the myristoylated G432 in filensin (GKISKAFK-LGKMIKEKVKGPK). This polybasic stretch of amino acids may form electrostatic interactions with acidic phospholipids in the membrane and act as a second signal that functions to enhance membrane association of myristoylated proteins.^{37,38} Therefore, myristoylation of G432 together with the cluster of basic residues ensures the membrane association of filensin C-terminal fragment.

Posttranslational N-acetylation has been reported for a very few proteins, such as actin³⁹ and β -endorphin⁴⁰; however, the unique N-terminal processing of actin seems actin-specific. The biological significance of N-terminal acetylation varies with the particular protein and the functional consequence of N-acetylation of filensin should be studied further. Both postproteolytic N-acetylation and N-myristoylation of filensin show similarities to co-translational N-acetylation and N-myristoylation. For example, N-terminal acetylation of filensin occurs on an alanine residue, which is one of the most frequently acetylated N-terminal residues.⁴¹ N-myristoylation of filensin occurs on glycine residues within a consensus sequence for N-myristoyl

transferase, G-X-X-Ser/Thr-Arg/Lys.⁴² Thus, it is anticipated that similar *N*-acetyltransferases or a myristoyltransferase are at work in the posttranslational processing of filensin.

Isomerization of aspartic acid residue to isoaspartate was detected in both filensin and CP49. Isomerization and racemization of aspartic acid residues is commonly observed in aged proteins of the lens, teeth, erythrocytes, and brain.^{43,44} Extensive isomerization and racemization of aspartic acid residues in α -crystallins has been reported where D-iso-Asp was formed.⁴⁵ Both D431 in filensin and D37 in CP49 were observed to be highly isomerized. The D- and L-stereoisomers of isoaspartic acid could not be distinguished by the current data. In general, the Asp residues most likely to be isomerized are those located in the flexible regions or those that are flanked by Gly or Ser at their C terminus.⁴⁶ Both D431 and D37 are followed by glycine; however, the isomerization in filensin shows site specificity. There are eight other Asp residues in filensin followed by Gly, and no isomerization of Asp was detected for those Asp residues. Truncation at susceptible asparagine and aspartic acid residues in the human may be due to age-related succinimide formation and spontaneous backbone cleavage.⁴⁷ That major truncation sites of D431 and D37 undergo isomerization suggests that the mechanism of truncation at these sites is due to nonenzymatic spontaneous chemical cleavage; however, the consensus caspase substrate motif of filensin 428–431 (DVPD) and subsequent *N*-myristoylation of G432 indicates a possible specific enzymatic cleavage process.

In summary, we systematically studied phosphorylation and truncation sites on filensin and CP49 and identified two unusual modifications, postproteolytic *N*-myristoylation and *N*-acetylation. These modifications provide detailed information to further our understanding of the structure and functions of these lens-specific intermediate filament proteins. In a retrospective examination, some of these modifications have been detected in human filensin; however, an exhaustive proteomics study remains to be performed.

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