Degradation of an Old Human Protein

AGE-DEPENDENT CLEAVAGE OF γS-CRYSTALLIN GENERATES A PEPTIDE THAT BINDS TO CELL MEMBRANES*[S]

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Background: Long-lived proteins degrade over time.
Results: Lens γS-crystallin is extensively modified with age, and truncation near the C terminus generates a peptide that binds tightly to cell membranes.
Conclusion: The peptide released from γS-crystallin can adopt an α-helical conformation and may alter permeability by interacting with cell membranes.
Significance: Peptide binding may explain why human lens membranes change with age.

Long-lived proteins exist in a number of tissues in the human body; however, little is known about the reactions involved in their degradation over time. Lens proteins, which do not turn over, provide a useful system to examine such processes. Using a combination of Western blotting and proteomic methodology, age-related changes to a major protein, γS-crystallin, were studied. By teenage years, insoluble intact γS-crystallin was detected, indicative of protein denaturation. This was not the only change, however, because blots revealed evidence of significant cross-linking as well as cleavage of γS-crystallin in all adult lenses. Cleavage at a serine residue near the C terminus was a major reaction that caused the release of a 12-residue peptide, SPAVQSFRRIVE, which bound tightly to lens cell membranes. Several other crystallin-derived peptides with double basic residues also lodged in the cell membrane fraction. Model studies showed that once cleaved from γS-crystallin, SPAVQSFRRIVE adopts a markedly different shape from that in the intact protein. Further, the acquired helical conformation may explain why the peptide seems to affect water permeability. This observation may help explain the changes to cell membranes known to be associated with aging in human lenses. Age-related cleavage of long-lived proteins may therefore yield peptides with untoward biological activity.

A number of sites in the human body contain long-lived cells (1–4); however, little is known about the longevity of individual proteins within these tissues. It is likely that the degeneration of abundant long-lived proteins, such as collagen (5), elastin (6), and components of the nuclear pore complex of postmitotic cells (7), contributes to age-related deterioration of tissues and may impact human fitness and health in old age (8). A useful tissue in which to examine such age-related changes is the human lens. The lens contains few major polypeptides, there is no protein turnover (9), and consequently post-translational modifications (PTMs)[2] accumulate in these life-long polypeptides over time. Abundant PTMs are racemization (10, 11), methylation (12, 13), and deamidation (14, 15). Some lens proteins, such as the α-crystallins (16–20) and aquaporin 0 (21–22), also undergo progressive and extensive truncation.

In lenses of experimental animals, some cleavages may be due to enzyme hydrolysis (23–25). In humans, it is unlikely that truncations are the result of protease activity because enzymes are inactive in the center of adult human lenses (26, 27), and this is presumably due to enzyme denaturation (26) following decades of exposure to body temperature. Knowledge of the processes involved in spontaneous peptide bond scission in proteins is incomplete, although details of cleavage on the C-terminal side of Asn residues and the involvement of a cyclic succinimide intermediate have been elucidated (28). Another amino acid that is susceptible to cleavage in old proteins is Ser (29), and several peptides in the lens contain N-terminal Ser (16, 30).

Although truncations increase with age, and this is accompanied by the formation of small peptides (16, 30, 31), little is known about the fate of these products. Some peptides originating from the chaperone α-crystallin have been implicated in protein aggregation and crystallin insolubility (16, 32) features associated with aged lenses (33, 34). In this work, we demonstrate that another protein, γS-crystallin, also undergoes substantial PTM with age, resulting in the formation of cross-linked as well as truncated polypeptides. Cleavage of γS-crystallin at a Ser residue near the C terminus yields a 12-residue peptide, SPAVQSFRRIVE, which was found to bind tightly to fiber cell membranes. Model studies showed that, once cleaved from γS-crystallin, SPAVQSFRRIVE adopts a markedly different shape from that in the intact protein and

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[2] The abbreviations used are: PTM, post-translational modification; WSP, water-soluble protein; USP, urea-soluble protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CAPS, 3-(cyclohexylamino)propane-sulfonic acid.

that this helical conformation may explain why it interacts so strongly with the membrane and also affects permeability. An analogous process may underpin the substantial alteration in water penetration that has been found to take place in the cell membranes of aged lenses (35).

EXPERIMENTAL PROCEDURES

Protein and Peptide Extraction—Human lenses across the age range were obtained from the Lions Eye Bank, Sydney Eye Hospital. Enucleation occurred within 12 h of death, and lenses were stored at −80 °C until use. Tissue was handled in accordance with the tenets of the declaration of Helsinki, and ethical approval was obtained from Sydney. Lenses were dissected into cortex and nucleus using a trephine with a diameter of 4.5 mm. A cold scalpel was used to remove ~1 mm from each end from the nuclear core. Tissue was homogenized in 500 μl of 10 mM phosphate, pH 7.0, containing protease inhibitor (Roche Applied Science) and centrifuged at 20,000 × g for 20 min at 4 °C. Supernatants were collected, and homogenization was repeated three times to yield water-soluble protein (WSP). The resultant insoluble pellet was homogenized in 10 mM Tris, pH 8.0, containing 8 M urea (500 μl) three times to produce urea-soluble protein (USP). The resultant urea-insoluble material is referred to as the membrane pellet. Peptides were extracted from the membrane pellets using 95% ethanol overnight at −20 °C. The ethanol was dried down, and the lipid film was solubilized in a small volume of 70% formic acid which was diluted to 1% formic acid prior to HPLC using lysine (5 mg/ml).

Gel Electrophoresis—WSP and USP concentration was determined using a micro-BCA assay (Pierce). Protein samples (10 μg) were loaded onto a 16% Tris-Tricine gel (Nusep, Bogart, GA). Prior to loading, an equal volume of 2× sample buffer was added to each sample (0.5 M Tris buffer, pH 6.8), glycerol (50%, v/v), SDS (10%, w/v), bromphenol blue (0.5%, w/v) and mercaptoethanol (5%, v/v) and heated for 5 min at 95 °C.

Western Blots—Proteins were transferred onto nitrocellulose membranes (Invitrogen) using CAPS buffer (10 mM, 10% (v/v) methanol, pH 11) at 100 V for 120 min. The membrane was blocked with Blotto, nonfat dry milk (5% w/v) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 45 min and then incubated with one of three different antibodies raised against either full sequence (Novus Biologicals, Littleton, CA; H00001427-B01, dilution 1:1500); C terminus (Santa Cruz Biotechnology, Inc.; sc103180, dilution 1:800), or the N terminus (Abcam; ab80041, dilution 1:400) of γS-crystallin for 16 h at 4 °C. Donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc.; sc-2020, dilution 1:4000), goat anti-rabbit IgG-HRP (Abcam; ab6721, dilution 1:4000), or goat anti-mouse IgG-HRP (Abcam; ab6789, dilution 1:4000) was added, respectively, for 2 h at room temperature. Immunoreactive proteins were enhanced using chemiluminescence (SuperSignal West Pico Substrate, Pierce) and visualized using GeneSnap software (Syngene) with a gel doc system.

HPLC—Reversed phase HPLC was performed on a Shimadzu system (Kyoto, Japan). Peptides were separated on a Phenomenex column (Jupiter; 5 mm, C18, 300 Å, 250 × 4.6 mm) with solvent A (aqueous 0.1% (v/v) TFA) and solvent B (100% (v/v) acetonitrile, 0.1% (v/v) TFA) using the following

mobile phase conditions: isocratic (10% solvent B) 0–10 min, gradient to 60% solvent B (10–60 min), gradient to 90% solvent B (60–70 min), isocratic (70–85 min). The flow rate was 1.0 ml/min with detection at 216 and 280 nm. A standard curve of the peptide SPAVQSFRRIVE (GLS peptide synthesis, Shanghai, China) was constructed to allow quantification.

Mass Spectrometry—An Axima MALDI TOF[2] mass spectrometer (Shimadzu, Kyoto, Japan) in linear and positive ion mode was used for peptide analysis. Peptides were prepared in α-cyano-4-hydroxycinnamic acid (8 mg/ml) in 50% (v/v) acetonitrile, 1.0% (v/v) trifluoroacetic acid. Each spectrum was acquired with 350 laser shots.

Vesicles—Vesicles were prepared from phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) using the method of Mozafari *et al.* (36). Briefly, the lipid was incubated for 2 h at 25 °C in 20 mM HEPES buffer, pH 7.2, containing 3% glycerol and then heated for 20 min at 120 °C. Laurdan was added to a final concentration of 0.5 μM and incubated for 1 h at 37 °C. This method most likely results in the formation of both multilamellar and unilamellar vesicles. Peptides and protein were dissolved separately (1.0 mg/ml) in 20 mM HEPES buffer (pH 7.2). Vesicles (195 μl, 0.1 mg/ml) were incubated for 30 min with the peptide (5 μl) at 37 °C. Samples were measured in a fluorometer (Safire 2, TECAN, Männedorf, Switzerland) at an excitation wavelength of 360 nm. A ratio of emission intensities from 440 and 490 nm was used to calculate general polarization values. Each point represents the mean of six replicates.

Circular Dichroism—Far-UV spectra were recorded on a JASCO J-810 spectropolarimeter using peptides (0.1 mg/ml) dissolved in MilliQ water and varying amounts of trifluroethanol. A path length of 1 mm was used, and data were acquired between 180 and 250 nm in wavelength scan mode with a bandwidth of 1 nm, a step size of 0.1 nm, and scan speed of 100 nm/min. A total of six scans were acquired, and the data were averaged.

RESULTS

Western Blots—In order to visualize the extent of age-related modification of γS-crystallin, samples of both WSP and water-insoluble proteins, which were solubilized with 8 M urea to generate USP, from individual human lenses across the age range were examined by gel electrophoresis and Western blotting. Three different antibodies to γS-crystallin were used: one to the full sequence, one specific for the N terminus, and one specific for the C terminus of the protein.

To determine antibody specificity, each antibody was first examined with fetal WSP because these proteins should show no age-related PTMs. Full sequence and C- and N-terminal antibodies gave a single band corresponding to the molecular weight of γS-crystallin (supplemental Fig. 1*a*).

Full Sequence Antibody—As seen in Fig. 1, the full sequence antibody typically gave rise to a single band at ~21 kDa in both WSP and USP (*i.e.* insoluble) fractions. A clear age-related effect was observed in lenses below the age of 40. Soluble γS-crystallin decreased, and there was a corresponding appearance of a 21 kDa immunoreactive band in the USP. Such a pattern is consistent with γS-crystallin undergoing conforma-
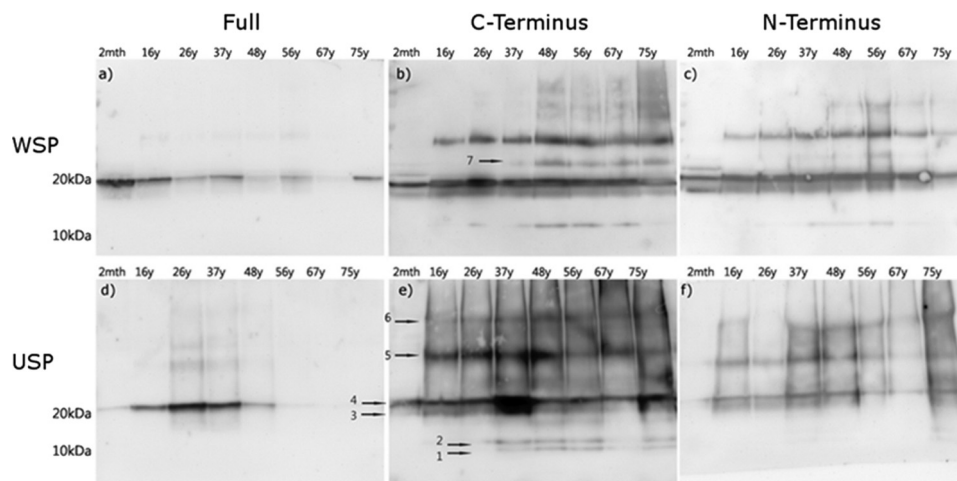
FIGURE 1. **Western blots using three different antibodies against γS-crystallin with WSP and USP from the nuclear region of human lenses.** Ages are shown above each *lane*. *a* and *d*, WSP and USP probed with a full sequence antibody. *b* and *e*, WSP and USP probed with antibody to the C terminus of γS-crystallin. *c* and *f*, WSP and USP probed with an antibody to the N terminus of γS-crystallin. *Numbered bands* are discussed in "Results."

tional changes that lead to protein insolubility, without the involvement of truncation.

Also apparent from the blots was that very little immunoreactive protein was seen in the older lenses, such that by the age of 50 there was little or no detection of any γS-crystallin band on the blots. One explanation is that modifications to γS-crystallin take place progressively in the lens, such that by middle-age, any full-length crystallin that remains is so extensively altered that it is rendered unable to bind to the full-sequence antibody.

It has been described previously that γS-crystallin undergoes truncation at both the N and the C terminus (16). To determine whether one reason for the observed loss of γS-crystallin immunoreactivity with age may be due to loss of one or both termini, two antibodies, one specific to the N terminus (γS-crystallin 19–37) and one to the C terminus (γS-crystallin 167–178), were utilized.

C Terminus Antibody—The WSP and USP fractions when probed with the C terminus antibody revealed significant differences from that of the full sequence antibody (Fig. 1, *b* and *e*). Even at the age of 2 months, a lower M_r band was present with an approximate mass of 19 kDa. This band at ~19 kDa corresponds with the expected mass of intact γS-crystallin minus an N-terminal peptide of ~2.5 kDa (band 3), which has been reported previously (16). This truncation was absent in the fetal lens, suggesting that loss of the N-terminal peptide begins soon after birth. After 16 years, a broadening of the γS-crystallin band (21 kDa) was observed, and this is most likely due to increasing PTMs with age (37). After age 37, there was a notable decrease in the amount of soluble intact γS-crystallin (band 4); by age 75, ~60% had been lost (supplemental Fig. 1*b*).

An immunoreactive band with a mass of ~10 kDa (band 2) was observed in both the WSP and USP fractions. This was first observed at the age of 26 and presumably corresponds to cleavage in the linker peptide between the two γS-crystallin domains (38). After the appearance of the 10-kDa peptide, another smaller peptide was detected in the USP fraction with a mass ~8–9 kDa (band 1). This peptide was detected only in USP fraction with the C-terminal antibody and only after the initial

cleavage of γS-crystallin in the linker peptide was observed. Increased loading of the gel prior to Western blotting did not lead to detection of the peptide in the WSP fraction.

Although truncation of γS-crystallin was apparent with age, higher M_r protein bands were also a consistent feature of the blots for both the WSP and USP fractions after the age of 16 (Fig. 1, *b*, *c*, *e*, and *f*). The major band corresponds in mass with that of a γS-crystallin dimer (band 5). A weaker higher mass band (~60 kDa) was also present (band 6). Previously, it has been reported that γS-crystallin can form both dimers and trimers via a unique non-disulfide cross-link that has not been identified (18). Interestingly, an immunoreactive ~26 kDa band was also observed, more clearly in the WSP, and this may represent a similar cross-linkage between γS-crystallin and a small peptide (band 7).

N Terminus Antibody—The results for the blots using the N terminus antibody largely mirrored those of the C terminus antibody; however, the N terminus antibody appeared to interact more weakly (Fig. 1, *c* and *f*). The pattern of cross-linking of γS-crystallin was almost identical. The lower mass bands at ~10 and ~7 kDa were less apparent with this antibody, perhaps reflecting its overall weaker interaction in Western blotting. Even in the 2-month lens, a lower mass band corresponding to loss of ~2–2.5 kDa from the intact γS-crystallin was clearly observed.

It should be noted that the full sequence antibody barely detected any of the cross-linked γS-crystallin species, suggesting that the epitope recognized by this antibody is either not exposed for detection or has been altered. On the other hand, both the C terminus (residues 167–178) and N terminus (residues 19–37) antibodies reacted strongly with these higher M_r forms of γS-crystallin, suggesting therefore that these parts of the protein are not involved in the cross-linkage. On this basis, the cross-link may be located within residues 43–166 of γS-crystallin.

Membrane-bound γ-Crystallin—Western blotting had shown that, with age, significant amounts of γS-crystallin, as well as both truncated and cross-linked versions, became insoluble (*i.e.* were in the USP fraction). To determine if there was
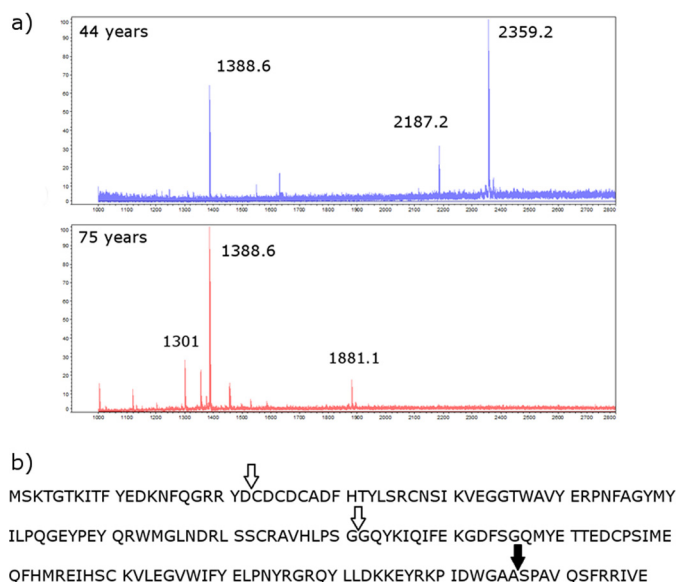
FIGURE 2. *a*, MALDI mass spectra of membrane pellets from the nuclear region of a 44- and a 75-year-old human lens following extraction with ethanol. MS/MS spectra confirmed the following: *m/z* 1301 (γS-crystallin 166–178), *m/z* 1388 (γS-crystallin 167–178), *m/z* 1881 (βA3-crystallin 200–215), *m/z* 2187 (αB-crystallin 2–18), and *m/z* 2359 (1–18 αB-crystallin). All peptides contain an RR sequence. *b*, reported sites of cleavage in γS-crystallin (16, 38).

any additional γS-crystallin associated with the membrane fraction, the pellet remaining after the 8 M urea extraction that contains cell membranes was extracted with 0.1 M NaOH and examined by SDS-PAGE. Following staining with Coomassie dye, no bands at the M_r of γS-crystallin were observed; however, intense staining was observed at the dye front (data not shown). When examined by MALDI mass spectrometry, a number of peptides were detected in the extract. In particular, one peptide with an *m/z* of 1388 was detected with high signal intensity and was found in all membranes examined. Other peptides were also observed (Fig. 2), but they tended to be more variable. MS/MS confirmed the *m/z* 1388 peptide to be SPAVQSFRRIVE, which matched the C-terminal peptide of γS-crystallin. Quantification of the γS-crystallin peptide by Western blot analysis was attempted using the antibody specific to the C terminus; however, peptide transfer was very inefficient.

Peptides Bound to Cell Membranes—To determine the amount of the C-terminal γS-crystallin peptide (SPAVQS-FRRIVE) that was bound to membranes, a more gentle extraction protocol was developed that avoided any possibility of base-catalyzed peptide bond cleavage. Membrane pellets from the nuclear region of lenses ranging in age from 19 to 75 years were extracted with 95% ethanol. This solvent had been shown to efficiently extract samples of the synthetic peptide. MS/MS of the peptide from the lens extracts confirmed that its sequence was SPAVQSFRRIVE (*i.e.* γS-crystallin 167–178). This was consistent in all samples examined (Fig. 2). Additional truncated forms of the γS-crystallin peptide were sometimes detected in samples in much lower abundance (*e.g.* PAVQS-FRRIVE, AVQSFRRIVE, and SFRRIVE). In some extracts, additional peptides were detected with masses of 2187 and 2389 *m/z* (Fig. 2*b*), which MS/MS showed correspond to N-terminal peptides 2–18 and 1–18 of αB-crystallin. Irregularly, ions were seen at 3389 and 3252 *m/z*, which MS/MS confirmed were due to the

C-terminal peptides of βA3-crystallin 188–215 and 189–215. All peptides contained an RR sequence.

RPHPLC was used to quantify the amount of SPAVQS-FRRIVE bound to lens membranes with age. Initial attempts, using several methods, to extract the peptide from the cell membranes and to quantify it reproducibly, yielded variable results. It appeared that an interaction between membrane phospholipids and the peptide was occurring; however, conventional extraction protocols to separate the peptide from lipids were unsuccessful. Previous studies have demonstrated tight binding of basic residues, such as arginine and lysine, in peptides with phospholipids (39–41). It was assumed that the double arginine present in the γS-crystallin peptide was binding tightly to the negatively charged phosphate moiety of the membrane phospholipids. To overcome this interaction, the lens ethanol extract was dissolved in 70% formic acid, and excess free lysine was added to compete with peptide binding sites on the lipid. Using this protocol, the γS-crystallin peptide in the extract eluted reproducibly at the same time as the standard SPAVQSFRRIVE (Fig. 3*a*). Other crystallin-derived peptides were found bound to the membrane using this protocol; however, they were not examined in detail in this study.

The amount of SPAVQSFRRIVE bound to the membrane was determined by HPLC and is shown in Fig. 3*b*. The γS-crystallin peptide was confirmed by MALDI MS/MS in all lens membrane extracts with the exception of the 2-month-old lens. Prior to the age of 37, it was below the detection limit. After the age of 37, the amount of γS-crystallin peptide increased steadily, reaching ~0.7 μg/mg tissue by the age of 75. On a molar basis, this corresponds to ~25% of the total γS-crystallin present in the lens.

The WSP and USP fractions were examined for the C-terminal γS-crystallin peptide. The urea-soluble fraction contained negligible amounts in all ages tested, whereas the amount in the water-soluble fraction varied with age.

Vesicle Experiments—To ascertain whether binding of the C-terminal γS-crystallin peptide may affect the properties of lens membranes, phospholipid vesicles were prepared, and the fluorescent dye Laurdan was incorporated into them. This reporter dye is sensitive to changes in the lipid microenvironment, and its fluorescence properties alter in response to water permeability in the lipid headgroup area (42). The shift in Laurdan emission can be quantified by general polarization and is defined as (440 nm − 490 nm)/(440 nm + 490 nm) in the range −1 to +1 (43). Vesicles treated with Laurdan were incubated with varying amounts of two peptides: SPAVQSFRRIVE and a related peptide in which the two arginine residues were replaced by threonine (SPAVQSFTTIVE). The γS-crystallin peptide had a pronounced affect on the vesicles (Fig. 4) with a marked decrease in the general polarization value. By comparison, the threonine-substituted peptide did not appear to alter the properties of the vesicles.

To further examine the mechanism by which the γS-crystallin peptide was interacting with lipid vesicles, SPAVQSFRRIVE was studied by circular dichroism (CD). In aqueous solution, SPAVQSFRRIVE appeared to exist as a mixture of random coil and β-sheet conformations (Fig. 4*b*). When the concentration of trifluoroethanol was increased to mimic the hydrophobic
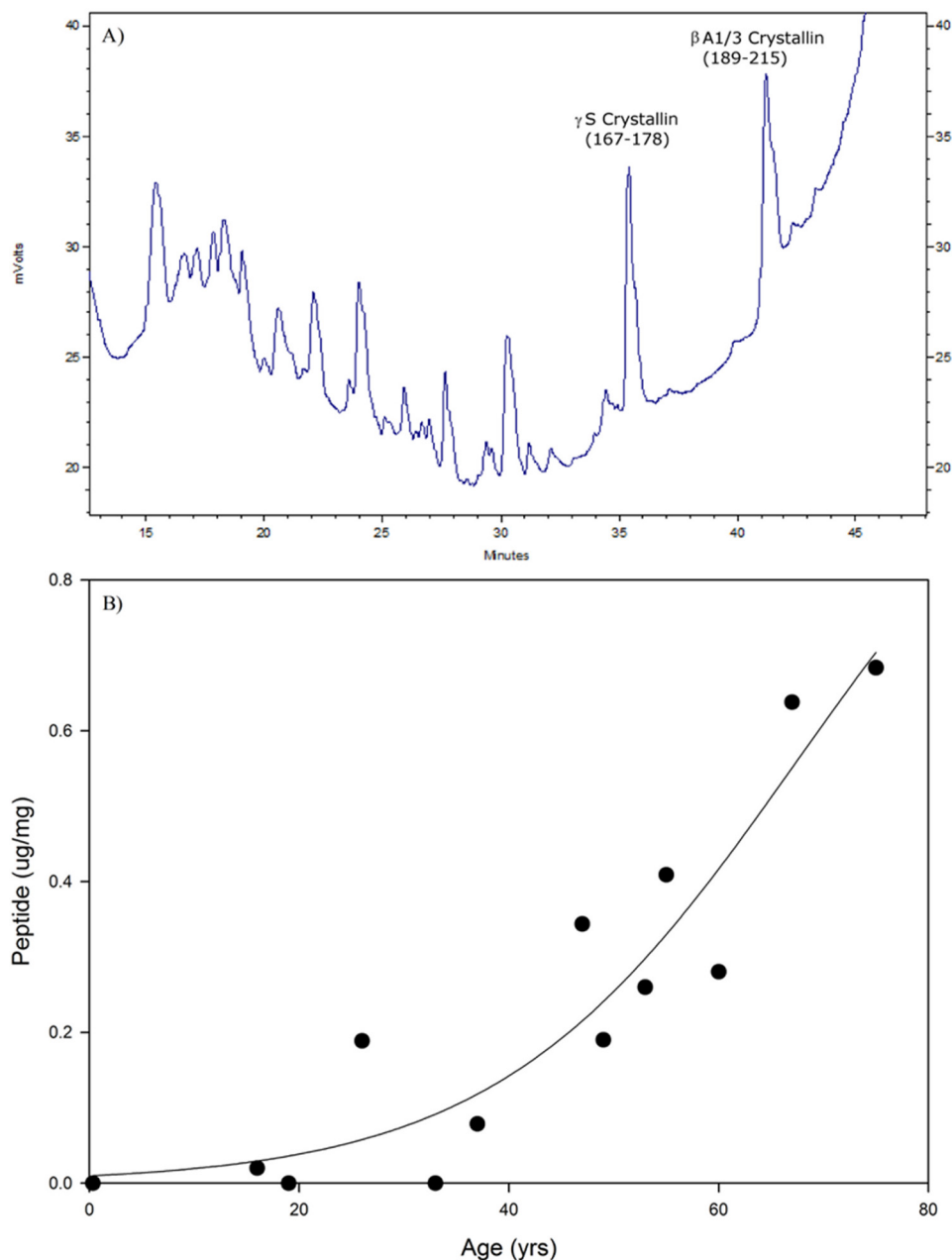
FIGURE 3. **Quantification of the γS-crystallin-derived peptide (SPAVQSFRRIVE) bound to lens membranes as function of age.** *a*, a typical HPLC profile of an older membrane extract with the γS-crystallin and βA3 peptide peaks indicated; *b*, the amount of SPAVQSFRRIVE bound to cell membranes from human lenses as determined by HPLC. Early eluting peaks appear to be mostly membrane lipid constituents. The amount of peptide was calculated as μg/mg of wet weight lens tissue.

environment of a membrane, there was a marked shift in the CD spectra. Deconvolution of the CD data using K2D3 software (44) indicated that in 80% trifluoroethanol, the α-helical content had increased to 66%, with little or no change in the amount of peptide in the β-sheet structure. This suggests that in a more hydrophobic environment, such as a phospholipid membrane, the γS-crystallin peptide adopts a more structured, α-helical, conformation.

Peptide Modeling—Because the γS-crystallin peptide appeared to interact strongly with lens cell membranes and in a vesicle system to alter membrane permeability, we modeled the structure of SPAVQSFRRIVE using a *de novo* peptide structure prediction program PEP-FOLD (45, 46). The three-dimen-

sional structure of the peptide and intact γS-crystallin were visualized in PyMOL (Fig. 5). The lowest energy conformation predicted for SPAVQSFRRIVE was an α-helical structure, and this is illustrated in Fig. 6 together with a water molecule located within the helix. The α-helical structure for SPAVQS-FRRIVE is in agreement with the CD data.

The structure of the released peptide is very different from that in the intact γS-crystallin (Fig. 5). It was hypothesized that binding of the peptide to the membrane was most likely mediated via interaction of the paired arginine residues with the negatively charged phosphate headgroup of the phospholipid, and one such arrangement is depicted in Fig. 6. It should be noted that another "reversed" mode is possible, which main-
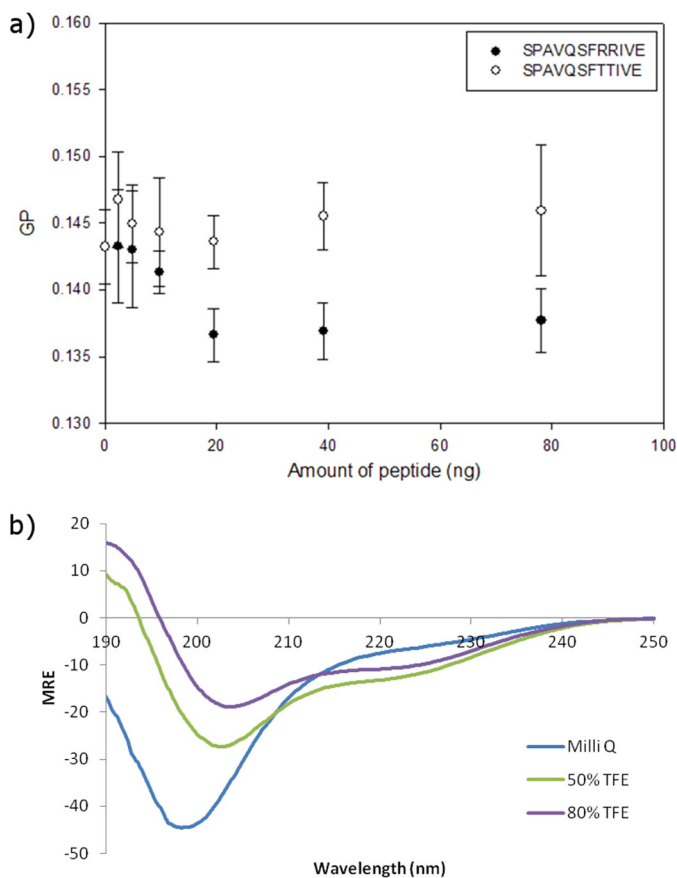
a)

b)

FIGURE 4. *a*, the interaction of peptides with lipid vesicles. SPAVQSFRRIVE (*closed circles*) and a sequence-related peptide lacking RR (SPAVQSFTTIVE) (*open circles*) were mixed with phosphatidylcholine vesicles containing Laurdan. (Each *point* represents mean ± S.D. (*error bars*), $n = 6$.) General polarization (*GP*) values reflect the microenvironment of the phospholipid headgroups. *b*, far-UV CD spectra of SPAVQSFRRIVE in water and different concentrations of trifluoroethanol.
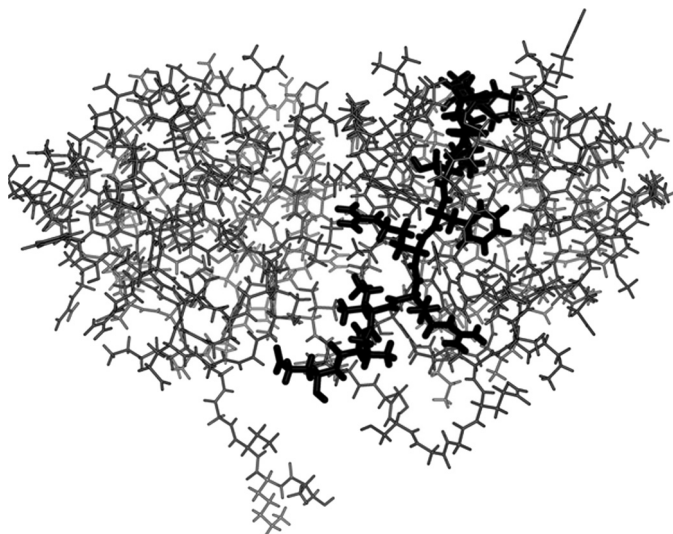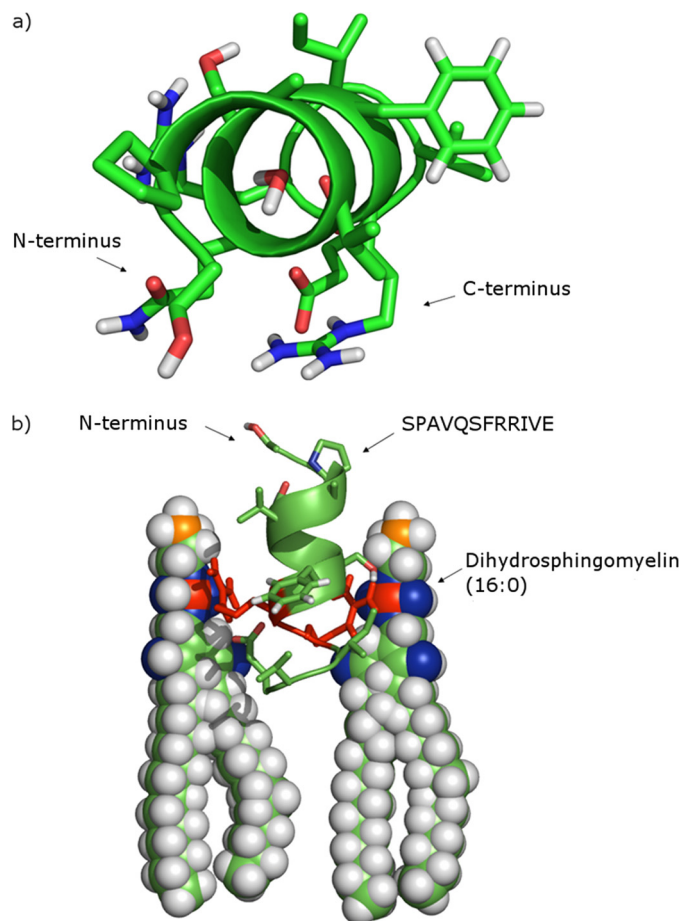
a)

b)

FIGURE 6. *a*, proposed structure of the C-terminal γS-crystallin-derived peptide (SPAVQSFRRIVE) following its release from the intact protein. The predicted structure, as determined by PEPFOLD, reveals an α-helix with a pore diameter of 4.3 Å. A water molecule is highlighted in the center of the pore. *b*, one potential mode of interaction of the γS-crystallin peptide, SPAVQS-FRRIVE, with phospholipid molecules (dihydrosphingomyelin (16:0)) present in human lens membranes. The arginine residues are shown aligned with the phosphate headgroup.

FIGURE 5. **A three-dimensional representation of γS-crystallin based on crystal structure (accession number P22914).** The *black residues* in *boldface type* correspond to the C-terminal peptide SPAVQSFRRIVE.

tains the arginine and phosphate headgroup alignment; however, the peptide is flipped with more of the α-helical portion of the peptide inserted into the membrane.

DISCUSSION

Long-lived and lifelong proteins are found at several sites in the human body (1–6); however, little is known about the processes that are chiefly responsible for their deterioration over time or the consequences of such deterioration on the function of cells, tissues, or indeed the aged individuals themselves. The human lens contains numerous proteins that do not turn over (9), and it can therefore act as a useful model to study the time course of such degradative processes. In this paper, we investigated the impact of age on one major lens protein: γS-crystallin.

Extensive age-dependent modification of γS-crystallin was observed in the human lens. As an initial analysis, three different antibodies to γS-crystallin were used to investigate modification of the protein. Up until the twenties, a significant process is insolubilization because full-length protein, which is originally confined to the water-soluble fraction in young lenses, begins to appear in the urea-soluble fraction (Fig. 1*a*). This may represent a generalized response of proteins to denaturation because there is a pronounced increase in the amount of insoluble protein in the lens as a function of age (34, 47). This process

may not be confined to the lens because other tissues also accumulate insoluble protein with age (48–50).

Another major PTM involved cross-linking of γS-crystallin. Previous investigators have reported dimeric forms of γS-crystallin and γS-crystallin cross-linked to other proteins (*e.g.* α- and β-crystallins) (18, 51). In the experiments performed here, dimers were observed using antibodies to both the N- and C-terminal regions of γS-crystallin. Dimers became noticeable in human lenses older than 2 months. Trimers were also found in older lenses, together with a ∼26 kDa band corresponding to the full-length 21-kDa protein plus an ∼5-kDa fragment. The reason for such cross-linking is not known, but it is very likely to involve non-disulfide bonds because the SDS gels were run in the presence of a reducing agent. It is possible that the cross-linking observed by Western blotting involves γS-crystallin bonded to other crystallin polypeptides (18, 51).

Truncation of γS-crystallin was another prominent process, and by middle age, little full-length protein could be detected with the full antibody (Fig. 1, *a* and *d*). Several sites of cleavage were indicated by Western blotting. One major site appears to involve the linker peptide between the two domains yielding 10–12-kDa fragments (Fig. 1, *b* and *c*). Such fragmentation of γS-crystallin has been observed previously, and some sites of peptide bond scission have been characterized (16, 38).

Other sites of cleavage were found to occur toward the end of the C-terminal domain of γS-crystallin. These were adjacent to serine residues, yielding two peptides, SPAVQSFRRIVE and sometimes SFRRIVE. Such peptide bond cleavage on the N-terminal side of Ser is an age-dependent phenomenon observed in a number of crystallins. One reason for this may involve an intein-like mechanism (52). This scission is a spontaneous reaction and does not require the involvement of proteases because it can be reproduced with model peptides (29). The effect of this cleavage on γS-crystallin structure and function is not known; however, truncations at the C and N terminus of αB-crystallin lead to significant changes in its structure (53).

Remarkably, peptides, such as SPAVQSFRRIVE, were found to bind tightly to the membranes of lens fiber cells, such that even extraction with 8 M urea was unable to remove them. The peptides could only be removed from the cell membranes by extraction with ethanol or 0.1 M NaOH. Quantification by HPLC revealed that significant concentrations of the peptides were present in the membranes and also that the amounts increased with age (Fig. 3*b*). Calculations based on the amount of γS-crystallin originally present in the lens (54) revealed that the amount of the peptide found associated with the cell membranes corresponded to ∼25% of the total protein in lenses aged above 70. This represents a substantial modification of total γS-crystallin present in the lens and could be expected to have a significant effect on the structure of the truncated γS-crystallin (Fig. 5) and possible interactions of this presumably denatured form with the molecular chaperone α-crystallin (55). Although this aspect was not investigated in detail in this study, other peptides were also found to be bound to lens membranes. These include βA3-crystallin 188–215 and αB-crystallin 1–18 and 2–18. All of these peptides also contain an RR sequence that is known to bind tightly to the phosphate headgroup of various membrane phospholipids (56, 57). The binding of these peptides to the membrane may act as a "seeding point" and facilitate crystallin association with the peptides on the membrane surface. Large scale binding of crystallins to lens membranes is implicated in the formation of the diffusional barrier in the aged human lens (58).

It may not be surprising if the levels of peptides present in the membranes of older lenses alter the properties of such membranes. Recently, it was found that the headgroup environment of cell membranes, which may reflect permeability to water, alters substantially in the center of human lenses in an age-dependent manner (35), and it was postulated that interaction of crystallins with phospholipids could be implicated in this alteration. The results of the current study provide an additional related mechanism for the apparent increase in membrane permeability. Modeling and CD studies showed that the C-terminal peptide can adopt an α-helical structure with a pore that is large enough to accommodate a water molecule (Fig. 6*a*).

A significant concentration of peptides embedded in the nuclear membranes of older human lenses may have other consequences. *In vitro* studies using peptides containing RR have demonstrated that binding to phospholipids can induce membrane aggregation, "puckering" of phospholipid vesicles, and membrane fusion. This alternative mechanism may account for changes in apparent membrane permeability with age (59). It is of interest that phenomena such as fusion and fragmentation have been observed in aged lenses (60). Membrane fusion is thought to result in a pathway that allows the movement of macromolecules from one lens cell to adjacent cells (60); however, it is likely that other processes also contribute to such age-dependent membrane alterations.

This work serves to illustrate that age-related cleavage of proteins in the body may have results that are not limited to a loss of function of the original protein. A similar phenomenon in the lens has been noted with α-crystallin, where the peptide fragments may promote protein aggregation (32). Peptides that result from degradation of long lived proteins over time may have biological activity that, in turn, can affect the properties of tissues.

CONCLUSIONS

Denaturation of long-lived proteins may arise from several sources (*e.g.* binding of reactive molecules, deamidation, racemization, and truncation). Most research to date has focused on the modified protein. The current study demonstrates that the consequences of age-dependent modification of proteins can be more wide ranging because peptides that result from spontaneous cleavage of old proteins may themselves have biological activity. In the lenses of adults, a peptide containing an RR sequence that was released by an incompletely characterized cleavage mechanism at a serine residue (61) in the C-terminal domain of γS-crystallin was found to adopt a different, helical conformation and to bind tightly to cell membranes. This interaction may alter the properties of cell membranes in older human lenses.

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