Developmental Truncations of Connexin 50 by Caspases Adaptively Regulate Gap Junctions/Hemichannels and Protect Lens Cells against Ultraviolet Radiation*

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Background: Gap junction-forming connexins are truncated during lens development; however, little is known about the mechanism of this truncation.

Results:Cx50 truncation by caspase-1/3 resulted in reduced gap junctions and hemichannel activities and increased resistance to UV radiation.

Conclusion: Development-associated truncations of Cx50 by caspases have cell protective function.

Significance: This is the first report showing the functional importance of naturally truncated connexins.

The primary function of the lens, an avascular organ, is to transmit light and focus it on the retina. The lens is composed of an anterior epithelial cell layer and fiber cells that form the bulk of the organ. Epithelial cells differentiate into fiber cells, accompanied by cell elongation and organelle loss; this differentiation process continues throughout the lifespan of the organism. Lens fiber cells do not turnover throughout the lifespan of the animal and rely on a large network of intercellular gap junction communications between fiber cells and cells at the lens surface for the exchange of metabolites and ions (1). Gap junctions are clusters of transmembrane channels between two adjacent cells that allow the passage of small molecules (molecular mass = 1 kDa), such as ions, metabolites, and second messengers (2). The structural units of gap junction channels are a family of proteins called connexins, which contains >20 members expressed in an overlapping, tissue-dependent pattern (3). Each connexin has four conserved transmembrane domains and two extracellular loops in which their cytoplasmic regions are variable, especially the C-terminal domain. Connexins expressed in most of the other tissues are highly dynamic proteins that undergo rapid turnover both in cell lines and in animal organs; their degradation has been shown to involve either the lysosome or the ubiquitin-proteasome pathway (4–7). In addition, the C-terminal domain of connexin 50 (Cx50)2 contains several predicted protease consensus sequences (8, 9).

Three connexins, Cx43, Cx46, and Cx50, have been identified in the vertebrate lens. Cx43 is mainly expressed in anterior epithelial cells and the lens bow region. Cx43 is down-regulated and replaced by the other two lens fiber connexins, Cx50 and Cx46, during differentiation of epithelial cells to new lens fibers (10). Cx50 plays an important role in maintenance of lens transparency and in normal lens development and epithelial fiber differentiation (11–14). Mutations in the Cx50 gene are correlated with the formation of cataracts. Cx50-deficient mice not only develop cataracts but also exhibit microphthalmia (11, 12, 15). The cytoplasmic C-terminal domain of Cx50 is involved in gating and permeability of gap junction channels (16, 17). In contrast to connexins in other organs, lens fiber connexins, like

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1 The abbreviations used are: Cx50, connexin 50; LY, Lucifer yellow; RD, rhodamine dextran; PI, propidium iodide; CEF, chicken embryonic fibroblast.
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Determining Cleavage Sites of Cx50 Using HPLC Electrospray Ionization-Tandem Mass Spectrometry—Three-month-old chick lenses were collected and rinsed three times in PBS. Membrane proteins were prepared following the procedure described previously (9). Briefly, chicken lenses were lysed and pelleted at 100,000 × g for 30 min at 4 °C, and the crude membrane pellet was washed twice with prechilled 20 mM NaOH. Proteins were separated by one-dimensional SDS-PAGE and stained with Coomassie Blue. Bands of interest were excised, and the proteins were digested in situ with trypsin (Promega, modified). The digests were analyzed by capillary HPLC-electrospray ionization-MS/MS on a Thermo Fisher LTQ fitted with a New Objective PicoView 550 nanospray interface. Online HPLC separation was accomplished with an Eksigent NanoLC micro-HPLC: column, PicoFritTM (New Objective; 75-μm inner diameter) packed to 11 cm with C18 adsorbent (Vydac; 218MSB5, 5 μm, 300 Å); mobile phase A, 0.5% acetic acid (HAc)/0.005% trifluoroacetic acid (TFA); mobile phase B, 90% acetonitrile/0.5% HAc/0.005% TFA; gradient, 2% to 42% B in 60 min; flow rate, 0.4 μl/min. MS conditions were as follows: electrospray ionization voltage, 2.9 kV; isolation window for MS/MS, 3; relative collision energy, 35%; scan strategy, survey scan followed by acquisition of data-dependent collision-induced dissociation spectra of the seven most intense ions in the survey scan above a set threshold. Mascot (Matrix Science) was used to search the uninterpreted collision-induced dissociation spectra searched against the Swiss-Prot database. Methionine oxidation was considered as a variable modification, with semi-trypsin as the proteolytic enzyme specificity. The Mascot search results were imported into Scaffold (Proteome Software), and a second search was then conducted within Scaffold by X! Tandem. The assignments were compiled and further processed by PeptideProphet and ProteinProphet for determination of the probabilities of peptide assignments and protein identifications. The reported peptide assignments were all ≥ the peptide 95% confidence level.

Preparation of Retroviral DNA Constructs and High-titer Retroviruses Containing Cx50, Cx50 Mutants, and Truncated Cx50—Retroviral constructs and high-titer RCAS(A) retroviruses were prepared based on the protocol described previously (26). With the wild-type RCAS(A)-Cx50 DNA construct as a template, other Cx50 mutants and truncated Cx50 were generated by using QuikChange site-directed mutagenesis kit according to the manufacturer’s instructions. The primers used were as follows: Cx50(D379A), 5′-CTGAACTTTGACCACGCG-GTGAGATCCCTCA-3′ (sense) and 5′-TGAGGATCTCACC-GCCGTGGCAAGTTCAG-3′ (antisense); Cx50(E368A), 5′-TGAGGCGATGAAGGCCCTGCCACTTCATCGCTCA-3′ (antisense); Cx50(368T), 5′-CTGCTGAGGGATCTTCAATCGGTGGCAAG-3′ (antisense); and Cx50(379T), 5′-CTGAACTTGCCACCGCG-GTGAGATCCCTCA-3′ (sense) and 5′-CTGAACTTTGACCACGCG-GTGAGATCCCTCA-3′ (antisense); Cx50(D365A), 5′-AGCTGTTGAGCGCGTGTAATG-3′ (antisense); Cx50(368T), 5′-AGCTGTTGAGCGCGTGTAATG-3′ (antisense); and Cx50(379T), 5′-AGCTGTTGAGCGCGTGTAATG-3′ (antisense).

Materials—Fertilized, incubated white Leghorn chicken eggs were purchased from Ideal Poultry (Cameron, TX) and incubated in a humidified incubator. The QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA), and the annexin V and propidium iodide (PI) staining kit from Clontech (Mountain View, CA). The anti-Cx50 antibody against the intracellular loop domain was generated as described previously (9). The polyclonal anti-casparase-1 antibody was purchased from Invitrogen, and polyclonal anti-casparase-3 antibody was from Cell Signaling (Danvers, MA). Caspase-1 and caspase-3-specific inhibitors and a Caspase Colorimetric Assay kit were purchased from EMD Chemicals (Gibbstown, NJ). Fetal bovine serum (FBS) was from Hyclone laboratories (Logan, UT). Tissue culture reagents, Lucifer yellow (LY) and rhodamine dextran (RD) were from Invitrogen. Cell proliferation reagent WST-1 was purchased from Roche Applied Science (Mannheim, Germany). All other chemicals were purchased from Sigma or Fisher Scientific (Pittsburgh, PA).

EXPERIMENTAL PROCEDURES

Lens fibers, also do not turnover during the lifespan of the lens. Cx50 and Cx46 proteins are truncated gradually during lens development. Truncated connexin fragments accumulate in the center core regions of the lens (9, 18). Cleavage of the C-terminal domain of Cx50 has been reported to result in a dramatic decrease of junctional conductance (17). In one report, truncation at the Cx50 C terminus was found to cause a loss of pH sensitivity (16), but the results of another study indicated that pH sensitivity only was reduced partially after C-terminal truncation of Cx50 (19). However, until now, the biological function of truncation of Cx50 in the lens was largely unknown.

Proteolysis plays very important roles in many aspects of lens development. It has been suggested that the loss of all cytoplasmic organelles during the differentiation of epithelial cells to lens fiber cells resembles apoptosis to a certain degree (20). Transcripts for each executioner caspases, caspase-1, -3, -6, and -7, have been identified in lens fibers although endogenous proteolytic activity has only been reported for caspase-3 (21). Until now, the mechanistic role of caspase activation in the lens has not been elucidated clearly. Therefore, it is of interest to understand the molecular targets of caspases activated during lens development. The eye lens is constantly exposed to UV radiation, which produces reactive oxygen species (22). The elevated oxidative stress has resulted from UV radiation has also been observed in older lenses (23) and is considered to be one of the major factors leading to the development of age-dependent cataracts (24, 25). We have previously shown that Cx50 is a likely target of a caspase-3-like protease (9). In this study, specific natural truncation sites of Cx50 were identified, and both caspase-1 and caspase-3 were determined as the major enzymes responsible for the cleavage of Cx50 in vivo. Furthermore, similar truncations were observed in younger lenses after exposure to UV radiation, along with increased activity of caspase-1/3. Importantly, as compared with full-length Cx50, we showed that truncation of Cx50 enhanced cell resistance against UV radiation.
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plate, the Cx50(E368A,D379A) mutant was generated. The primers used were as follows: 5′-CTGAGCTTGGAGTTGGAC-3′ (sense) and 5′-TCAGTGATCTCA-CGCCGCTGGAGTTCCAG-3′ (antisense). All constructs were sequenced at the University of Texas Health Science Center, San Antonio, Institutional DNA Sequencing Facility. The wild-type and mutant proteins were prepared as described previously (26).

Immunoﬂuorescence Staining of Chick Lens Tissue Sections—Frozen sections prepared from postnatal day 1 chick lens were washed three times with PBS for 5 min each and were then incubated with blocking solution containing 2% normal goat serum, 2% fish skin gelatin, 0.5% Triton X-100, and 1% bovine serum albumin in PBS for 1 h at room temperature. Polyclonal anti-caspase-1 (1:200 dilution) or anti-caspase-3 (1:200 dilution) antibody in blocking solution was then added, and the mixture was incubated at 4 °C overnight. Sections were washed three times with PBS for 5 min each and then incubated with FITC-conjugated goat anti-rabbit IgG (1:750) for 2 h at room temperature. After three washes for 5 min each in PBS, sections were then incubated with DAPI (1:20,000 dilution) in PBS for 5 min at room temperature. Sections were then washed with PBS for 5 min and mounted on a coverslip with mounting medium. The specimens were analyzed using an epifluorescence microscope (Olympus Optical, Tokyo, Japan).

CEF Cells and Retroviral Infection—CEF cells were seeded at 3 × 10⁵ cells in 60-mm tissue culture plates in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal calf serum, 2% chick serum, and 5% sodium pyruvate. Cells were infected on the second day with high-titer retroviruses (>1 × 10⁹ colony-forming units/ml, 15–20 μl/plate). After reaching confluence, CEF cells were digested with 0.05% trypsin and plated into 35- or 60-mm culture plates.

Preparation of Lens Organ Culture—To prepare for lens organ cultures, intact lenses were isolated from chick embryo at day 12 and washed with PBS three times. Chick lens was incubated in M199 medium containing 10% FBS and 1% penicillin/streptomycin at 37 °C, 5% CO₂.

In Vitro Cleavage of GST-Cx50 C terminus and Lens Membrane Extract by Caspase-1/3—GST fusion proteins containing a portion of the C-terminal region of Cx50 (F1, residues 307–389) and its corresponding mutant Cx50F1(D379A) were expressed in Escherichia coli and purified by binding to glutathione-coupled agarose beads according to published procedures (27). Briefly, embryonic day 12 chick lens or CEF cells infected with recombinant retroviruses were lysed in lysis buffer (5 mM Tris, pH 8.0, 5 mM EDTA/EGTA) plus 2 mM PMSF, 10 mM N-ethylmaleimide, and 100 μM leupeptin. The crude membrane extract was pelleted at 50,000 rpm for 30 min at 4 °C (Beckman TLA 100.3 rotor). The GST fusion protein (150–200 ng) or membrane extract was subjected to enzymatic digestion with caspase-1 or caspase-3 in the absence or presence of corresponding caspase-specific inhibitors (caspase-1 inhibitor, Ac-YVAD-CHO, 0.05 μg/μl; caspase-3 inhibitor, Ac-DEVD-CHO, 0.05 μg/μl). The cleavage reaction was performed in 20 μl of reaction buffer (50 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM succrose, 0.1% Triton X-100, and 5% glycerol) with or without 50 units of caspase-1 or caspase-3 for different time periods. The reaction was terminated by the addition of 5x electrophoresis sample buffer (50 mM Tris, pH 6.8, 1% SDS, 2% β-mercaptoethanol, and 35% glycerol) and boiling for 5 min.

Dephosphorylation of the crude membrane extract was performed according to a published protocol (27). Briefly, 100–500 μg of protein from the chick lens membrane extract was dissolved in 1× New England Biolab buffer 3 and incubated with 7.5 units of alkaline phosphatase at 37 °C for 1.5 h. The reaction was terminated by addition of 5x sample buffer and then boiling for 5 min.

Activity Assay of Caspase-1 and Caspase-3—The activities of caspase-1 and caspase-3 were measured according to the protocol provided in the Caspase Colorimetric Assay kit (EMD Chemicals) with minor modifications. Briefly, supernatants of lysates prepared from CEF cells or chick lenses were incubated with caspase-1-specific substrate (Ac-YAVD-pNA) or caspase-3 specific substrate (Ac-DEVD-pNA) in reaction buffer at 37 °C for 6 h. Caspase activities were determined using a microtiter plate reader to assay absorbance at 405 nm. Values were normalized based on the total protein in each sample.

Dye Uptake and Scrape Loading Dye Transfer Assay—The dye uptake assay was performed as described previously (28) with some modifications. Briefly, CEF cells expressing exogenous Cx50 or mutants generated by retroviral infection or RCAS(A) vehicle-infected control cells were grown at low cell density to minimize physical contact among the cells. LY (457 Da) was used as a tracer to determine hemichannel activity; RD (10 kDa) was employed as a negative control. Cells were washed with Ca²⁺–free minimum essential medium three times and then mechanically stimulated to induce the opening of hemichannels by dropping Ca²⁺–free MEM from a pipette at a fixed distance. Then, cells were incubated in the presence of 0.4% LY plus 0.4% RD for 5 min. Cells were washed with Hanks’ balanced salt solution buffer and fixed in 2% PFA. Dye uptake was calculated as the percentage of fluorescent cells divided by the total number of cells. Scrape-loading dye transfer was performed according to the published procedure (29) with modifications. Briefly, CEF cells expressing exogenous Cx50 and its mutants were grown to confluence to maximize physical contact. Cells were washed five times with Hanks’ balanced salt solution buffer and fixed in 2% PFA. Dye uptake was measured with a fluorescence microscope in which LY and RD were detected by using fluorescein and RD filters. Acquisition conditions were kept consistent for all measurements, and no threshold adjustments were applied. The extent of dye transfer was calculated by measuring the ratio of the LY transfer distance from the scrape lines to the RD transfer distance.

UV Radiation Treatment and Cell Viability Assay Using WST-1—CEF cells expressing Cx50 and mutants or RCAS(A) vehicle control as described above were seeded at 5 × 10⁴ cells in a 96-well plate in DMEM plus 10% fetal calf serum, 2% chick serum, 5% sodium pyruvate and were cultured at 37 °C, 5% CO₂.
for 24 h. Cells were then treated with 200 ml/cm² UV radiation (254 nm, Fisher Biotech FBUV XL-1000 Microprocessor Controlled UV Crosslinker) and then cultured in 100 ml of fresh medium for another 18 h. Live cells were stained with WST-1 reagent according to the procedure provided by the manufacturer. Briefly, 10 ml of WST-1 reagent was added to each well, and then cells were incubated for 4 h at 37 °C, 5% CO₂. After vigorous shaking, a microplate reader was used to measure the absorbance of the samples at 450 nm immediately before UV treatment and 18 h after exposure. Values for cell viability were calculated as the percentage of absorbance at 18 h after UV exposure divided by the absorbance before treatment.

UV Radiation and Quantification of Cell Apoptosis by Flow Cytometry—CEF cells expressing Cx50 and mutants or RCAS(A) vehicle control were seeded at 8 x 10⁵ cells in 60-mm dishes in DMEM plus 10% fetal calf serum, 2% chick serum, and 5% sodium pyruvate and cultured at 37 °C, 5% CO₂ and grown to confluence. Cells were then treated with 200 ml/cm² UV radiation (254 nm, Fisher Biotech FBUV XL-1000 Microprocessor Controlled UV Crosslinker) and then cultured in 3 ml of fresh medium for another 18 h. Flow cytometry analysis was performed subsequently according to the protocol provided in the kit. Briefly, cells were trypsinized and gently washed once with serum-containing media, then rinsed with 1× binding buffer. Cells (0.1–1 x 10⁶) were resuspended in 200 μl of 1× binding buffer. Five microliters of annexin V and 10 μl of PI were added, and then cells were incubated at room temperature for 10 min in the dark. Binding buffer was used to bring the reaction volume to 500 μl, and the cells were analyzed by flow cytometry (LSR-II, high-speed, multicolor, digital analyzer, BD Biosciences). The signal generated by annexin V was detected in the FITC signal channel, and the signal generated by PI was monitored by the detector for phycoerythrin emission.

Statistical Analysis—All data were analyzed using GraphPad Prism software (version 5.04, GraphPad Software, La Jolla, CA). A paired Student’s t test was used for comparison between two groups, and one-way analysis of variance followed by a Student Newman Keul’s test were used for comparison of more than two groups. Unless otherwise specified in the figure legends, the data are presented as the mean ± S.E. of at least three determinations. Asterisks indicate the degree of significant differences (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

RESULTS

Identification of Cleavage Sites on Cx50 Protein during Lens Development—To elucidate the development-associated truncation of Cx50, membrane extracts isolated from chick lenses at embryonic days 8, 12, 15, and 18 and postnatal days 1 and 60 were immunoblotted with affinity-purified Cx50 antibody, which recognizes the intracellular loop domain region. The full-length Cx50 was adjusted to be at the same level. As compared with full-length Cx50, the level of truncated Cx50 (Cx50T) increased with lens development.

To locate the potential truncation sites of lens Cx50, mass spectrometry analyses were conducted of tryptic digests of Cx50 protein isolated from chick lenses. Based on database searches in which semitryptic cleavage was allowed (i.e. cleavage at lysine or arginine was only required to be present at one end of the peptide), high-confidence assignments were made for peptides terminating after Glu-368 and Asp-379 (Fig. 2, A and B); both of these sites are located in the C-terminal domain of Cx50 (Fig. 2C).

Increased Activities of Caspase-1 and Caspase-3 during Lens Development—To elucidate the consequences of exposure of Cx50 to caspase(s), membrane extracts prepared from chick lens at embryonic days 8, 12, 15, and 18 and postnatal days 1 and 60 were immunoblotted with anti-p20, the active subunit of caspase-1 or caspase-3, antibody. The p20 active form of caspase-1, started to appear at embryonic day 8, whereas the p20 form of caspase-3 appeared at embryonic day 12. Compared with younger lenses, there was a dramatic increase of p20 of both caspase-1 and caspase-3 in postnatal day 60 lenses (Fig. 3A). These results suggest that the activation of caspase-1 and caspase-3 parallels the progression of lens development. The cleavage of Cx50 as shown in Fig. 1 is associated with the increased level of active caspase-1 and caspase-3, suggesting that these two enzymes could be responsible for the truncation of Cx50 during lens development.

Tissue sections prepared from postnatal day 1 chick lens were immunolabeled with anti-caspase-1 or anti-caspase-3 antibody (Fig. 3B). Both caspase-1 and -3 were detected in the cortex regions of the lens but not in the central core region. These results were further verified through analysis of isolated tissue fractions enriched in the cortex or central core of postnatal day 1 lenses. By immunoblotting, caspase-1 and caspase-3 were only detected in the whole lens and cortex-enriched fractions, but not in core fraction of the lens (Fig. 3C, left panel).
FIGURE 2. Cx50 is cleaved after residues Glu-368 and Asp-379. Cx50 derived from three-month-old chick lenses was purified by immunoprecipitation and digested with trypsin before being subjected to HPLC-electrospray ionization-tandem mass spectrometry. A and B, assignment of the collision-induced dissociation fragments of semitryptic peptides KAEEEVVSDEVEGPSAPAELATD and AEEEVVSDEVE are shown, with annotations for the b-fragment (N-terminal) and y-fragment (C-terminal) ions. Both of these peptides contain an N terminus derived from cleavage by trypsin; two specific C-terminal truncation sites were identified: Asp-379 and Glu-368. C, topological structural and protein sequence of Cx50. Location and putative proteases correlated to this two truncation sites are indicated.

FIGURE 3. Expression and activation of caspase-1 and caspase-3 are associated with lens development. A, chick lens were isolated at embryonic day (E) 8, 12, 15, and 18 and postnatal day (P) 1 and 60, and lens lysates were immunoblotted with anti-p20 subunit of caspase-1 or anti-p20 subunit of caspase-3 antibody. The expression level of active forms of caspase-1 (CASP1, p20 subunit) and caspase-3 (CASP3, p20 subunit) were normalized to GAPDH (n = 3). B, postnatal day 1 lens sections were immunolabeled with anti-caspase-1 or anti-caspase-3 antibody and subsequently labeled with FITC-conjugated goat anti-rabbit IgG. The nucleus was counterstained by DAPI. The corresponding images were merged (MERGED) to show the location of caspase-1 and caspase-3 in the lens. C, the exterior portions of postnatal day 1 chick lens containing epithelial cells and outer cortex fibers were separated from the central core region containing mature lens fibers. Caspase-1 and caspase-3 were immunoblotted by their corresponding antibodies and normalized to GAPDH. The full-length Cx50 and truncated Cx50 (Cx50T) were immunoblotted with affinity-purified anti-Cx50 loop domain antibody.
Although a low level of the truncated Cx50 was detected, the majority in the Cx50 in the cortex was found to be full-length (Fig. 3C, right panel). Truncated Cx50 was detected predominately in the central core region of the lens. Consistent with this, our previous study showed that in postnatal day 60 chick lens, truncated Cx50 completely disappeared in the cortex region (9).

**Caspase-1 and Caspase-3 Are Likely to Be Responsible for Development-associated Cleavage of Cx50**—To determine whether caspase-1 is the protease responsible for the cleavage of Cx50, membrane extracts of embryonic chick lens were subjected to enzymatic digestion by caspase-1. Full-length Cx50 was found to be partially cleaved into two fragments, one with apparent molecular mass $/H1101149$ kDa, and the other with apparent molecular mass $/H1101146$ kDa. This cleavage was completely blocked by a caspase-1-specific inhibitor, Ac-YVAD-CHO (Fig. 4A). Treatment with caspase-3 also yielded two fragments (Fig. 4B). The smaller fragments generated by both caspase-1 and -3 migrated at the same apparent molecular weight, whereas the bigger fragment from caspase-1 cleavage appeared to be larger than the fragment from caspase-3. The smaller fragment generated by both enzymes and the larger fragment by caspase-1 appear to be a similar size as natural truncation products of Cx50 in embryonic day 8 and 12 lenses (Fig. 4B). To test the possibility that the mobility shift as a result of phosphorylation modification of Cx50 may lead to some of different protein bands shown on SDS-PAGE (27), we performed a dephosphorylation assay. Membrane extracts from embryonic day 12 or postnatal day 60 lenses with and without treatment with caspase-1 or caspase-3 were then dephosphorylated with alkaline phosphatase (Fig. 4C). Consistent with our previous report, dephosphorylation led to decreased migration of full-length Cx50 to $/H1101158$ kDa (27). The larger fragment detected in embryonic day 12 lenses was also shifted to a lower apparent molecular weight; this band appeared to be present after cleavage by caspase-1 in the presence of alkaline phosphatase. Interestingly, after alkaline phosphatase treatment, the smaller fragment generated by caspase-1 treatment, which was different from the one with similar molecular weight by caspase-3, disappeared and merged into a single protein band. The size of this band was similar to a naturally truncated fragment found in embryonic day 12 lenses. Conversely, the size of the smaller fragment from caspase-3 cleavage did not get altered in the presence of alkaline phosphatase and this was the major fragment accumulated in P60 lens. These results suggest that generation of the truncated Cx50 observed during lens development is likely to be mediated by caspase-1 and -3. The predominant fragment observed in the fully developed lens is produced by the action of caspase-3; this fragment does not appear to be phosphorylated (or it does not exhibit a gel shift when in the phosphorylated form). To determine whether Cx50 is a direct substrate of caspase-1, a GST fusion protein containing part of the C-terminal domain (amino acids 307–389) of Cx50 (Cx50F1) was exposed to caspase-1 for 6 and 12 h (Fig. 4D). Cx50F1 was cleaved partially
by 6 h and almost cleaved completely after 12 h. This cleavage was inhibited by preincubation with caspase-1 inhibitor. Moreover, the fragment generated by caspase-1 cleavage could also be cleaved by caspase-3 (Fig. 4E). Based on the size of cleaved fragments, these results further confirmed that caspase-1 cleavage site is located closer to end of C terminus than caspase-3.

Asp-379 Is Specific Cleavage Site by Caspase-1—To confirm that the truncation sites detected by mass spectrometry are indeed caspase-1 or -3 sites, GST fusion proteins, GST-Cx50F1, and mutants Cx50F1(D379A) and Cx50F1(E368A) (Fig. 5A) were subjected to in vitro enzymatic digestion by caspase-1 or caspase-3 (Fig. 5B). GST-Cx50F1 was cleaved by caspase-1 or caspase-3 into closely migrating protein bands, whereas Cx50F1(D379A) was completely resistant to caspase-1 digestion but not to caspase-3. Surprisingly, another mutant, Cx50F1(E368A), which we have shown previously to be a caspase-3-resistant mutant (9), could not be cleaved by caspase-1 as well. One of the possible reasons is that when Glu-368 is mutated to an alanine in the Cx50F1 fusion protein, access to Asp-379 on Cx50 may be hampered due to changes in protein conformation. To avoid this possibility, we used full-length Cx50 and its corresponding site-specific mutants: Cx50(D379A), Cx50(E368A), and Cx50(D365A) expressed in CEF cells through retroviral infection. Crude membrane extracts were isolated and treated with caspase-1 or caspase-3 for 6 h and were immunoblotted with anti-Cx50 antibody.

Development-associated Cleavage of Cx50 Could be Reproduced by UV Treatment—To determine whether Cx50 can be cleaved by activated caspase-1 or caspase-3 in lens in situ, embryonic day 12 chick lenses were pretreated in the absence or presence of caspase-1 or caspase-3-specific membrane-permeable inhibitors for 24 h, subjected to UV radiation, and followed by post-UV incubation. As compared with the non-UV-treated control, more Cx50 protein was truncated upon UV treatment, and no additional truncated fragments were generated. The increased cleavage of Cx50 was blocked significantly by caspase-1- or caspase-3-specific inhibitors (CASP1i or CASP3i, membrane-permeable) (Fig. 6A). Exposure to UV radiation also significantly increased the activity of caspase-1 and caspase-3 in comparison with non-UV-treated controls (Fig. 6B). The increased activity of caspases could be responsible for the increased cleavage of Cx50 in UV-treated lens. To further determine whether cleavage induced by UV radiation targets on caspase-specific sites on Cx50, CEF cells expressing caspase-1- or caspase-3-resistant mutants: Cx50(D379A) and Cx50(D365A), respectively, were exposed to UV radia-
tion. Interestingly, Cx50(D379A), the caspase-1-resistant mutant, still could be cleaved, but not Cx50(D365A), a caspase-3-resistant mutant (Fig. 7A). The activities of caspase-1 and caspase-3 were also determined in CEF cells 8 h after UV treatment. The results showed that unlike the activation of both caspase-1 and -3 in the lens, only caspase-3 activity was elevated, but not caspase-1, which remained at similar level as untreated cells (Fig. 7B). Although caspase-1 was not activated in CEF cells upon UV radiation, the activated caspase-3 was responsible for the specific cleavage of its corresponding substrate site on wild-type and caspase-1-resistant mutant of Cx50.

**Truncation of Cx50 Leads to Reduced Gap Junction Coupling and Complete Loss of Hemichannel Function**—To determine whether truncated Cx50 affects gap junction coupling, truncated Cx50 mutants, Cx50(368T), and Cx50(379T), caspase-resistant mutant, Cx50(D368A,E379A) as well as full-length Cx50 as illustrated in Fig. 8A were expressed in CEF cells via retroviral infection. A scrape-loading dye transfer assay was used to assess gap junctional coupling. CEF cells infected with retroviral vector, RCAS(A), were used as a negative control. Similar to wild-type Cx50, Cx50(368T), Cx50(379T), the two truncated mutants co-expressed, and Cx50(E368A,D379A) as well as full-length Cx50 as illustrated in Fig. 8A were expressed in CEF cells via retroviral infection. A scrape-loading dye transfer assay was used to assess gap junctional coupling. CEF cells infected with retroviral vector, RCAS(A), were used as a negative control. Similar to wild-type Cx50, Cx50(368T), Cx50(379T), the two truncated mutants co-expressed, and Cx50(E368A,D379A) were able to form functional gap junctions (Fig. 8B, left panels). However, in contrast to WT and the caspase-resistant mutant, gap junction coupling between the CEF cells expressing two truncated mutants was decreased significantly. Hemichannel activity was measured by a LY dye uptake assay (Fig. 8B, right panels). Similar to the RCAS(A) vehicle control, CEF cells expressing these two truncation mutants failed to uptake LY (Fig. 8C, left panels). Contrary to gap junctions, dye uptake analyses showed that these truncated mutants completely lost the ability of full-length Cx50 in forming functional hemichannels (Fig. 8C, right panel). Interestingly, a small reduction of hemichannel uptake was observed in cells expressing a caspase-resistant mutant. Together, our results suggest that truncation
of Cx50 by caspase-1 or caspase-3 dramatically decreases gap junction coupling and totally abolished hemichannel function.

**Truncated Cx50 Enhances Resistance to UV Radiation-induced Cell Death**—Elevated oxidative stress associated with UV exposure is one of the major factors in the development of age-dependent cataracts (23–25). To examine the influence of truncated Cx50 on this process, CEF cells expressing full-length Cx50, truncated Cx50, caspase-resistant mutant, Cx50(E368A,D379A) were subjected to UV radiation, and cell viability and apoptosis were determined by the WST-1 cell viability assay and annexin V/PI staining in conjunction with flow cytometry analyses, respectively. Interestingly, results from the WST-1 study showed that cells expressing WT Cx50 as well as caspase-resistant mutants exhibited the decrease of cell viability as compared with vehicle control. The truncated Cx50 significantly enhanced resistance to UV radiation compared with those expressing full-length Cx50 (Fig. 9A). Consistently, the flow cytometry results showed that the numbers of cells expressing truncated mutants either at an early stage of apoptosis (indicated by annexin V-positive/PI-negative cells) or apoptotic cells (indicated by both annexin V-positive/PI-positive cells) were significantly decreased (Fig. 9B), which is similar to vehicle control. However, WT and the caspase-resistance mutant enhanced cells under apoptosis. Together, these results suggest that cells with altered gap junctions and hemichannel function as a result of Cx50 truncation exhibit a higher degree of resistance to UV radiation than those with full-length Cx50. This is a likely mechanism for protecting inner, mature fiber cells against an accumulated level of oxidative stress during lens development.

**DISCUSSION**

In this study, we detected truncation sites at the C terminus of Cx50 by using mass spectrometry and showed that caspase-1 and caspase-3 were the likely enzymes responsible for the cleavage. Interestingly, the truncation of Cx50 associated with lens...
development could be mimicked by the activation of caspases that results from exposure to UV radiation. As compared with channels formed by full-length Cx50, those composed of caspase-1/3-truncated Cx50 had distinctive activities: reduced gap junctional coupling and completely impaired hemichannel function. Moreover, cells with truncated Cx50 remained more viable after UV radiation compared with those with full-length Cx50. Our data revealed a novel mechanism connecting caspase activation associated with lens aging and UV radiation and specific truncation by caspases, channel activity, and cell protection function of truncated Cx50.

During lens development, older cells are retained, and younger cells continually arise in the equator and outer cortex layers. Most cells in the central core region are differentiated fully mature lens fibers. Connexins, like several other proteins expressed in lens fibers, become progressively truncated during vertebrate lens development and in response to oxidative stress (23). The C-terminal truncation of Cx50 was first reported in ovine lens (30), which contains a 70-kDa full-length Cx50 located in the cortex and a 38-kDa fragment enriched in the core region. This truncation, resulting in the removal of the majority of the C terminus of Cx50, is generated by a calcium-activated protease, calpain (8). Two major truncated fragments of Cx50 accumulate during chick lens development. The larger fragment (∼49 kDa) is present at early embryonic stages and decreases dramatically in the later embryonic stage, at which time the smaller fragment (∼45 kDa) predominates. Our previous study indicated that a caspase-3-like protease likely was to be involved in the cleavage of Cx50, resulting in the generation of the smaller fragment in which Glu-368 was the likely cleavage site (9). Here, by mass spectrometry analysis, we confirmed that cleavage at Glu-368 did occur in the C terminus of Cx50. However, the presence of a larger fragment suggests that there is another protease besides caspase-3 that catalyzes truncation of Cx50. We identified another cleavage site at Asp-379 located within a caspase-1 substrate consensus sequence. Cleavage by caspase-1 generated the fragment with the identical size as the large fragment shown by nature truncation in the lens. More importantly, mutation of Asp-379 prevented cleavage by caspase-1. Moreover, we showed that the fragment generated after caspase-1 digestion could serve as a substrate for caspase-3. Therefore, it is not surprising that the amount of the larger fragment resulting from caspase-1 is much less abundant in the lens than the smaller one cleaved by caspase-3. In the late embryonic and postnatal adult lens, almost no caspase-1-cleaved fragment could be detected, which is possibly a result of increased caspase-3 activity. We observed that the levels of active forms of caspase-1 and 3 are elevated greatly (3–4-fold) in postnatal day 60 as compared with that in newborn lenses.

In vitro treatment of Cx50 with caspase-1 and 3 generated fragments of the same sizes as those detected in lens in situ; this cleavage was not observed when caspase-1 and -3 cleavage sites of Cx50 were mutated or in the presence of caspase-specific inhibitors. Interestingly, some of the truncated fragments of
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Cx50 migrated very close to one another on SDS-PAGE. We suspected that some of those fragments generated by either of caspase digestion could be the same origin but be phosphorylated differently. Cx50 is a phosphoprotein, but unlike most other proteins, phosphorylated Cx50 migrates at a higher apparent molecular weight than the unphosphorylated form (27). After dephosphorylation, the truncated fragments from late embryonic and postnatal lenses showed a similar pattern as those cleaved by caspase-3. Intriguingly, the intensity of the slower migrating band after dephosphorylation appeared to decrease greatly. This could be caused by partial dephosphorylation or/and additional cleavage fragment(s), and the latter possibility could be triggered by the initial cleavage by caspase-1/3 possibly resulting in protein destabilization. Contrary to caspase-3, dephosphorylation converts the doublets of caspase-1-cleaved fragments into a single fragment, suggesting the single cleavage by this enzyme.

We showed that cells expressing truncated Cx50, as compared with full-length, exhibited lower levels of gap junction coupling and total abolishment of hemichannel function. Our data are consistent with a previous paper that truncation of Cx50 leads to reduction in mean macroscopic gap junction conductance as compared with full-length Cx50 (17). However, a recent paper shows that cleavage of last seven amino acids of the Cx50 C terminus eliminates gap junction coupling and the formation of plaques in HeLa cells (31). This possibly could be explained by the difference in cell types being assayed and the dependence of ZO-1 in this cell type in forming connexin channels.

The activation of several caspases has been observed during lens development (21); this activation has long been hypothesized to be involved in degeneration of organelle and nucleus in mature lens fibers during lens development. However, mouse lenses that are deficient in caspase-3 develop marked cataracts but do not exhibit any abnormality in the degeneration of organelles and development of the lens (21). The results of this study suggest that caspase-3 plays an important role in maintaining lens transparency but may not be necessarily involved in lens development. In this study, caspase-associated cleavage of Cx50 was also detected after UV treatment; this cleavage was diminished significantly by inhibitors of caspase-3 and caspase-1. These results are consistent with the observation of elevated activity of both caspases after exposure to UV radiation. Analogous to the aging process of the lens, activation of these two caspases after UV radiation appear to be responsible primarily for the truncation of Cx50.

Extended exposure to the UV rays of the sun is one of the leading factors leading to the development of cataracts, possibly due to increased level of oxidative stress (32). Accumulative oxidative damage in old lenses is proposed as an underlying cause for age-related cataracts (33). Lens gap junction coupling is implicated to be responsive to oxidative stress. Activation of PKCγ by oxidative stress results in phosphorylation of Cx50 and inhibition of gap junctions, whereas uncontrolled opening of gap junctions resulting from PKCγ deficiency leads to fiber cell damage (34). Our study, for the first time, showed that truncation of Cx50 by caspase-1 or -3 can protect lens cells against UV radiation. This may be correlated with reduced gap junctional activity and/or loss of hemichannel function. Interestingly, cells expressing caspase-resistant mutants exhibited the similar sensitivity to UV-induced cell death as wild-type Cx50. Although this mutant has similar level of gap junction activity, the hemichannel function is lower than that of wild-type. The reduced hemichannel activity may partially protect cells, compensating for the consequence of a total loss of Cx50 truncation by caspases in response to UV. This may explain the comparable responses between this mutant and wild-type to UV-induced cell death. These results indicate that hemichannels are likely to play a major role in UV-induced cell death. In addition, reduced level of gap junction activity may be cell protective to eliminate the passage of apoptotic signals.

The close relationship between gap junctions and cell death has been reported. Gap junction channels are considered as conduits for signals, such as Ca^{2+} and ATP, which modulate the fate of the surrounding cells through a “bystander effect;” however, the precise mechanism for this has not been elucidated fully (35, 36). In addition, the blockage of gap junction channel function could also slow down the propagation and amplification of cell injury (37). Aberrant hemichannel function has been suggested to be involved in cell death. Opening of the hemichannels induced by oxidative stress has been indicated as the cause of death of Marshall cells (38). In addition, a recent study showed that Cx43 hemichannels increased the sensitivity of the cell to cadmium-induced oxidative stress and cell injury (39). These studies are in accordance with our data of altered activities of gap junction and hemichannels in response to oxidative stress. However, our study is distinct from other previous observations involving full-length connexins. Reduced gap junction channels and closed hemichannels formed by truncated connexins are unlikely to eliminate the progression of death signals but would reduce the passage of these signals and thus reduce the cell death. At the mean time, gap junction coupling formed by truncated connexins are still able to maintain homeostasis of the inner fibers where metabolic activity is lower as compared with outer cortical fibers expressing full-length connexins. Therefore, functions of gap junctions and hemichannels, through the natural truncation of connexin molecules, were regulated adequately to protect the lens cells against accumulation of oxidative stress in the mature differentiated lens fibers. This could be a possible self-protective mechanism against multiple insults accumulated over the years to maintain lens transparency and homeostasis. Therefore, studies of the interplay between connexin channels and caspase-like proteases might provide useful information for better understanding about how lens tissues respond and deal with stress.

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