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Phosphorylation and Truncation Sites of Bovine Lens Connexin 46 and Connexin 50

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Abstract

Connexins 46 and 50 combine to form the gap junctions in ocular lens fiber cells. These proteins are known to be modified with fiber cell age; however, limited work has been done to characterize specific lens connexin modifications. In this report, bovine lens membrane proteins were isolated, digested by multiple enzymes, and analyzed by HPLC-tandem mass spectrometry. Automated database searching revealed the locations of both phosphorylation and truncation sites. The results confirmed the full sequence of connexin 46 and 99% of the connexin 50 sequence. Eighteen phosphorylation sites on connexin 50 and nine phosphorylation sites on connexin 46 were identified, all on serine or threonine residues. All but three phosphorylation sites on connexin 50 were located the cytoplasmic C-terminus. All of the truncation sites of connexin 50 were localized in the cytoplasmic C-terminus (region 280–304). Truncation sites in connexin 46 were found in four different regions including: the N-terminus (residues G2), the cytoplasmic loop (residues 121–124), the cytoplasmic C-terminus (residues 251–285), and the distal C-terminus (residues 344–395). In an analysis of dissected lenses some truncation sites were specific to nucleus samples and others were detected in both nucleus and cortex samples.

Keywords

Lens fiber cells; connexin50; connexin 46; phosphorylation; truncation; mass spectrometry; proteomics

Introduction

Connexin proteins combine to form gap junctions; one of the two types of network junctions in the lens which directly couple the cytoplasm of neighboring cells to facilitate the intercellular transport of essential ions and metabolites. Of the three lenticularly expressed connexins, Cx43, Cx46 and Cx50, the latter two are expressed in fiber cells while Cx43 is expressed in lens epithelial cells. Connexin 46 and connexin 50 have been shown to be important in normal lens growth and maintenance of lens transparency (Gong et al., 1997; Rong et al., 2002; Sellitto et al., 2004). Specifically, mutations in the Cx46 and Cx50 genes are associated with the development of cataracts (Graw et al., 2001; Mackay et al., 1999). Moreover, disrupting the

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Cx46 gene results in the development of nuclear cataracts (Gong et al., 1997) and mice deficient in Cx50 not only develop cataracts, but also exhibit microphthalmia (White et al., 1998).

Both Cx46 and Cx50 are phosphorylated proteins (Jiang et al., 1993; Voorter and Kistler, 1989) and undergo truncation in the lens core (Kistler et al., 1990; Lin et al., 1997). Connexin phosphorylation has been implicated in the regulation of intercellular communication. For example, phosphorylation of connexin 43 has been found to be involved in gap junctional communication through a number of mechanisms, including connexin biosynthesis, trafficking, assembly, membrane insertion, internalization, and degradation (reviewed by Lampe and Lau, 2004). Phosphorylated carboxy terminal serine residues of connexin 45 were found to stabilize the mouse gap junction protein connexin 45 against degradation (Hertlein et al., 1998) and phosphorylation of connexin-32 prevented its proteolysis by calpain (Elvira et al., 1993). However, phosphorylation of connexin 45.6, the chicken homologue of human Cx50, was found to promote in its degradation (Yin et al., 2000) and inhibit cleavage of the connexin via a caspase protease (Yin et al., 2001). Moreover, phosphorylation of connexin 50 was found to regulate the cell-to-cell communication by disassembling the channels (Berthoud et al., 2000; Zampighi et al., 2005).

Connexin 43 is the most studied connexin regarding phosphorylation; however, little is known about the specific phosphorylation sites of Cx50 and Cx46 and their functional roles. The chicken homologue of human Cx50 can be phosphorylated at S363 by casein kinase II (Yin et al., 2001). The chicken homologue of Cx46 has been shown to be phosphorylated at S118 and S493 in cells and activation of PKC led to increased phosphorylation at S118 and decreased communication in a PKCγ dependent manner (Berthoud et al., 2000). However, S363 in Cx 45.6 and S118 and S493 in Cx 56 are not conserved sites in different species and many sites in both Cx50 and Cx46 are predicted to be potential phosphorylation sites.

The C-terminal regions of Cx50 and Cx46 may play important roles in channel gating and permeability (DeRosa et al., 2006; Xu et al., 2002); however, the exact roles are not entirely elucidated. For example, cleavage of the C-terminus of Cx50 has been reported to greatly reduce pH sensitivity and decrease junctional conductance; a phenomenon that could link the endogenous cleavage of Cx50 to the observed 50% decrease in junctional coupling during fiber maturation (DeRosa et al., 2006; Bennett et al., 1991; Xu et al., 2002). In contrast, other data have shown the persistence of pH gating after C-terminal truncation (Stergiopoulos et al., 1999). One possible reason that could contribute to these conflicting results is the different Cx50 isoforms studied. Calpain cleavage of Cx50 in sheep lenses occurs at residues 290 and 300 (Lin et al., 1997) while truncation at residue 365 was reported for chicken Cx50 (Yin et al. 2001). Cleavage of the C-terminus of Cx46 was reported to result in changed gap junction plaque distribution and dye coupling in rat lens (Jacobs et al., 2004); however, little is known about the sites of truncation in Cx46.

As described above, phosphorylation and truncation likely play important roles in the regulation and function of lens connexins; therefore, it is crucial to elucidate sites and potential functional roles of these modifications. Recently, several posttranslational modifications of bovine lens fiber connexins were reported (Shearer et al., 2008). Several phosphorylation and truncation sites were identified; however, protein sequence coverage was poor in the C-terminus of both connexins where extensive phosphorylation and truncation was expected to occur. In the present study, multiple enzymatic digestions and liquid chromatography (LC) - electrospray tandem mass spectrometry (ESI/MS/MS) analysis was employed to map connexin sequences and to identify phosphorylation and truncation sites. This study provides a comprehensive analysis of connexin modifications from bovine lens fiber cells using state-of-the-art proteomics methods.

Material and Methods

Materials

Bovine lenses (one year old or older) were obtained from PelFreez Biologicals (Rogers, AK). Ammonium bicarbonate, sodium fluoride, EDTA, urea, DTT, sodium hydroxide, trifluoroacetic acid (TFA), sequence-grade endoproteinase Lys-C and pepsin were purchased from Sigma (St. Louis, MO). All HPLC grade solvents were purchased from Fisher (New Jersey, NJ). Sequence-grade modified trypsin was obtained from Promega (Madison, WI). Sequence-grade endoproteinase Glu-C was obtained from Roche Diagnostics Corporation (Indianapolis, IN).

Lens Membrane protein preparation

Lens membrane proteins were prepared from whole lens homogenates according to the procedure of Goodenough with slight modification (Goodenough, 1979). In one experiment a frozen bovine lens was decapsulated and dissected into cortex and nucleus regions prior to homogenization. Tissue was homogenized in 50mM ammonium bicarbonate buffer containing 5 mM EDTA, 10 mM NaF and 1 mM DTT, pH8 and centrifuged at 88,000 *g* for 30 mins and the supernatant was discarded. The pellets were washed three times with 50mM ammonium bicarbonate buffer containing 7M urea, 5 mM EDTA, 10 mM NaF and 1 mM DTT, pH8 followed by triplicate water washes. The remaining pellets were reduced and alkylated by incubating the pellets for 45min. at room temperature with 6 μL tributyl phosphine and 6 μL 4-vinylpyridine in 400 μL 1:1 n-propanol:1.5 M Tris (pH7.3). The excess reagents were removed by triplicate water washes of the pellets. The pellets were then delipidated in 95% ethanol at −20°C overnight followed by water wash (3X).

Enzyme digestion

Trypsin digestion was done in 10% acetonitrile / 90% ammonium bicarbonate buffer (pH8.0) as described by Han & Schey (Han and Schey, 2004). 0.5 μg of trypsin was used to digest membrane proteins derived from 1/3 of the bovine lens cortex sample. The digestion was performed at 37 °C for 24 hours. For Glu-C digestion, 1μg of Glu-C was added to membrane proteins from 1/3 of the lens cortex sample in 200μL 50mM ammonium bicarbonate buffer, incubated at RT for 24 hours. For Lys-C digestion, 0.5 μg of Lys-C was added to membrane proteins from 1/3 of the lens cortex sample in 200μL 50 mM ammonium bicarbonate buffer, incubated at 37 °C for 24 hours. For pepsin digestion, 10μL of formic acid was added to the membrane proteins from 1/3 of the lens cortex sample, vortexed and 90 μL of water was then added. 2 μg of pepsin was then added to the proteins in 10% formic acid. The solution was incubated at RT for 6 hours. Trypsin, Glu-C, and Lys-C digestions were stopped by adding TFA to a concentration of 0.1% and all of the digests were dried on the SpeedVac. For truncation site identification, nucleus samples were digested by trypsin or Glu-C. 0.5 μg of trypsin was used to digest membrane proteins derived from 1/3 of bovine lens nucleus sample and 1μg of Glu-C was added to membrane proteins from 1/3 of the lens nucleus sample. The digest conditions were the same as described above. All of the digestion was done in a dry bath incubator with occasional shaking.

NanoLC-ESI/MS/MS

Enzyme digested peptides were reconstituted in H_2O (0.1% TFA) and analyzed by HPLC-ESI-MS. Peptides were separated on an Ultimate Nano-LC system equipped with a Switchos unit for sample loading and desalting (Dionex, Germany). Samples were either desalted on a trap column (PepMap, C18, 5μm particle size, 300 μm i.d. × 5mm, 100 Å pore size) for 6 mins with 40 μL/min flow rate prior to loading on the analytical column (PepMap C18, 150 mm × 75 μm, 3 μm particle size, 100 Å pore size, Dionex, Germany) or directly loaded onto the analytical

column without desalting to observe very hydrophilic peptides. The following gradient was used for HPLC separation: 2–50% solvent B (ACN/0.02% HFBA) from 0–80 min. and 50–95% solvent B from 80–100 min. balanced with solvent A (0.02%HFBA in water) at a flow rate of 0.18 μL/min. The eluate was directly infused into a nano-ESI-LTQ mass spectrometer (ThermoFisher, San Jose, CA). The mass spectrometer was operated in data dependent mode with the top 5 most abundant ions in each mass spectrum being selected for subsequent MS/MS scans. Mass spectra were typically acquired over a m/z range of 360–2000. Dynamic exclusion (repeat count 2, repeat duration 0.5 min., exclusion duration 3 min) was enabled to allow detection of less abundant ions in order to detect lower abundance proteins. Major peptides derived from aquaporin 0, the major intrinsic membrane protein in lens fiber cells, were excluded from the analysis by placing their m/z values on an exclusion list in the Xcalibur (ThermoFisher) method file.

Data analysis

All MS/MS spectra were searched using the Sequest algorithm as part of the Bioworks 3.3 software package (Thermo Finnigan, San Jose, CA) using a bovine lens protein NCBI database. Peptide identifications were made according to the following criteria: cross-correlation values were at least 1 for +1 charged ions, 1.5 for +2 charged ions and 2 for +3 charged ions. Potential posttranslational modifications including acetylation, deamidation, phosphorylation, Met oxidation, and truncation were considered. All the identified peptides and posttranslational modifications presented in this paper were manually verified. To identify the truncation sites, raw data were subjected to Sequest searches with the partial enzymatic cleavage setting.

Results

Protein coverage of connexin 50 and connexin 46

Although connexins are not the most abundant proteins in the lens membrane protein mixture, under our experimental conditions i.e. by excluding AQP0 peptides, analysis of the trypsin digestion mixture gave 87% coverage for Cx50 and 99% coverage for Cx46. Due to this extensive sequence coverage no additional purification steps, which could cause loss of modifications, were carried out. However, the N-terminal tryptic peptides of Cx50 are large hydrophobic membrane spanning peptides that are potentially difficult to reconstitute in aqueous solution. Moreover, the distal C-terminus of both proteins (about 20 amino acids), contains many trypsin cleavage sites which results in very small peptides that can also be challenging to detect. Also there very hydrophilic peptides, such as 137–143 of Cx50, that could only be detected in the absence of a trap column. Because of these issues multiple enzymes, including trypsin, Glu-C and Lys-C, were employed to digest the proteins to achieve maximum sequence coverage. Ultimately, the combined analyses of trypsin, Glu-C and Lys-C digestions, produced no sequence coverage of the Cx50 region containing residues 24–76; therefore pepsin was used to digest the membrane protein preparation and the water-insoluble pellet remaining after trypsin digestion. N-terminal peptides, including 17–26, 31–37, 33–42, 43–51, 52–66 and 67–77, were detected in the pepsin digest of the membrane protein mixture. These peptides were also present in water-insoluble pellet remaining after trypsin digestion. Therefore, the absence of this hydrophobic region in the tryptic digest results is due to its hydrophobicity and lack of trypsin cleavage sites. The sequence coverage of connexin 50 increased from 88% to 99%, compared to 47% coverage reported by Shearer et al. (Shearer et al., 2008), when tryptic digest results were combined with pepsin digestion results.

The protein coverage maps of Cx46 and Cx50 in bovine lens cortex are shown in Figure 1 and Figure 2, respectively. Figure 1 and Figure 2 show predominantly tryptic peptides because only the peptides that were not covered by trypsin digestion are shown for the alternative enzyme digestions. Note that, trypsin digestion of Cx46 generates some very large peptides such as

243–313 and 332–388 which lie outside of the instrumental mass range for the +2 or +3 charge states; but could be detected in the +4 or +5 charge state. Endoproteinase Glu-C and Lys-C digestion gave good sequence coverage of the whole cytoplasmic C-terminus of Cx50 and the distal C-terminus of Cx46, but did not give good sequence coverage for the membrane spanning regions.

Phosphorylation sites of connexin 50 and connexin 46

The bovine Cx46 sequence contains 29 serine residues (7.1%), 19 threonine residues (4.7%) and 10 tyrosine residues (2.5%). Based on the predicted sequence of Cx50, it contains 40 serine (9.1%), 19 threonine (4.3%) and 10 tyrosine (2.3%) residues. Many of these serine and threonine residues are predicted to be phosphorylated by NetPhos 2.0 Server (www.cbs.dtu.dk/services/NetPhos/). To determine which sites are indeed phosphorylated *in vivo*, lens membrane proteins were prepared from bovine lenses and subjected to trypsin digestion. To identify phosphorylated peptides in Cx50 and Cx46, the raw data files from the LC-ESI/MS/MS experiments were subjected to a Sequest database search that included phosphoserine, phosphothreonine and phosphotyrosine as potential modifications. The identified phosphorylation sites in Cx50 and Cx46 are summarized in Table 1 and Table 2, respectively. In these tables, the sequence column lists the shortest peptides identified containing the corresponding phosphorylated serine or threonine residues. The tandem mass spectra of all of the peptides in Table 1 and Table 2 can be found in the supplemental materials. All sequence assignments that passed Sequest analysis criteria were manually verified. Three criteria were used for a positive assignment: 1) the presence of the neutral loss of H_3PO_4; 2) 90% of the abundant peaks (> 20% relative abundance) in a tandem mass spectrum matched either b- or y-ions of the peptide; and 3) the presence of at least 3 ions in a continuous bor y-ion series.

There are several peptides in both connexins that possess more than one phosphorylation site. When phosphorylation occurs at different sites of the same peptide, these phosphorylated peptides may coelute and, in this case, one tandem mass spectrum is generated from a mixture of different phosphorylated peptides. For example, the singly phosphorylated tryptic peptide 110-125 of Cx50 was observed with evidence of phosphorylation at both S115 and S118. In addition, the well-documented dominant neutral loss of H_3PO_4 from phosphorylated peptides may result in insufficient fragmentation of the phosphopeptide backbone in the MS/MS mode. Therefore, an element of uncertainty can arise when assigning the site of phosphorylation to a specific residue. For example, the singly phosphorylated Lys-C peptide 395-407 of Cx46 was observed with possible phosphorylation at either residue 395 or 396. When this occurs, careful assignment of the site of phosphorylation was made using the criteria that each site was supported by at least two fragments which were clearly above baseline noise. Further confirmation was achieved by employing MS^3 experiments of the ($MH\text{-}H_3PO_4$) ions.

A total of 18 phosphorylation sites were identified on bovine Cx50 including S115, S118, S134, S258, S261, S265, S266 S297, S300, T326, S364, T404, S410, S424, S427, S430, S431 and S435. Most of these sites were confirmed in digests from more than one enzyme. Sites S115, S118, and S134 are located in the cytoplasmic loop of Cx50 and the others are all in the cytoplasmic C-terminal region. A total of 11 phosphopeptides of Cx46 were observed and the data allow confident assignment of 9 sites to specific residues including T238, S241, S245, S329, S334, T339, S345, S365 and S393. All of these sites are in the C-terminal cytoplasmic region. Phosphorylation on S329, T339, S345, and S365 were confirmed by more than one enzyme.

There are still several phosphorylation sites that cannot be confidently assigned to specific residues even after combining data from trypsin, Glu-C and Lys-C digestion. For example, the tandem mass spectrum of singly phosphorylated peptide 395-407 of connexin 46 indicates

phosphorylation may occur either on S393 or S395. The same situation was observed for the triply phosphorylated peptide 376-407 of connexin 46. The MS/MS spectrum of this triply phosphorylated peptide showed one phosphorylation on S393, one on S389 or S390, and one on S395 or S396. The data also support phosphorylation on serine residues in the region 138–142 of Cx50 and some residues in the region 272-283 of Cx46, but the tandem mass spectra did not provide significant information to unambiguously assign the phosphorylation sites (data not included).

Identified truncation sites of Cx50 and Cx46

To identify the truncation sites of connexins, multiple enzymes were utilized and the MS/MS spectra were searched using Sequest with partial enzymatic cleavage, i.e. one end of the peptide results from non-specific cleavage. Multiple enzymes were used because truncation C-terminal to lysine or arginine residues would not be detectable after tryptic digestion. Trypsin, Lys-C, and Glu-C were used to digest cortical samples and trypsin and Glu-C were used to digest nucleus samples. The results are summarized in Table 3 and Table 4 by listing the shortest peptides observed. Truncation of Cx50 was observed in both cortex and nucleus samples. The truncation sites identified in cortical samples included H284, E290, S300, and F304. Truncation sites identified in the nucleus include H284, H286 and E290. E290 was identified as the major site of truncation and truncation at E290 was found significantly higher in the nucleus than in the cortex. The ratio of the peak area of the Cx50 peptide 272–290 compared to the non-truncated Cx50 peptide 272–310 was 250 times higher in the nucleus samples than in the cortex samples. The selected ion chromatograms of these peptides showing integrated peak areas are presented in the supplemental materials. The signals for truncation at S300 and F304 were weak in the cortex and were absent in the nucleus probably because the protein has been extensively truncated at E290 in the nucleus.

Both C-terminal and N-terminal truncation were detected in Cx46. As expected, truncation of Cx46 in the nucleus was found to be much more abundant than in cortical samples. Truncation occurs in three regions of Cx46 in the nucleus including: N-terminal at G2, the cytoplasmic loop at G121, H122 and D124, and in the region 251–285. Truncation at G2 was only detected in the nucleus samples, but not in the cortex samples. Truncation at G2 has been confirmed by both trypsin and Glu-C digestion. Truncation at G121, H122 and D124 in the cytoplasmic loop was occasionally detected in the cortex, but repeatedly detected in the nucleus. The variability in the cortex may be due to the variability in the manual dissection of the lens. The signals of non-tryptic peptides Cx46 108–121 and 108–122 were weak, but truncation at these sites was confirmed by both trypsin and Glu-C in the nucleus. A significant amount of truncation at D124 was found in the nucleus. Non-tryptic peptides corresponding to cleavage at E344, G347 and Q352 at the distal C-terminus were also detected in the cortex; however, truncation at these sites has not been confirmed by other enzymes. A series of truncation sites were identified in the region of 251–285 in the nucleus but not in the cortex suggesting that this specific location on the protein is highly susceptible to cleavage. In fact, this may be the major truncation region of Cx46 in the nucleus because expected C-terminal peptides were present in very low amounts in the nucleus. The selected ion chromatograms of the corresponding peptides (with phosphorylation on S245) are presented in the supplemental materials. Signals for corresponding peptides without S245 phosphorylation were not always detected. Extensive truncation of Cx46 in the region of 251–285 in the nucleus may also explain why truncation at E344, G347 and Q352 was detected in the cortical samples but not in the nucleus.

Besides phosphorylation and truncation, other connexin modifications were observed including: oxidation of Met293 in Cx50, deamidation of N121 in Cx50 and N13 in Cx46, as well as partial acetylation of the N-terminal Gly in both Cx50 and Cx46 as reported by Shearer et al.(Shearer et al., 2008).

Discussion

Connexins are transmembrane proteins which are normally difficult to handle because of their hydrophobic properties. Furthermore, the presence of the abundant AQP0 protein in the mixture provides added difficulty to the study. In this report, crude fiber cell membrane proteins were analyzed without further purification of connexins and the entire sequence of Cx46 and 99% of the Cx50 sequence were mapped by multi-enzyme digestion and mass spectrometry. The results in this report confirm most of the predicted sequence of bovine Cx50. Based on the present study of multiple enzymes, trypsin produced the most extensive coverage for both connexins; however, Lys-C provided better C-terminal coverage due to the fewer AQP0 peptides produced and the longer connexin peptides produced compared with trypsin. Endoproteinase Glu-C digestion provided some complementary C-terminal sequence coverage of both connexins. Based on a previous report on the advantages of pepsin digestion of membrane proteins (Han and Schey, 2004) pepsin was used to digest the connexin samples and the previously undetected N-terminal transmembrane domain was detected.

Shearer et al. (2008) recently reported phosphorylation on 3 serine residues in cytoplasmic loop and 6 serine residues in the C-terminus of Cx50. Results of the current study confirm all of these sites. Of these nine phosphorylation sites identified in Cx50, most are conserved among human, bovine and mouse including: S134, S258, S261, S265, S266 and S297. S118 and S300 are conserved between bovine and mouse. In addition, the current work provides additional phosphorylation sites on the C-terminus of Cx50; a region believed to play important roles in channel gating and permeability (DeRosa et al., 2006; Xu et al., 2002). Most of these new phosphorylation sites are highly conserved among different species (T326, S410, S424, S427, S430 and S435) and are expected to occur in other species. Shearer et al. (2008) also reported 5 phosphorylation sites and 3 possible phosphorylation sites on Cx46. The present results confirm phosphorylation on T238, S241, S245 and S329; however, phosphorylation on T300 and T303 was not observed with confidence. The results presented in this paper add 7 additional phosphorylation sites; two (T339 and S393) on highly conserved sites among different species. Phosphorylation sites identified in the present work were observed in the bovine cortex. All of the phosphorylation sites interior to the major truncation sites in both connexins were also detected in the nucleus samples. S300 and S297 phosphorylation in Cx50 was also detected in the nucleus samples.

The C-terminal cytoplasmic region of the connexins is considered to be a regulatory domain as it is the most variable region between different connexins and contains consensus sequences for several protein kinases (Bennett et al., 1991; Saez et al., 1993). The results in this paper expand the number of modified connexin forms and provide important information for further investigation aimed at studying the regulation of intercellular communication by connexin phosphorylation. For example, cleavage of the C-terminus and simultaneous removal of the C-terminal phospho-regulatory sites in both connexins may have profound functional consequences.

Cleavage of the carboxy terminus of lens fiber cell connexins has been proposed to occur naturally during maturation of lens fiber cells and as an artifact of sample preparation. (Yin et al., 2001; Lin et al., 1997) Studies of ovine Cx50 have identified calpain as the enzyme that removes a 32-kDa portion of the carboxy tail of Cx50 and identified E290 and S300 as two cleavage sites (Lin et al., 1997). In contrast, truncation at residue 365 by a caspase-3-like protease was reported in the chicken homologue of Cx50 while truncation at E290 and S300 was suggested to be due to sample preparation (Yin et al., 2001). Recently, Shearer et al (2008) reported bovine Cx50 truncation at H284, F286 and E290. The results in this report confirm H284, F286, E290 and S300 truncation sites in bovine Cx50. Truncation sites were identified in this paper by identifying non-tryptic or non-Glu-C peptide termini which are

assumed to result from *in vivo* cleavage; however, cleavage due to non-specific enzymatic digestion during sample preparation is possible. An argument can be made against artifactual degradation in that non-tryptic peptides identified in this paper clearly showed different distributions in the nucleus samples compared with in the cortex samples. For example, a significantly higher percentage of truncation at E290 was observed in the nucleus than in the cortex, suggesting that this truncation event is not due to sample preparation. Except S300, all observed truncation sites are located in a single, highly conserved region of Cx50 which suggests that this region, between residues 280 and 300, is highly susceptible to enzymatic cleavage.

Until recently, very little was known about the truncation sites in Cx46. Shearer et al. reported truncation of Cx46 at D124 and L255. In this paper, the truncation sites of Cx46 were systematically mapped. Cleavage at D124 and L255 was confirmed; however, additional truncation sites were observed. Similar to Cx50, truncation of Cx46 starts to occur at some sites in the cortex of the lens; whereas unlike Cx50, Cx46 possesses a region where truncation occurs only in the nucleus. For example, truncation at D124 is much higher in the nucleus than in the cortex and truncation in the region 251–285 and G2 was not detected in the cortex even though all of the samples were treated in the same way. Several truncation sites identified in this paper are not in a highly conserved region such as G121, H122, L255, E344, and Q352; therefore, it is hard to predict whether truncation occurs in the similar sites of other species based on these results; however, several new truncation sites identified in this report are conserved between human and bovine such as I267, F269, Y273, A274, and A285; therefore, similar truncation in human Cx46 may be expected.

In this study, bovine lenses were dissected into cortex and nucleus regions; therefore, the results reported here are representative of a mixture of different regions of the lens. Further investigation with more precisely dissected lens regions is needed to explore the spatial distributions of connexin modification with fiber cell age. In summary, the number of sites of bovine ocular lens connexin modification has been greatly expanded to include many phosphorylation sites as well as age-related truncation sites. These results provide a wealth of information to connexin researchers, in the lens and in other tissues, to further explore the functional consequences of connexin modification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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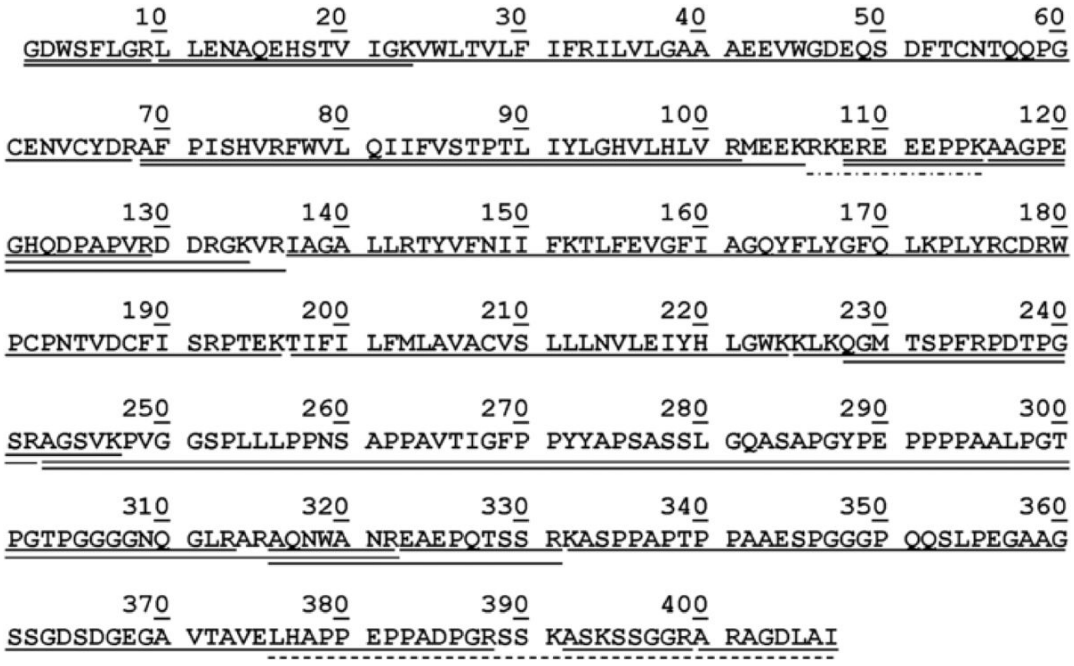

Figure 1.

The protein sequence coverage map of Connexin 46.

Trypsin peptides that were detected are indicated by solid lines (_____). Sequences that are not covered by trypsin, but by Glu-C (_ _ _) and by Lys-C (__ . _) are also included.

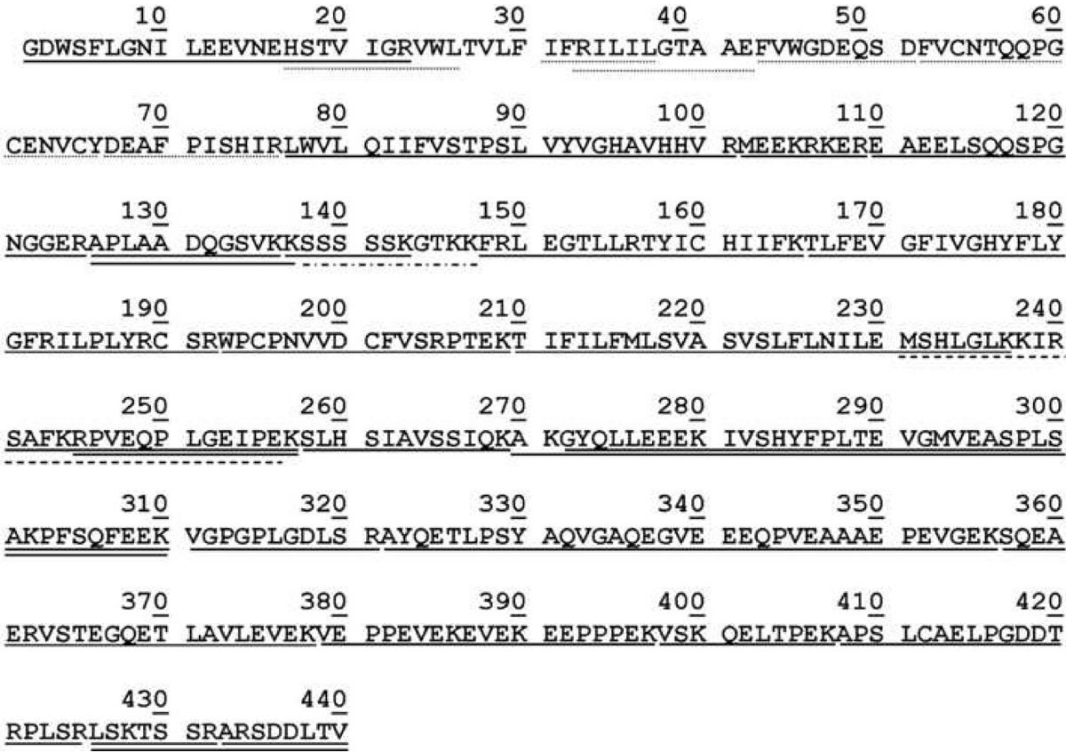

Figure 2.

The protein sequence coverage map of Connexin 50.

Trypsin peptides that were detected are indicated by solid lines (_____). Sequences that are not covered by trypsin, but by Glu-C (_ _ _), by Lys-C (__ . _) and by pepsin ( ) are also included.

Table 1

Identified phoshorylated peptides in Cx50

Phosphorylation sites	Observed Peptides	Enzyme
S115 singly	110-125	Trypsin
S118 singly	114-124	Glu-C
	108-127	Lys-C
S115 + S118 doubly	110-125	Trypsin
S134	126-136	Trypsin
S258+261 doubly	245-269	Trypsin
S265	258-269	Trypsin, Lys-C
S266	258-269	Trypsin, Lys-C
	257-278	Glu-C
S266 + S261 doubly	258-269	Trypsin, Lys-C
S297	281-310	Trypsin, Lys-C
	296-309	GluC
S300	281-310	Trypsin, Lys-C
	296-309	Glu-C
S297 + S300 doubly	281-310	Trypsin, Lys-C
	296-309	Glu-C
T326	311-356	Lys-C
S364	357-378	Trypsin, Lys-C
	360-369	Glu-C
T404	398-407	Lys-C
	403-414	Glu-C
S410	408-425	Trypsin, Lys-C
	407-414	Glu-C
T404 + S410 doubly	401-425	Trypsin
	403-414	Glu-C
S410 + S424 + S427 triply	408-428	Lys-C
S430	426-432	Trypsin
S435	433-440	Trypsin
	429-440	Lys-C
S430 + S431 + S435 triply	426-440	Trypsin

Table 2

Identified phosphorylation sites in Cx46:

Phosphorylation sites	Observed Peptide	Enzyme
T238	228-242	Trypsin
S241	228-242	Trypsin
T238 +S241 doubly	228-242	Trypsin
S245	243-255 (Truncated)	Trypsin
S329	323-331	Trypsin
	324-344	Glu-C
T339	324-344	Glu-C
S334 +S345 doubly	332-347 (truncated)	Trypsin
S334 + T339 doubly	332-344 (truncated)	Trypsin
S334 + T339 + S345 triply	332-352 (truncated)	Trypsin
S339 + S345 doubly	332-388	Trypsin
	333-391	Lys-C
	324-356	Glu-C
S365	357-375	Glu-C
	332-388	Trypsin
S 395 or S396	395-407	Lys-C
S389 or S 390 + S393 + S395 or S396 triply	376-407	Glu-C
S395 or S396	376-407	Glu-C
S395 or S393	392-407	Lys-C

Table 3

Truncation sites of Cx50

Truncation sites	Source	
	Cortex	Nucleus
H284	Trypsin (272-284), Lys C (272-284)	Trypsin (272-284), Glu-C (278-284)
F286	N/D	Trypsin (272-286)
E290	Trypsin (281-290), Lys C (281-290)	Trypsin (281-290), Glu-C (N/A)
S300	Trypsin (281-300), Lys C (281-300)	N/D
F304	Trypsin (281-304)	N/D

N/D: not detected; N/A: enzyme cleavage site, therefore, cannot be used to detect truncation at this site

Table 4

Truncation sites of Cx46:

Truncation sites	Source	
	Cortex	Nucleus
G121	Trypsin (108-121)	Trypsin (108-121), Glu-C (109-121)
H122	Trypsin (108-122)	Trypsin (108-121), Glu-C (109-122)
D124	Trypsin (116-124), Lys C (116-124)	Trypsin (116-124), N/A
G251	N/D	Trypsin (243-251)
L255	N/D	Trypsin (243-255)
N259	N/D	Trypsin (243-259)
I267	N/D	Trypsin (243-267)
F269	N/D	Trypsin (243-269)
Y273	N/D	Trypsin (243-273)
A274	N/D	Trypsin (243-274)
S278	N/D	Trypsin (243-278)
S284	N/D	Trypsin (243-284)
A285	N/D	Trypsin (243-285)
E344	Trypsin (332-344), Glu C (N/A)	N/D
G347	Trypsin (332-347)	N/D
Q352	Trypsin (332-352)	N/D
G2	N/D	Trypsin (3-9), Glu-C (3-12)

N/D: not detected; N/A: enzyme cleavage site, therefore, cannot be used to detect truncation at this site