Caspase remodeling of the spectrin membrane skeleton
during lens development and aging

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Terminal differentiation of lens fiber cells resembles the apoptotic process in that organelles are lost, DNA is fragmented, and changes in membrane morphology occur. However, unlike classically apoptotic cells, which are disintegrated by membrane blebbing and vesiculation, aging lens fiber cells are compressed into the center of the lens, where they undergo cell-cell fusion and the formation of specialized membrane interdigitations. In classically apoptotic cells, caspase cleavage of the cytoskeletal protein α-spectrin to Mr~150 kD fragments is believed to be important for membrane blebbing. We report that caspase(s) cleave α-spectrin to Mr~150 kD fragments and β-spectrin to Mr~120 and Mr~80 kD fragments during late embryonic chick lens development. These fragments continue to accumulate with age, so that in the oldest fiber cells of the adult lens, most, if not all, of the spectrin is cleaved to discrete fragments. Thus, unlike classical apoptosis, where caspase cleaved spectrin is short-lived, lens fiber cells contain spectrin fragments which appear to be stable for the lifetime of the organism. Moreover, fragmentation of spectrin results in reduced membrane association, and thus may lead to permanent remodeling of the membrane skeleton. Partial and specific proteolysis of membrane skeleton components by caspases may be important for age-related membrane changes in the lens.
INTRODUCTION

The spectrin-actin membrane skeleton underlies the plasma membranes of all cells, and is important for cellular shape, membrane stability and deformability, as well as the formation of membrane subdomains (1). The major component of the membrane skeleton, spectrin, is composed of an α/β heterodimer that self-associates head-to-head to form a 200 nm extended tetramer filament. Spectrin crosslinks actin filaments into an isotropic meshwork. This spectrin-actin meshwork is attached to the membrane by direct interactions of β-spectrin with membrane proteins and indirect interactions of β-spectrin with membrane attachment proteins, such as ankyrin (2).

Proteolysis of α-spectrin (αII-spectrin, nonerythroid spectrin, or fodrin) to discrete fragments is implicated in changes in cell shape and membrane morphology that occur in many cell types. During platelet activation, which includes a cell shape transformation from discs into irregular spheres, spectrin is cleaved to ~150 kD fragments by the calcium-dependent protease, calpain (3). α-Spectrin cleavage by calpain has also been implicated in cellular hypoxia (4), neuronal injury and degeneration (5), and neuronal growth cone formation (6). However, in apoptotic cells, α-spectrin proteolysis to ~150 kD fragments is mediated by caspases; in these cells, spectrin proteolysis is thought to be important for the disintegration of the plasma membranes via formation of vesicular ‘apoptotic bodies’ (7-12). While calpain cleavage of spectrin is known to affect its ability to bind membranes or actin filaments (13,14), the detailed consequences of caspase cleavage of spectrin have not been studied.

The terminal differentiation and aging of lens fiber cells is marked by dramatic membrane morphological changes. As new cells arise on the outside of the lens, older cells are pushed inward, where organelles are lost and cells fuse to form a syncytium (15,16). Since these cells are never lost from the lens, the most central cells are as old as
the organism! In addition, as lens fiber cells mature, specialized membrane interdigitations develop, which are regularly distributed on the lateral membranes (17). These age-related changes in membrane morphology have been compared to the formation of apoptotic bodies (18). In addition, they are believed to be important for lens transparency by reducing light scattering at cell boundaries and by allowing for protein turnover and ion homeostasis (16).

We have previously shown that spectrin and other components of the membrane skeleton are associated with the plasma membranes of young and old lens fiber cells (19). Here, we report that α-spectrin is cleaved to Mr ~150 kD fragments during terminal differentiation and aging of lens fiber cells. In addition, β-spectrin, which dimerizes with α-spectrin, is also proteolyzed to Mr~120 and Mr~80 kD fragments. Fragmentation of spectrin progresses with lens and fiber cell age, so that in the oldest fiber cells of the adult lens, most, if not all, of the spectrin is fragmented. The spectrin-binding and membrane-binding protein, ankyrin, is also partially proteolyzed with lens fiber cell age. N-terminal amino acid sequencing of the spectrin fragments reveals that caspase cleavage is responsible for lens spectrin proteolysis. Moreover, subcellular fractionation of lens fiber cells indicates that caspase-cleaved spectrin fragments display reduced association with lens membranes. The specific proteolysis of membrane skeleton components by caspases may be important for age-related membrane changes in the lens.
EXPERIMENTAL PROCEDURES

Antibodies

Affinity-purified rabbit polyclonal antibodies to bovine brain α-spectrin (fodrin) were prepared as described (R6017) (20). Rabbit polyclonal antibodies (PAb-10D) were raised against a recombinant peptide representing residues 1676 to 2204 of human βII spectrin, corresponding to repeat unit 13 near the middle of the COOH-terminal domain III (7,21). Monoclonal antibodies to actin (C4) were a generous gift from J. Lessard (Children’s Hospital Research Foundation, Cincinnati, OH). Rabbit polyclonal antibodies to repeats 13-24 in the N-terminal ankyrin repeats domain of human red blood cell ankyrin were a generous gift from P. S. Low (Purdue University, West Lafayette, IN).

Isolation and subcellular fractionation of lens cells

Whole lenses were harvested from chickens or rats and contaminating ciliary epithelium was removed by careful dissection. For Fig. 3, cortical fiber cells were isolated from nuclear fiber cells, which were more tightly compacted, by dissection of adult chicken lenses (6-8 weeks old). Using fine forceps on the nuclear fiber cells, the outer nuclear fiber cells were peeled away, in layers, from the inner nuclear fiber cells. Whole lenses or isolated fiber cells were rinsed in PBS with 10 mM EGTA and homogenized in lens buffer (100 mM NaCl, 25 mM Heps, pH 7.4, 4 mM MgCl₂, 10 mM EGTA, 1 mM DTT) at 30 mg/ml, using a Dounce homogenizer (8-10 strokes with the tight pestle). Subcellular fractionation was performed as described in (19). Briefly, lens fiber cell homogenates (T) were centrifuged at 30000g for 20 minutes at 4°C to separate the cytosol supernatant (S1) from the membrane pellet. The pellet was washed by two more rounds of resuspension and centrifugation to prepare washed membranes, which were either resuspended in lens buffer (P) or extracted in lens buffer with 1% Triton for 1 hour on ice. Triton extracts were subsequently centrifuged at 30000g for 20 minutes at 4°C (S2,
All procedures were performed on ice in the presence of the following protease inhibitors: PMSF (100 µg/ml) (Sigma, St. Louis, MO), aprotinin (1 µg/ml), leupeptin (15 µg/ml), pepstatin A (5 µg/ml) (Boehringer Mannheim, Indianapolis, IN) and tosyl-L-lysyl chloromethyl ketone (100 µg/ml) (Calbiochem, San Diego, CA).

**Electrophoresis and Western blotting**

SDS-PAGE was performed on large pore-10% polyacrylamide gels according to Dreyfuss et al. (22) and gels were transferred to nitrocellulose in Tris-glycine transfer buffer (23) with the addition of 0.01% SDS and the omission of methanol (this was optimal for efficient transfer of spectrin). Broad range molecular weight standards were purchased from Biorad (Hercules, CA). The relative mobility ($M_r$) of each fragment was estimated based on standard Rf analysis. Unless otherwise indicated, Western blotting was performed as described (24), except that antibodies were detected with protein A-horseradish peroxidase (Sigma), followed by standard chemiluminescence detection methods. To reprobe blots using a different antibody, blots were first stripped in 62.5 mM Tris, pH 6.5, 2% SDS, and 100 mM 2-mercaptoethanol at 65°C for 30 minutes. Stripped blots were washed extensively with PBS with 1% Triton, and then blocked and probed as usual. For the quantitation of the ratio of full-length α-spectrin to fragments, autoradiographic films were scanned into NIH Image. The total number of pixels in each band was quantified in arbitrary units. To correct for non-specific binding, pixels from an unlabeled part of the film were subtracted. Similar results were obtained from direct labeling of the blots with $[^{125}\text{I}]$-Protein A followed by $\gamma$-counting (data not shown).

**Purification of fragments and N-terminal sequencing**

Adult chicken lenses (200) were homogenized using a Dounce homogenizer at 500 mg/ml in 10 mM NaHPO$_4$, pH 7.4, 100 mM KCl, 5 mM EDTA, 5 mM EGTA, 0.5 mM caspase cleaves lens spectrin
DTT (lens buffer). Homogenates were centrifuged at 30000g to obtain a membrane pellet (water insoluble fraction), which was washed twice in lens buffer, and subsequently extracted in 10 mM NaHPO4, pH 7.4, 1.5 M KCl, 5 mM EDTA, 5 mM EGTA, 0.5% Triton, 0.5 mM DTT. Extracts were then centrifuged at 30000g and the supernatant, containing fragments and full-length spectrin, was retained (high salt extract). The high salt extract was dialyzed into 10 mM Tris, pH 8.0, 20 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM DTT and then loaded onto a 5 ml Resource Q anion exchange column (Pharmacia, Piscataway, NJ). Bound proteins were eluted using a 94 ml linear gradient from 20 to 500 mM NaCl; the elution position of fragments was determined by SDS-PAGE and Coomassie staining. Fractions enriched in α-spectrin fragments were selected, and the proteins were TCA precipitated, and run on two-dimensional gels (25), followed by transfer to PVDF membranes in 10 mM CAPS, 10% methanol, 0.01% SDS. Anion exchange fractions containing the β-spectrin 120 kD fragment were pooled separately, TCA precipitated, solubilized in 0.1% SDS (with 20 mM Tris, pH 8.0, 2.5 mM EDTA, 2.5 mM EGTA), and loaded onto a Sepharose CL-4B gel filtration column. Bound proteins were loaded and eluted in the presence of 0.1% SDS as described in Speicher, 1982 (26). Fractions enriched in the β-spectrin 120 kD fragment were loaded on a one-dimensional SDS-PAGE and transferred to PVDF membranes. Proteins were eluted from PVDF and subjected to standard N-terminal sequencing by J. Leszyk (U of Massachusetts Medical School, Shrewsbury, MA). Molecular weights of fragments were estimated based on amino acid composition using the ProtParam tool at

http://expasy.cbr.nrc.ca/tools/protparam.html.
RESULTS

Spectrin is cleaved to discrete fragments during lens development and aging

We have previously shown that α-spectrin is partially proteolyzed to three Mr~150-160 kD fragments in the nuclear (oldest) fiber cells of the adult chicken lens (19). These results suggested either that spectrin fragmentation occurs only during terminal differentiation (i.e. organelle loss) of fiber cells in the embryonic lens, or that spectrin fragmentation progresses with aging of fiber cells in posthatched lenses. To determine when α-spectrin proteolysis occurs, Western blotting was performed on proteins from chicken lenses at different stages of embryonic and post-hatched development (Fig 1A). Of particular interest were the day 6 embryo, when the lens first appears transparent (27), the day 8-10 embryo, when organelle breakdown begins (28), and the day 12 embryo, when an organelle free zone is first observed. When non-crystallin proteins were equivalently loaded (Fig 1A, actin), α-spectrin fragments were barely detected at day 17 of embryonic lens development; moreover, no fragments smaller than Mr~150 kD were detected. Strikingly, scanning quantitation of blots indicated that the ratio of α-spectrin fragments to full-length α-spectrin increased dramatically with age after hatching (Fig. 1B). In the representative experiment shown, fragments were first detected at day 17 of embryonic development (Fig. 1A). However, when proteins from twice as much tissue (wet weight) were loaded from day 12 embryonic lens proteins, α-spectrin fragments were barely detected (data not shown). Nonetheless, fragments of α-spectrin were not detected from samples of day 6, 9, or 10 embryonic lens proteins, even when 3x wet weight of tissue was loaded (data not shown). These results suggest that α-spectrin fragmentation may be initiated simultaneously with the formation of an organelle free zone. However, the extent of fragmentation of α-spectrin continues to increase in post-hatched lenses relative to late embryonic lenses, suggesting that proteolytic processing of α-spectrin progresses with lens age.
To determine whether β-spectrin, which forms a heterodimer with α-spectrin in most mammalian cells, is also proteolyzed, blots were stripped and reprobed with a β-spectrin antibody (7,21) (Fig. 1A). Two fragments were detected with the β-spectrin antibody, which was raised against a peptide from the C-terminal half of the protein (see Experimental Procedures). A Mr~120 kD fragment first appeared in day 17 embryonic lenses, when α-spectrin fragments were first detected. In contrast, a smaller Mr~80 kD fragment was not detectable in day 17 embryonic lenses, and was first detected in older lenses. These results suggest either that the Mr ~80 kD fragment arises from cleavage at a site which is digested less readily than the site for the Mr~120 kD fragment, or that the Mr~80 kD fragment arises from cleavage of the Mr~120 kD fragment. An additional band at Mr~70 kD was also barely detectable in the 18 month lens. We also observed that, similar to α-spectrin, more β-spectrin fragments relative to full-length were observed with increasing age of the lens.

Fragments of spectrin were also found to be present in the rat lens (Fig. 2). As for the chicken lens, the amount of fragmentation progressively increased with the postnatal age of the rat lens. The ratio of intensities of α-spectrin fragments to full-length α-spectrin at postnatal day 3 in the rat lens was similar to that of the day 17 embryonic chicken lens (data not shown; see Fig 1A); this is consistent with the later development of an organelle-free zone in rodent lenses as compared to chicken lenses (28,29). However, in addition to Mr, 150-160 kD α-spectrin fragments, an additional α-spectrin fragment at Mr~110 kD was found in the adult rat lens. Moreover, in addition to Mr, ~120 and ~80 kD β-spectrin fragments, another fragment at Mr~50 kD was also detected. Similarly-sized α-spectrin fragments were also observed in the newborn bovine lens (data not shown). No α-Spectrin fragments smaller than ~110 kD or β-spectrin fragments smaller than ~50 kD were detected in bovine or rat lenses (data not shown). These results indicate that both α and β-spectrin are cleaved to discrete fragments during development and aging of the chicken, rat, and bovine lens.
Spectrin is completely cleaved to discrete fragments in the oldest fiber cells of the adult lens

The progressive increase in spectrin fragmentation in older chicken and rat lenses suggested that spectrin might be progressively fragmented with the age of the lens fiber cell. To investigate this possibility, we compared the amount of spectrin fragmentation in fiber cells of different ages in the 6-8 week old adult chicken lens (Fig. 3). The youngest (~0-4 weeks old), newly differentiated cortical fiber cells (C) constitute the outermost shell of the lens fiber cell mass, and are easily peeled away from the remaining, older (nuclear) fiber cells. The outer nuclear fiber cells (oN; ~4-6 weeks old) were then separated from the inner nuclear fiber cells (iN), the oldest fiber cells of the lens. Thus, for this experiment, the iN cells were approximately 6-8 weeks old. We estimate that our iN preparation (~ 1 mm diameter core) contained ~8000 cells out of 1.5 million total lens cells (30), based on the average cross-sectional area of the human embryonic nuclear fiber cell (31).

As expected, in newly differentiated fiber cells (C), spectrin fragments were barely detectable relative to full-length by Western blotting (Fig 3). In older fiber cells (oN), the ratio of fragments to full-length spectrin was ~1:1. In contrast, full-length α or β-spectrin was barely detectable in the oldest, iN fiber cells (Fig 3, left and middle panels). Strikingly, Mr,~150 kD α-spectrin and Mr,~120 kD β-spectrin fragments were easily detectable in the iN cells, suggesting that spectrin fragments were not further degraded and lost from the lens. In some iN preparations, which presumably contained a smaller core of central fiber cells, only spectrin fragments and no full-length spectrin at all could be detected by Western blotting or silver staining (data not shown). As expected, in cortical fiber cells, Western blotting revealed that the majority of the α- and β-spectrin was full-length (Fig. 3; also see (19)). While it is technically difficult to isolate the undifferentiated epithelial cell layer from adult chicken lenses, in the undifferentiated
epithelial cells of day 15 embryonic lenses, most of the α-spectrin was full-length (data not shown).

To investigate whether the spectrin-binding and membrane-binding protein, ankyrin, is also proteolyzed during lens fiber cell aging, Western blotting was performed on cortical and nuclear fiber cells of the adult chicken lens (Fig. 3, right panel). In both cortical and nuclear fiber cells, multiple ankyrin antibodies recognized bands corresponding to the full-length ankyrin (220 kD). However, in nuclear fiber cells, an additional doublet at ~190 kD was recognized by antibodies raised against brain ankyrin, human erythrocyte ankyrin (data not shown), or an N-terminal peptide of human erythrocyte ankyrin (Fig. 3, third panel). These results suggest that ankyrin is cleaved during lens fiber cell aging. Furthermore, since the antibodies raised against an N-terminal peptide of ankyrin recognize the 190 kD cleavage product, the cleavage site is likely to be located near the C-terminus.

These results indicate that spectrin and ankyrin are progressively cleaved during fiber cell aging. Moreover, the oldest fiber cells of the adult lens are likely to contain only spectrin fragments, and no full-length spectrin.

A caspase is responsible for lens spectrin cleavage

Both calpains and caspases have been shown to cleave α-spectrin to ~150 kD fragments in vitro (7,32). However, the lens β-spectrin fragment sizes are similar to that reported for in vitro caspase cleavage of bovine brain β-spectrin (~110 and ~85 kD) (7). In contrast, calpain cleavage of β-spectrin results in 165 kD, 125 kD, and 120 kD fragments (32). The sequences of caspase-3 cleavage sites of αII- and βII-spectrin have been identified (7) and are distinct from the sites of calpain cleavage of αII and βII spectrin (32).

To determine directly whether a caspase is responsible for lens spectrin...
proteolysis, spectrin fragments were purified from adult chicken lens membranes (Fig. 4A; see Experimental Procedures) and subjected to N-terminal sequencing (Fig 4B). No sequence could be obtained from the α160 kD fragment, suggesting that this fragment contained the blocked N-terminus (7,32). The N-terminal sequence of both of the smaller α-spectrin fragments (M_r α155 and α150) mapped to the sequence DETD^{1185}*SKTASP (with * representing the site of cleavage and the beginning of the amino acid sequence obtained). This sequence is the previously reported site of cleavage of bovine brain α-spectrin II by caspase-3 in vitro (7). However, this amino acid sequence differs slightly from the GenBank sequence for chicken α-spectrin, which reports the sequence S^{1186}KTYS^{33}. RT-PCR amplification of chicken stomach and lens spectrin cDNA followed by DNA sequencing confirmed that the correct DNA and amino acid sequences, respectively, for that region are TCTAAGACAGCSTCTCT^3489 and KTA^1190. Interestingly, the caspase cleavage site in α-spectrin is located in repeat 11, just nine residues upstream of the calmodulin-binding site (32) (Fig 4B).

The N-terminal sequence of the β-spectrin M_r 120 kD fragment was XKRLTVEKKFLE (Fig 4A). Although the DNA or amino acid sequence of chicken β-spectrin II is not known, this N-terminal sequence of the M_r 120 kD fragment is homologous to the human β-spectrin II sequence DEVD^{1457}*SKRLTVQTKFME in repeat 11 (34). Importantly, this sequence is also the same site as that reported for caspase-3 cleavage of bovine brain β-spectrin II in vitro (7). Interestingly, this site was not located near regions important for ankyrin, ankyrin-independent membrane (MAD), or actin filament binding (1) (Fig 4B). Unfortunately, we were unable to obtain sufficient amounts of the β-spectrin M_r, 80 kD fragment for N-terminal sequencing.

In conclusion, the cleavage sites of lens spectrin, as well as the sizes of the α and β spectrin fragments, indicate that a caspase is responsible for spectrin cleavage in the lens. In particular, the sequences of the cleavage sites strongly implicate caspase-3.
Spectrin fragments are partially dissociated from lens plasma membranes

To investigate the biochemical consequences of caspase fragmentation of lens spectrin, subcellular fractionation of adult lens fiber cells was performed (Fig. 5). Immunoblotting of cytosol and membrane fractions indicated that α-spectrin fragments were considerably more abundant in the 30000g supernatant fractions (66.3 ± 5.97% in S1) than in the membrane pellet. In contrast, full-length α-spectrin was more abundant in the membrane pellet (only 15.6 ± 10.0 % in S1), as expected (19,35). Similarly, the majority of β-spectrin fragments remained in the 30000g supernatant (58.1 ± 3.82% in S1), while full-length β-spectrin was more abundant in the membrane pellet (only 16.8 ± 2.56 % in S1), as previously shown (19,35). However, while all of the Mr, ~80 kD β-spectrin fragment remained in the supernatant (99.5 ± 1.53% in S1), a significant proportion (~40%) of the Mr, ~120 kD β-spectrin fragment was pelleted (59.7 ± 4.78% in S1). This difference in membrane association of the two fragments may be a result of the Mr, 80 kD fragment missing the C-terminal membrane association domain (MAD 2), as suggested in Fig. 4B.

However, the small proportion of α- and β-spectrin fragments that did pellet with the membranes (P1) appeared to be tightly associated with the membranes. These fragments were not extracted in 1% Triton (S2), and were only partially extracted by 1M NaCl or by 1% Triton with 1 M NaCl (data not shown). Thus, spectrin fragments that are membrane-associated might constitute a different population from the cytosolic fragments.

Interestingly, all three of the α-spectrin fragments in the supernatant appeared to fractionate together on gel filtration (data not shown), suggesting that they were tightly associated in a complex. On the other hand, the β-spectrin fragments did not fractionate together, suggesting that they had dissociated from one another and from the remaining full length α-spectrin. Thus, our results collectively indicate that caspase fragmentation
caspase cleaves lens spectrin

of spectrin leads to partial dissociation of both spectrin subunits from lens membranes.
DISCUSSION

This is the first report of caspase cleavage of membrane skeleton proteins to discrete and stable fragments during cellular maturation and aging. We have shown that α-spectrin is cleaved to Mr ~150 kD fragments and β-spectrin to Mr ~120 and Mr ~80 kD fragments during lens fiber cell maturation and aging. The spectrin-binding protein, ankyrin, is also cleaved to Mr ~190 kD fragments. These fragments appear to be extremely stable, and indeed, accumulate with age. In contrast, in other cell types, caspase cleavage of spectrin precedes cell death, thus ensuring a short half-life for the spectrin fragments (7-12). Moreover, when cleavage of spectrin does not lead to cell death, (i.e. neuronal remodeling), a brief period of accumulation of fragments is followed by a decrease in the proportion of fragments to full-length spectrin polypeptides, suggesting replacement of spectrin fragments with newly-synthesized full-length spectrin (36). Our results indicate that in lens fiber cells, which do not undergo cell death, the accumulation of caspase cleavage products of spectrin and ankyrin may lead to a permanent remodeling of the membrane skeleton.

The cleavage of membrane skeleton components in the lens is specific. In contrast to spectrin and ankyrin, other components of the membrane skeleton, tropomodulin, tropomyosin, and actin, do not appear to be proteolyzed during lens fiber cell aging (19). However, there are reports of other membrane-associated lens proteins being proteolyzed to discrete and stable fragments. Partial proteolysis of the major lens membrane protein, MP-26 from a Mr ~26 kD to a Mr ~20 kD protein, has been reported to occur during fiber cell maturation (37). The gap junction protein, connexin 50 (α8) has been shown to be cleaved by calpain, leading to removal of the C-terminal tail of the protein from the plasma membranes (38). Connexin 46 (α3) may also be cleaved in mature fiber cells (39). The lens-specific intermediate filament protein, filensin, is also proteolyzed to a discrete Mr ~53 kD band in maturing fiber cells in the bovine lens (40).
and multiple bands in the chicken lens (19). Finally, Mr ~150 kD $\alpha$-spectrin fragments have been observed in the rabbit and guinea pig lens (41), in addition to our observations in the chicken, rat, and cow lens. Partial proteolysis of particular membrane-associated proteins (and not others) to discrete and stable fragments may be important for membrane remodeling during lens fiber cell aging.

The timing of spectrin fragmentation also implies roles in mediating membrane morphological changes during lens fiber cell aging. Spectrin fragments are first detected late during embryonic development, coincident with formation of the organelle-free zone (28), but continue to accumulate during development and after hatching. In the adult lens, spectrin fragmentation appears to be restricted to older fiber cells, and the amount of spectrin fragmentation increases with the age of the lens fiber cell. Unfortunately, it is difficult to compare the timing of spectrin fragmentation with that of other membrane-associated proteins, because a similar developmental analysis has not been performed with other proteins. However, a functional syncytium of cells within the organelle-free zone is first detected at day 12 of embryonic development, and expands with age (16). In addition, membrane protrusions are not present in the day 7 embryonic lens, but are first observed later in the day 10 embryonic lens; these membrane protrusions become more elaborate with age (42). Moreover, in the adult chicken lens, newly differentiated cortical fiber cells exhibit smooth profiles, while older fiber cells display numerous membrane protrusions (43). In support of a role for spectrin fragmentation in the development of membrane protrusions, antibodies which recognize both full-length and fragments of $\alpha$-spectrin stain protrusions of nuclear fiber cells (19) as well as blebs decorating differentiated lens cells in culture (B. Fischer & V.M. Fowler, unpublished observations).

This is the first report of caspase-mediated cleavage of membrane-associated components in the lens. Our data suggests that caspase-3 may be involved in proteolysis
of spectrin during lens fiber cell maturation and aging. The sequence of the cleavage site for chicken lens α-spectrin is identical to that obtained by in vitro cleavage of bovine brain α-spectrin II by caspase-3. In addition, the sequences of cleavage for both chicken lens α- and β- spectrins (DETD*S, DEVD*S) match well with the consensus DXXD*S cleavage site identified for other caspase-3 substrates (44). In rat and cow lenses, the presence of the ~110-120 kD α-spectrin fragment generated by caspase 3 cleavage in vitro also suggests cleavage of endogenous lens α-spectrin by caspase-3 (7,45). The lack of a ~120 bp fragment of α-spectrin in the chicken lens may be due to conformational differences between chicken and mammalian spectrins.

Caspase activity has previously been shown to be necessary for loss of nuclei during lens fiber cell differentiation (46-48). In addition, members of the caspase family (1,2,3,4,6), and the caspase substrates, DNA fragmentation factor and poly-(ADP-ribose) polymerase (PARP), have been identified in the lens (46). Like spectrin, PARP has also been reported to be cleaved late in lens development, after organelle loss (46).

While our data suggests that caspase cleavage of the membrane skeleton occurs following organelle loss, others have reported that caspase activation is required for organelle loss. This apparent discrepancy may be explained by the idea that different caspases may be active at different times during lens fiber cell maturation. According to Wride et al., 1999 (46), particular caspases (i.e., caspase-3) are present early during lens fiber cell differentiation, while others (i.e., caspase-1) are predominant in older lens fiber cells. Moreover, while inhibitors of caspases-1, 2, 6, 9 appeared to inhibit nuclear loss in lens cell cultures, inhibitors of caspase-3, 8 were ineffective. Both of these observations suggest that, while the caspase pathway has not been precisely delineated for lens cells, particular caspases are important at different times during lens fiber cell differentiation. Thus, it is possible that specific caspases (particularly caspase-3) are important for selective proteolysis of the membrane skeleton following organelle loss, while other
Caspases are important for initiating organelle loss.

What are the molecular consequences of caspase cleavage of spectrin and ankyrin? We have shown that spectrin fragments are partially dissociated from lens membranes. This may be due to cleavage of ankyrin, which binds to both β-spectrin and integral membrane proteins. Calpain-cleaved ankyrin, which, like lens ankyrin, is also a ~190 kD fragment derived from cleavage near the C-terminus, exhibits an 8-fold weaker affinity for erythrocyte membranes (49). Alternatively, cleavage of β-spectrin could lead to reduced membrane association. However, the initial site of cleavage near the middle of the protein is not within its ankyrin-binding site, actin-binding site, or within any of the ankyrin-independent membrane binding domains (MAD) (1). Thus, β-spectrin cleavage is unlikely to interfere with membrane association by direct interference with its membrane binding sites, but perhaps indirectly by affecting the conformation of these sites.

Cleavage of lens spectrin by caspase may lead to lowered membrane affinity as well as calmodulin down-regulation of spectrin activities. The cleavage site on α-spectrin is within 9 amino acids of the calpain cleavage site, and proximal to the calmodulin binding site (Fig 4B). When calmodulin is bound to α-spectrin during the action of calpain, both α- and β- spectrin subunits are cleaved, and the hetero-tetramer irreversibly dissociates into its component fragments (14). This results in complete loss of tetramer formation, F-actin binding or membrane binding. Such a dissociation of spectrin fragments is reminiscent of the dissociation reported here that results from caspase-mediated cleavage of both subunits in the intact heterodimer.

Interestingly, α and β spectrin are both cleaved at repeat 11, at sites that interact in the α,β-spectrin heterodimer (50), suggesting that spectrin may be fully assembled before cleavage. In contrast, in other types of cells (1), calpain may either cleave the α- or β- spectrin subunits separately, or together in the intact hetero-tetramer, thus leading
to targeted loss of tetramer assembly, F-actin binding, or membrane binding (13,14).
Thus, while other cell types may use a multi-step process involving calpain and calmodulin to accomplish an incrementally regulated disassembly of the spectrin cortical skeleton, lens cells may use predominately caspase and calmodulin to disassemble their membrane skeleton in a single-step process.

We have developed a speculative model (Fig. 6) to describe how specific and partial proteolysis of membrane skeleton proteins could lead to membrane morphological changes in the lens. In young (cortical) fiber cells (Fig 6A), as in other non-erythroid cells, α/β spectrin tetramers are likely cross-linked to short actin filaments. This two-dimensional meshwork is then anchored to the membranes via spectrins interactions with ankyrin and other membrane proteins (1,51). In older fiber cells (Fig 6B), localized caspase activity could result in proteolytic cleavage of specific regions of the membrane skeleton, loosening constraints on the membranes, and thus, allowing for membrane blebbing in specific sites. The extent and placement of membrane blebbing could be regulated by whether ankyrin, α-spectrin, and/or β-spectrin are cleaved, or by calmodulin-binding. With age, more caspase activity, and thus, more proteolytic processing would occur. This could result in the increased density of membrane protrusions with fiber cell age (17). Membrane blebbing could lead to repositioning of integral membrane proteins, such as ion channels, which might be necessary for age-related changes in ion and water flow (52). Moreover, membrane blebbing might lead to cell-cell fusion events which have been observed in maturing lenses (16,17).

Interestingly, cleavage of α-spectrin by calpain, not by caspase, has been associated with a number of cataract models (41,53). Inhibition of calpain inhibits cataract formation and spectrin cleavage (54), and human lenses with age-related nuclear cataracts display a higher density of finger-like membrane projections than transparent lenses of the same age (55). Thus, it is possible that aberrant cleavage of spectrin by
calpain may lead to uncontrolled or incomplete membrane furrowing and opacification of the lens, while specific cleavage of spectrin by caspase may be important for normal physiological functioning of the lens.

In conclusion, partial and specific proteolysis of spectrin and ankyrin by caspases appears to effect an apoptosis-like program of membrane changes during lens aging. Further characterization of lens membrane morphology and membrane skeleton proteolysis in caspase knockouts, as well as additional biochemical analysis of the lens membrane skeleton, will lead to greater insight in the importance of caspase cleavage of membrane skeleton components during lens development and function. It is likely that the transparency of the aging lens nucleus may depend not only on organelle loss but also on membrane skeleton remodeling. We anticipate that these changes in the membrane skeleton, induced by caspase mediated but limited proteolysis, will modulate ion homeostasis, the positioning of ion channels, the frequency of intercellular fusion events, and lens deformability.

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FIGURE LEGENDS

Fig. 1. α and β-Spectrin are cleaved to discrete fragments during development and aging of the chicken lens. A. Western blotting of lens proteins harvested from chickens at different days of embryonic development (day 6, 9, 10, 12, 15, 17) or ages after hatching (1 day, 6 weeks, 5.5 months, 18 months). α-Spectrin is partially proteolyzed to Mr, ~150-160 kD fragments in the older embryonic and post-hatched chicken lens. Simultaneously, β-Spectrin fragments at Mr, ~120 kD and Mr, ~80 kD are also detected during late embryonic development. Western blotting for actin indicates that lanes were relatively evenly loaded for non-crystallin proteins (the concentration of non-crystallin proteins decreases with lens age (56). Wet weight of tissue loaded is as follows: 0.7 mg (6E, 9E, 10E, 12E), 1.2 mg (15E, 17E, 1D, 6 wks, 5.5 mo.), and 0.92 mg (18 mo). B. NIH Image quantitation of the ratio of intensities of the full-length α-spectrin band relative to the intensities of the α-spectrin fragments. The average and standard error of two experiments is shown. Comparison of Coomassie staining (data not shown) and Western blotting indicates that our transfer and Western blotting procedures slightly overestimate the amount of fragment to full-length by 1.1 fold.

Fig. 2. Spectrin fragmentation occurs during rat lens development and aging. Western blotting of lens proteins from postnatal day 3, day 10, and adult (6 weeks) rats using antibodies raised against α- and β-spectrin. Note the presence of an α-spectrin fragment at Mr, 110 kD. α-Spectrin is partially proteolyzed to one Mr, ~120 kD and three Mr, ~150-160 kD fragments. Simultaneously, β-Spectrin fragments at Mr, ~120 kD, Mr, ~80 kD, and Mr, ~50 kD are also detected during late embryonic development.
Fig. 3. Spectrin is cleaved to discrete fragments during lens fiber cell aging. A,B. Western blotting for spectrin of lens proteins from cortical (C), outer nuclear (oN), and inner nuclear (iN) fiber cells of the adult chicken lens (6-8 weeks). We were unable to use actin as a loading control, because the high amounts of δ-crystallin in inner nuclear fiber cells interfered with the detection of actin. However, proteins corresponding to 0.6 mg cortex (1x), 1.2 mg or 1.7 mg outer nucleus (2x, 3x), and 1.2 mg or 4.7 mg inner nucleus (2x, 7x) were loaded. These are roughly equivalent estimates of non-crystallin protein, because δ-crystallin protein concentration increases (and thus, non-crystallin concentration decreases) by ~3-fold from cortex to outer nucleus, and an additional ~1.5-fold from outer nucleus to inner nucleus (57). Moreover, the ratio of intensities of the full-length to fragment bands for each sample did not change when different amounts of sample were loaded. This experiment was repeated three times with similar results; a representative experiment is shown. C. Western blotting for ankyrin of lens proteins from cortical and nuclear fiber cells of the adult chicken lens (6-8 weeks).

Fig. 4. N-terminal sequencing indicates a caspase is responsible for lens spectrin cleavage. A. α- and β-Spectrin fragments were purified from adult chicken lens membranes and subjected to N-terminal sequencing. For α-spectrin, sequence was determined from RT-PCR followed by DNA sequencing of chicken lens and stomach α-spectrin RNA. For β-spectrin, the human (34) and bovine (7) sequences were used. B. Caspase cleavage sites for α- and β-spectrin are drawn. Depicted is the domain structure of spectrin, with boxes identifying each ~106 residue repeat. The placement of unknown cleavage sites (?) was estimated based on the fragment size and known sites for caspase-3 digestion of spectrin in vitro (7). The location of binding sites for calmodulin (CaM), ankyrin, actin, and ankyrin-independent membrane binding sites 1-3 (MAD1,2,3) are also shown (1). The β-spectrin antibody used (pAb10D) was raised against a peptide
Fig. 5. Spectrin fragmentation results in partial dissociation from lens membranes. A.
Adult chicken lenses (6-8 weeks) were homogenized in a physiological buffer followed by centrifugation at 30000g to obtain cytosol-enriched supernatants (S1) and membrane pellets (P1). Membranes were then extracted to obtain 1% Triton-soluble (S2) extracts and Triton-insoluble pellets (P2). Proteins were subjected to Western blotting with anti-\(\alpha\) and anti-\(\beta\) spectrin antibodies. B. Histogram indicates fraction of total full-length spectrin or spectrin fragments in S1. The percentage of soluble spectrin or spectrin fragments was calculated by dividing the amounts in S1 by the sum of S1 and P1. For \(\alpha\)-spectrin and \(\beta\)-spectrin, respectively, the average percentage and standard deviation of 6 or 3 experiments is shown.

Fig. 6. Caspase cleavage of spectrin and ankyrin may lead to cell-cell fusion and the formation of membrane protrusions during lens fiber cell aging (a model). A. In the cortex, the spectrin-actin membrane skeleton is intact, contributing to the stability and shape of the plasma membrane. Spectrin tetramers are linked to short actin filaments, which are capped by tropomodulin (58). Integral membrane proteins are positioned in the membrane skeleton via interactions with ankyrin and also through direct binding to \(\beta\)-spectrin at MAD domains. Sites for caspase cleavage of \(\alpha\)- and \(\beta\)-spectrin are located in regions of contact in the heterodimer (50). B. In the nucleus, localized caspase activity leads to cleavage of \(\alpha\)- and \(\beta\)-spectrins, and ankyrin. When both \(\alpha\)- and \(\beta\)-spectrins are cleaved, complete loss of actin and membrane binding abilities are likely, and the membranes are no longer constrained (A). Fragmentation of ankyrin reduces its affinity for membranes (B). In the presence of calmodulin (CAM), and when only \(\alpha\)-spectrin is...
cleaved, the ability to form tetramers, and bind actin filaments and membranes is lowered (C). The spatially restricted reduction of membrane affinity allows for membrane blebbing and repositioning of integral membrane proteins (D). Membrane blebbing may lead to cell-cell fusion and the formation of membrane protrusions.
caspase cleaves lens spectrin
### A. N-terminal sequencing

| Fragment | N-terminal sequence | Predicted cleavage site | Assumed C-terminus | Calc M_r |
|----------|---------------------|-------------------------|--------------------|----------|
| α160     | Blocked             | N-terminus, assumed     | D_{1185}           | 136087   |
| α155     | SKTASPW             | DETD_{1185}^*SKTASPW    | N_{2477}           | 148834   |
| α150     | SKTASPW             | DETD_{1185}^*SKTASPW    | ?                  | ?        |
| β120     | XKRLTVEKKFLE        | DEVDD_{1457}^*SKRTLTVQTGFME (human, bovine) | K_{2364}          | 104605   |

### B. Caspase digestion sites

![Diagram of α-spectrin and β-spectrin cleavage sites]

- **α-Spectrin**
  - 160 kD
  - 155 kD
  - 150 kD

- **β-Spectrin**
  - 165 kD
  - 120 kD
  - 80 kD
A. Western

|   | T | S1 | P1 | S2 | P2 |
|---|---|----|----|----|----|
| α-Spectrin |   |    |    |    |    |
| β-Spectrin |   |    |    |    |    |

B. Quantitation

|         | % soluble (S1) |
|---------|----------------|
|         | full-length    | frags         |
| α-Spectrin | 15.6 +/- 10.0  | 66.3 +/- 5.97 |
| β-Spectrin | 16.8 +/- 2.56  | 58.1 +/- 3.82 |
A. Cortex

β-Spectrin
α-Spectrin
Ankyrin
Actin
Tropomodulin
caspase cleavage

B. Nucleus

C
D
A
B
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