Mass Measurements of C-terminally Truncated $\alpha$-Crystallins from Two-dimensional Gels Identify Lp82 as a Major Endopeptidase in Rat Lens*

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Molecular chaperone activity of lens $\alpha$-crystallins is reduced by loss of the C terminus. The purpose of this experiment was to 1) determine the cleavage sites produced in vitro by ubiquitous m-calpain and lens-specific Lp82 on $\alpha$-crystallins, 2) identify $\alpha$-crystallin cleavage sites produced in vivo during maturation and cataract formation in rat lens, and 3) estimate the relative activities of Lp82 and m-calpain by appearance of protease-specific cleavage products in vivo. Total soluble protein from young rat lens was incubated with recombinant m-calpain or Lp82 and 2 mM Ca$^{2+}$. Resulting fragmented $\alpha$-crystallins were separated by two-dimensional gel electrophoresis. Eluted $\alpha$-crystallin spots were analyzed by mass spectrometry. Cleavage sites on insoluble $\alpha$-crystallins were determined similarly in mature rat lens nucleus and in cataractous rat lens nucleus induced by selenite. In vitro proteolysis of $\alpha$A-crystallin by Lp82 and m-calpain produced unique cleavage sites by removing 5 and 11 residues, respectively, from the C terminus. In vivo, the protease-specific truncations removing 5 and 11 residues from $\alpha$A were both found in maturing lens, whereas only the truncation removing 5 residues was found in cataractous lens. Other truncation sites, common to both calpain isoforms, resulted from the removal of 8, 10, 16, 17, and 22 residues from the C terminus of $\alpha$A. Using uniquely truncated $\alpha$-A-crystallins as in vivo markers, Lp82 and m-calpain were both found to be active during normal maturation of rat lens, whereas Lp82 seemed especially active during selenite cataract formation. These C-terminal truncations decrease chaperone activity of $\alpha$-crystallins, possibly leading to the observed increases in insoluble proteins during aging and cataract. The methodology that allowed accurate mass measurements of proteins eluted from 2D gels should be useful to examine rapidly other post-translational modifications. Molecular & Cellular Proteomics 1:357–365, 2002.

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$\alpha$-Crystallins in lens are related to the small heat shock family of proteins (1). Because lens proteins have very little turnover, $\alpha$-crystallin functions as a molecular chaperone to prevent aggregation of other lens proteins (2). The C terminus is essential, because loss of 16 amino acid residues of C terminus from $\alpha$A-crystallin caused a loss of 50% of its chaperone activity (3).

Truncation of lens crystallins is a common feature of both aging and cataract formation in rodents. A number of experiments revealed that calcium-activated proteases (calpains) were involved in formation of cataracts induced by selenite (4), galactose (5), diamide (6), and the hypocholesterolemic drug U18666A (7) and in hereditary rat Shumiya cataract (8). One of the ubiquitous calpains, m-calpain, has been credited with the proteolysis of $\alpha$- and $\beta$-crystallins during cataractogenesis in rodents. Incubation of $\alpha$A-crystallin with m-calpain reduced chaperone activity and produced truncated forms of $\alpha$A that migrated during two-dimensional electrophoresis (2-DE) to positions similar to truncated forms of $\alpha$A observed in cataractous lenses (9). A recently discovered lens-specific calpain, a splice variant of calpain 3 termed Lp82, may also be activated during formation of rodent cataracts (10).

The primary truncation sites on $\beta$-crystallins in rodent lenses in vivo are at the N terminus where the cleavage sites can be determined by Edman sequencing (11, 12). In contrast, determinations of the exact $\alpha$-crystallin truncation sites have not been performed because of the lack of a convenient C-terminal sequencing method. To solve this problem, mass spectral analysis of peptides from in-gel digests (13) and accurate mass measurement of whole proteins eluted from gels (14) have become powerful tools to determine C-terminal truncation sites. Therefore, the purposes of the present report were to use mass spectrometry to 1) determine in vitro cleavage sites produced by m-calpain and Lp82, 2) compare the C-terminal cleavage sites to those produced in vivo during aging and cataract formation in rats, and 3) use this information to determine whether the activity of one rat lens calpain isoform is dominant in vivo.

*The abbreviation used is: 2-DE, two-dimensional electrophoresis.
**α-Crystallin Truncation**

**EXPERIMENTAL PROCEDURES**

*Preparation of Rat Lens Proteins—* Lenses for protein isolation were obtained from normal 12-16-day-old or 6-week-old Sprague-Dawley rats (BK International, Fremont, CA) or cataract lenses induced by injection of 30 μg of sodium selenite/kg body weight in 11-day-old rats (15). Treatment of animals conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The lenses were dissected into cortex and nucleus as described previously (11), and tissue was homogenized in 20 mM sodium phosphate (pH 7.0), 1.0 mM EGTA containing protease inhibitor (Complete mini protease inhibitor mixture; Roche Molecular Biochemicals) dissolved at 10 m of buffer/tablet. Lens proteins isolated for incubation with recombinant m-calpain and Lp82 were pooled from 10 rat pups selected randomly from three litters of 12-day-old animals. Insoluble proteins from the lens nucleus of 16-day-old rats were isolated from 12 non-injected control animals and 10 animals receiving selenite injections at 11 days of age. Insoluble proteins from the lens nucleus of normal 6-week-old rats were isolated from five animals. Protein content was then assayed using the BCA method (Pierce). Lens proteins were stored frozen at -70 °C until use.

*Expression and Purification of Recombinant Lp82—* The Lp82 cDNA from rat was cloned into a pFASTBAC HTb vector (Invitrogen) with a His tag on the N terminus. This plasmid was used to transform DH10Bac competent cells containing a bacmid and helper plasmid (Invitrogen). Recombinant bacmid DNA containing Lp82 was isolated and used to transfect insect cells. Transfection of Spodoptera frugiperda insect cells (Sf9 cells; Invitrogen) was performed with amplified recombinant Lp82 baculovirus (10⁸ plaque forming units/ml). After infection at 37 °C, followed by incubation at 37 °C for 3 h. A control incubation containing soluble proteins eluted directly from spots excised from duplicate 2-DE gels were deconvoluted using Xcalibur software with BioWorks (ThermoFinnigan). Mass accuracy of better than 0.01% was confirmed using horse myoglobin. Experiments using carboxic anhydrate eluted from SDS-PAGE gels estimated that a minimum of 3.5 pmol of protein was required to obtain an accurate mass using this procedure. Use of a smaller 0.5 × 150-mm column with identical packing material and a 10 μl/min flow rate was unsuccessful in increasing sensitivity because of overloading with SDS and elution of SDS-protein complexes.

*Assignment of Protein Cleavage Sites and Calculation of Isoelectric Points—* After mass determination, the truncation sites in proteins were determined using PAWS software (prowl.rockefeller.edu/software/contents.htm) to match measured masses with calculated masses of truncated species. For further confirmation of truncation sites, the isoelectric points of identified truncated species were calculated using GeneWorks 2.5 software (Accelrys, San Diego, CA). Mass spectra of proteins eluted from the C4 column were deconvoluted using Xcalibur software with BioWorks (ThermoFinnigan). Mass accuracy of better than 0.01% was confirmed using horse myoglobin. Experiments using carboxic anhydrate eluted from SDS-PAGE gels estimated that a minimum of 3.5 pmol of protein was required to obtain an accurate mass using this procedure. Use of a smaller 0.5 × 150-mm column with identical packing material and a 10 μl/min flow rate was unsuccessful in increasing sensitivity because of overloading with SDS and elution of SDS-protein complexes.

*Assay of m-Calpain and Lp82 Activity—* Activity of recombinant m-calpain and Lp82 was determined by a fluorescence assay (EnzChek protease assay kit E-6638; Molecular Probes, Eugene, OR) as described previously (17). The calcium activation requirements of the two enzymes were determined by varying the final concentration of free calcium in the incubation mixture from 0 to 1000 μM.
RESULTS

In Vitro Hydrolysis of α-Crystallins by m-Calpain and Lp82—To compare the in vitro breakdown patterns of α-crystallins, total soluble lens proteins containing intact α-crystallins were incubated with activated m-calpain and Lp82. m-Calpain and Lp82 readily hydrolyzed α-crystallins to 8 (Fig. 1b) and 10 (Fig. 1c) major fragments, respectively. The general breakdown profile of α-crystallins appeared similar on 2-DE, except Lp82 caused the additional appearance of spots 8 and 9, as well as a triplet of spots marked with an asterisk (Fig. 1c).

To examine the specific cleavage sites producing the truncated α-crystallins, the species described above were isolated from 2-DE gels and analyzed by mass spectrometry. A summary of the measured masses is given in Table I, along with the indicated missing residues. All Lp82 and m-calpain cleavage sites in αA and αB were at the C termini. Although they have very similar positions on 2-DE gels, spot 1 produced by m-calpain (Fig. 1b) and spot 2 produced by Lp82 (Fig. 1c) were distinct. The m-calpain-specific spot 1 (mass = 18,735 Da) was produced by removal of 11 residues from the C terminus of αA (αA$_{1-162}$), whereas the Lp82-specific spot 2 (mass = 19,461 Da) was produced by removal of five residues (αA$_{1-168}$). The deconvoluted mass spectra for these truncated αA-crystallins are shown in Fig. 2.

Two other potential unique Lp82 markers were observed (Fig. 1c, spots 8 and 9). However, the αA fragment in spot 8 was nearly identical in mass to the αA fragment in spot 2. The masses of both these species matched αA$_{1-168}$ (Table I). Whereas the pI of spot 2 matched the calculated pI for αA$_{1-168}$, the pI of spot 8 was higher (more basic) than expected. A modification causing an increase in pI, but no significant change in mass, is unknown. Spot 9 was also analyzed, but its concentration was too low to determine its mass.

The remaining partially truncated α-crystallins produced by m-calpain and Lp82 were identical. αA-crystallin missing 8, 10, 16, 17, and 22 residues from its C terminus and αB-crystallin missing 5 and 12 residues from its C terminus were produced by both enzymes (Fig. 1, b and c, spots 3–7, 10, 11). However, m-calpain and Lp82 differed in their preferred cleavage sites. The major truncation product of m-calpain was αA$_{1-163}$, followed by αA$_{1-162}$ and αA$_{1-157}$. In contrast, αA$_{1-168}$ was the dominant truncation product produced by Lp82, and αA$_{1-163}$ was a relatively minor species.

In general, the measured isoelectric points of the partially degraded α-crystallins matched their calculated isoelectric points. The two exceptions were the fragment identified in spot 8 discussed above and the fragment in spot 4 matching the mass of αA$_{1-165}$. This fragment was expected to migrate to a pI nearly identical to intact αA but migrated to a more acidic position. The cause of the pI shifts of these truncated species is unknown. However, minor species of αA were found in the undigested control sample (Fig. 1a) with shifts in...
\(\alpha\)-Crystallin Truncation

**TABLE I**

**Masses and identities of truncated \(\alpha\)-crystallins produced by incubation with Lp82 or m-calpain**

Truncated \(\alpha\)-crystallins produced by incubation with Lp82 or m-calpain and numbered in Fig. 1, b and c were eluted from duplicate 2-DE gels, and their masses were determined by electrospray ionization mass spectrometry. \(\alpha\) A fragments in bold were unique in that they were Lp82- or m-calpain-specific.

| Spot No. | \(\alpha\)-Crystallin* | Residues missing from the C terminus | Measured mass (m-calpain-incubated) | Measured mass (Lp82-incubated) | Calculated massb | Calculated pl |
|----------|------------------------|-------------------------------------|------------------------------------|--------------------------------|-----------------|--------------|
| 1        | \(\alpha\) A1-162      | 11                                  | 18,735                             | ND                              | 18,735          | 5.50         |
| 2        | \(\alpha\) A1-168      | 5                                   | ND                                 | 19,461                          | 19,462          | 5.52         |
| 3        | \(\alpha\) A1-163      | 10                                  | 18,891                             | 18,890                          | 18,892          | 5.72         |
| 4        | \(\alpha\) A1-165 (acidic)c | 8                                  | 19,149                             | 19,147                          | 19,149          | 5.54         |
| 5        | \(\alpha\) A1-157      | 16                                  | 18,267                             | 18,267                          | 18,267          | 5.50         |
| 6        | \(\alpha\) A1-156      | 17                                  | 18,111                             | 18,112                          | 18,112          | 5.27         |
| 7        | \(\alpha\) A1-151      | 22                                  | NA                                 | 17,629                          | 17,630          | 5.29         |
| 8        | \(\alpha\) A1-168 (basic)c | 5                                  | ND                                 | 19,460                          | 19,462          | 5.52         |
| 9        | Unknown                | Unknown                             | ND                                 | NA                              |                 |              |
| 10       | \(\alpha\) B1-170      | 5                                   | 19,635                             | 19,635                          | 19,635          | 6.14         |
| 11       | \(\alpha\) B1-163      | 12                                  | 18,880                             | 18,880                          | 18,880          | 6.33         |

* The subscript numbers give the amino acid residues remaining following incubation.

b Calculated masses of C-terminally truncated \(\alpha\)-crystallins. Intact \(\alpha\) A (SwissProt number P02490) contains 173 residues with an average mass of 19,891 because of \(N\)-acetylation (+42) and carbamidomethylation of its single cysteine residue (+57), and intact \(\alpha\) B (SwissProt number P23928) contains 175 residues with an average mass of 20,131 because of \(N\)-acetylation (+42).

c ND, not detected.

d These species migrated to pl values either more acidic or basic than their predicted pl values (last column).

e NA, not analyzed or insufficient protein recovery for mass determination.

pl to both basic and acidic sides of the major \(\alpha\)A species. This suggested that the truncated \(\alpha\)A species in spots 4 and 8 were derived from the pl-shifted \(\alpha\)A species that existed before incubation. It was also interesting to note that the relative molecular weights estimated for \(\alpha\)A1-163 and \(\alpha\)A1-162 by SDS-PAGE did not agree with their actual molecular weights determined by mass spectrometry. \(\alpha\)A1-163 migrated faster than \(\alpha\)A1-162 during SDS-PAGE. This result illustrated the inherent inaccuracy of molecular weight estimation by SDS-PAGE.

**Fragmentation of \(\alpha\)-Crystallin in Vivo—Analysis of water-insoluble lens proteins from the nucleus of normal 16-day-old rats by 2-DE indicated that \(\alpha\)-crystallins became fragmented at an early age (Fig. 3a). This fragmentation was largely absent from the water-soluble fraction (data not shown). Cataract produced by an overdose of selenite in age-matched lenses increased dramatically the concentration of fragmented \(\alpha\)-crystallins. Intact \(\alpha\)A was almost absent in the insoluble fraction of the nucleus following cataract formation (Fig. 3b, circle), the density of most \(\alpha\)-fragments increased (Fig. 3b, spots 1–17), and the % of total protein that was insoluble increased 2.5-fold (Fig. 3d). Aging from 16 days to 6 weeks in normal lenses also increased the abundance of \(\alpha\)-crystallin fragments (compare Fig. 3, a and c). However, the fragmentation occurring with age was not as extensive as in cataract.**

Intact \(\alpha\)A was still observed in the insoluble fraction of the nucleus of 6-week-old rats, and the amount of insoluble protein only increased by 35% from 16 days to 6 weeks of age (Fig. 3d). The correlation between fragmentation of \(\beta\)-crystallins and formation of insoluble protein has been documented in both cataractous lenses from young rats and mature normal rat lens (11). The present results indicated that breakdown of \(\alpha\)A-crystallin was also associated with accelerated crystallin insolubilization.

The positions of \(\alpha\)-crystallin fragments on the 2-DE gels of insoluble protein from the nucleus of 16-day control, 16-day cataractous, and 6-week-old normal lenses were very similar (Fig. 3). Their positions were also very similar to the \(\alpha\)-crystallin fragments produced by Lp82 and m-calpain incubation (Fig. 1). All of the \(\alpha\)-crystallin fragments produced in vitro during incubation with Lp82 and m-calpain were also found in vivo. However, the abundance of specific fragments in cataractous and aged lenses suggested that Lp82 activity was more prevalent during maturation and cataract formation in young rats and that proteolysis by m-calpain did not become significant in normal lenses until after 6 weeks of age. The extent of proteolysis in vivo was also greater. Many of the minor products produced by Lp82 incubation in vitro, which were too low in concentration to be analyzed by mass spectrometry, were present in sufficient quantities in vivo for analysis. Several additional species not produced by either enzyme in vitro were also observed. The masses and identities of the various spots marked in Fig. 3 are summarized in Table II.

The Lp82-specific product \(\alpha\)A1-168 was one of the major forms of insoluble \(\alpha\)A-crystallin in both normal and cataractous lenses from 16-day-old animals (Fig. 3, a and b, spot 2). Fig. 4a shows the deconvoluted mass spectrum of the protein isolated from spot 2 of the 2-DE gel shown in Fig. 3b that was identified as \(\alpha\)A1-168. The mass spectrum of the protein from control lenses isolated from spot 2 of Fig. 3a was nearly identical (data not shown). Neither of these spots contained a
protein matching the mass of \( \beta_9251^{1-162} \), the unique m-calpain-produced fragment. In contrast, spot 1, observed in the insoluble protein isolated from normal 6-week-old lens nucleus (Fig. 3c), contained a major species with a mass of 19,735 (Fig. 4b). This matched the mass of \( \beta_9251^{1-162} \), the unique m-calpain-produced fragment. A second species corresponding to \( \beta_9251^{1-168} \) (mass = 19,462) was also observed, because of the incomplete resolution of spots 1 and 2. These data indicated that Lp82 was most active in both normal and cataractous lenses of 16-day-old rats and that products produced by m-calpain were only present in appreciable quantities in 6-week-old lenses.

Additional spots numbered 9, 14, 15, 16, and 17 in Fig. 3, corresponding in position to unique species produced by Lp82 in vitro, were also analyzed. These were identified as forms of \( \alpha_A \) missing 54, 26, 43, 47, and 55 residues from its C terminus, respectively (Table II). Other truncated forms of \( \alpha \)-crystallins common to both Lp82 and m-calpain proteolysis were also found (spots 3, 4, 5, 6, 7, 10, 11). \( \beta \)-crystallin truncation was not as extensive in both 16-day-old normal and cataractous lenses as was \( \alpha \)-crystallin fragmentation. However, \( \beta_B^{1-170} \) and \( \beta_B^{1-163} \) (spots 10 and 11) and their phosphorylated forms (spots 13 and 12) accumulated in older rat lens (Fig. 3c). \( \beta_B^{1-170} \) and \( \beta_B^{1-163} \) were also found in human lens fiber cells growing in lens capsules following cataract surgery (21). This suggested that calpains are also active in human lens.

The mass of spot 18 did not match the mass of any possible \( \alpha \)-crystallin fragment. Therefore, this protein was identified by MS/MS analysis of its peptide fragments as the recently characterized protein GRIFIN (galectin-related inter-fiber protein) (22).

Calcium Requirement for m-Calpain and Lp82—Calcium requirements of recombinant Lp82 and m-calpain were tested. Activity of m-calpain and Lp82 reached \( \frac{1}{2} \) of maximum at \( \approx 120 \) and \( 20 \, \mu M \) calcium, respectively (Fig. 5). Maximum activity of Lp82 was reached at 50 \( \mu M \) calcium, whereas m-calpain required 500 \( \mu M \) calcium for maximum activity.
**α-Crystallin Truncation**

TABLE II

| Spot No. | Identified protein | Residues removed from C terminus | Measured mass (16-day-old normal) | Measured mass (16-day-old cataract) | Measured mass (6-week-old normal) | Calculated mass | Calculated pl | Abundance (16-day-old normal) | Abundance (16-day-old cataract) | Abundance (6-week-old normal) |
|----------|-------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------|--------------|------------------------------|-------------------------------|-------------------------------|
| aAaA1−173| intact            | 19,891                           | NA                               | 19,891                           | 19,891                           | 5.52           | ++           | +                           | +                             | +                             |
| 1        | aA1−162           | 11                               | ND                               | 18,735                           | 18,735                           | 5.50           | +            | +                           | +                             | +                             |
| 2        | aA1−168           | 5                                | 19,461                           | 19,461                           | 19,462                           | 5.52           | ++           | ++                          | ++                            | ++                            |
| 3        | aA1−163           | 10                               | 18,890                           | 18,890                           | 18,892                           | 5.72           | +++          | +++                         | +++                           | +++                           |
| 4        | aA1−165 (acidic)  | 8                                | 19,149                           | 19,149                           | 19,150                           | 5.54           | ++           | +                           | +                             | +                             |
| 5        | aA1−157           | 16                               | 18,267                           | 18,267                           | 18,268                           | 5.50           | +            | +                           | +                             | +                             |
| 6        | aA1−156           | 17                               | 18,111                           | 18,111                           | 18,112                           | 5.27           | ++           | ++                          | ++                            | ++                            |
| 7        | aA1−151           | 22                               | 17,629                           | 17,630                           | 17,631                           | 5.29           | ++           | +++                         | +++                           | +++                           |
| 8        | NA                | –                                | –                                | –                                | –                                | –              | –            | –                           | –                             | –                             |
| 9        | aA1−119           | 54                               | NA                               | 14,365                           | 14,365                           | 5.79           | +            | +                           | +                             | +                             |
| 10       | aB1−170           | 5                                | NA                               | 19,634                           | 19,635                           | 6.14           | +            | +                           | +                             | +                             |
| 11       | aB1−163           | 12                               | NA                               | 18,881                           | 18,880                           | 6.33           | +            | +                           | +                             | +                             |
| 12       | aB1−163 (phosphorylated)  | 12                             | NA                               | 18,961                           | 18,960                           | –              | +            | +                           | +                             | +                             |
| 13       | aB1−170 (phosphorylated)  | 5                               | ND                               | 19,715                           | 19,715                           | –              | +            | +                           | +                             | +                             |
| 14       | aA1−147           | 26                               | 17,257                           | 17,257                           | 17,257                           | 5.54           | +            | +                           | +                             | +                             |
| 15       | aA1−130           | 43                               | 15,477                           | 15,477                           | 15,477                           | 5.54           | +            | +                           | +                             | +                             |
| 16       | aA1−126           | 47                               | NA                               | 15,119                           | 15,119                           | 5.54           | +            | +                           | +                             | +                             |
| 17       | aA1−118 (basic)   | 55                               | NA                               | 14,209                           | ND                               | 14,209         | 5.54         | +                           | +                             | +                             |
| 18       | GRIFIN            | 15,833                           | NA                               | 15,832                           | 15,896                           | 5.28           | +            | +                           | +                             | +                             |

**DISCUSSION**

Major findings of this study were as follows: 1) extensive C-terminal truncation of α-crystallins occurred in rat lenses during maturation and cataract formation, 2) these truncated α-crystallins were insolubilized selectively, and 3) the calcium-dependent proteases Lp82 and m-calpain were likely responsible, with the activity of Lp82 predominating over m-calpain in the nucleus of young rat lens. The last finding was based on the detection of unique, truncated α-A-crystallin products of both Lp82 and m-calpain in lens, with the predominant accumulation of the Lp82-specific α-A products.

Previous studies in our laboratory did not analyze fragmented α-crystallins in detail, because, unlike β-crystallins, which all undergo cleavage within their N-terminal extensions, fragmented α-crystallins in rats remain intact at their N terminus (9). This complicates the analysis of their cleavage sites by Edman sequencing. The experiments in the current study avoided these limitations by isolating whole proteins from 2-DE gels and accurately measuring their masses. The methodology, based on dispersion of gel pieces into 20-μm particles, allowed more rapid extraction and analysis of proteins than previous methods using passive elution (14, 23) and represents an improvement in the analysis of proteolytically modified proteins. The technique should also be useful to detect other post-translational modifications in 2-DE-separated proteins. Future studies will determine the utility of the method to isolate and measure masses of a wider variety of 2-DE-separated proteins.

m-Calpain has long been implicated as the major protease responsible for the processing of rodent crystallins, during both lens maturation and cataract formation (11, 24). However, because of its abundance and stability, the more recently discovered lens protease, Lp82, may also play an important role (25). Because there was no previous direct evidence that Lp82 was responsible for degradation of endogenous substrates in rat lens, the first goal in the present study was to identify biological markers that could estimate the relative activities of m-calpain and Lp82 in vivo. The α-A-fragment, αA1−168, missing five residues from its C terminus, was shown to be an Lp82-specific product, whereas the α-A-fragment, αA1−162, missing 11 residues from its C terminus, was shown to be an m-calpain-specific product. This finding agrees with the studies of Yoshida et al. (26), who showed that purified bovine lens m-calpain could remove 10 and 11 residues, but not five residues, from the C terminus of αA-crystallin. Earlier studies using purified Lp82 and m-cal-
pain from rat and bovine lens also confirmed these m-calpain cleavage sites and showed that Lp82 removed uniquely five residues from the C terminus of \(\alpha\)A (10, 17). In the present study, using 2-DE gel separation to isolate the various truncated forms of \(\alpha\)-crystallins, we were able to demonstrate for the first time that \(\alpha\)A1-168 appeared in lens before \(\alpha\)A1-162. This suggested that Lp82 is activated much earlier than m-calpain during lens maturation and that Lp82 may be responsible for the majority of excess crystallin proteolysis associated with experimental cataract formation. Evidence for m-calpain activity was only found in the lens nucleus of normal 6-week-old rats. m-Calpain may exhibit relatively greater activity in mature lenses, because, unlike Lp82, enzymatic activity and m-RNA for m-calpain are maintained in lens with age (27). m-Calpain may function in mature lens to provide very slow but sustained proteolytic activity, whereas Lp82 may function only during the period of rapid postnatal growth.

Three recent findings may explain the greater activity of Lp82 compared with m-calpain in young rat lens. Unlike m-calpain, Lp82 is relatively insensitive to calpastatin, the endogenous inhibitor of calpains (28). Lp82 also undergoes autolytic inactivation more slowly than does Lp82 (17). Finally, the calcium requirement for activation of Lp82 is much lower than the calcium requirement of m-calpain. The lower calcium requirement of Lp82 compared with m-calpain was demonstrated for both enzymes purified from bovine lens (17) and the recombinant forms of rat Lp82 and m-calpain used in the present study. Although the 108 \(\mu\)M free calcium concentration in the nucleus of young rats developing selenite-induced cataracts is high enough to activate Lp82, the mechanism for Lp82 activation in normal rat lens containing 1 \(\mu\)M free calcium is unknown (29). One possibility is that Lp82 is activated following association of the enzyme with lens membranes. This hypothesis is supported by the preferential association of Lp82 with the insoluble fraction of the lens (27).

The C-terminal truncation of \(\alpha\)-crystallins may have great biological significance in lens. Loss of C-terminal regions of \(\alpha\)-crystallins has been shown previously to diminish the chaperone-like properties of \(\alpha\)-crystallins (3, 9). The present study also demonstrated that truncated \(\alpha\)-crystallins were insolubilized selectively. Similar selective insolubilization of truncated \(\alpha\)-crystallins was also observed during analysis of mouse lens proteins by 2-DE (30). Carver and Lindner (31) have postulated that the flexible, solvent-exposed, hydrophilic C-terminal extensions of \(\alpha\)-crystallins may function to keep \(\alpha\)-crystallin-denatured protein complexes in solution. Selenite cataract may form because of insolubilization of truncated \(\alpha\)- and \(\beta\)-crystallin complexes that are unable to stay in solution because of a loss of the solubilizing C-terminal extensions of \(\alpha\)-crystallins. Similar complexes of truncated \(\alpha\)- and \(\beta\)-crystallins may not form opacities during normal aging of lens, because their slower accumulation could allow a more ordered arrangement within the lens cytosol.

The increased rate of proteolysis observed during formation of selenite-induced cataract in the present study is a common
response of rodent lenses to stress. For example, similar accelerated truncation of α-crystallins have been reported following cataract formation induced by galactose feeding (5), treatment with an inhibitor of cholesterol synthesis (7), and inherited mutations (8, 32). In contrast, α-crystallins in human lenses undergo less proteolysis. The majority of αA and αB examined from the water-insoluble fraction of normal aged human lenses remained intact (33). Additionally, unlike rodent lenses, the major αA-crystallin truncation product found in human and bovine lenses is only missing the C-terminal serine residue (33, 34). Whereas small quantities of αA1–168, missing five residues from its C terminus, have also been detected in human lens (35, 36), the protease causing this cleavage is unknown, because no Lp82 activity is present in human lens (37). Thus, until the truncation states of α-crystallins are examined more closely in cataractous human lenses, or additional calpain isoforms are discovered, caution should be used in extrapolating the current findings in rodents to cataracts in man.

During the course of this study, we identified unexpectedly the recently discovered lens-specific protein GRIFIN (galectin-related inter-fiber protein). Unlike other members of the galectin family, GRIFIN does not bind to β-galactoside, and its function is unknown (22). However, the current study found significant amounts of GRIFIN in the insoluble fraction of lens. This suggested that GRIFIN could be a peripheral membrane protein, with a function similar to another recently characterized member of the galectin family, galectin-3. Galectin-3 was postulated to act as a cell adhesion molecule in lens (38). The localization of GRIFIN by immunofluorescence to the interface between lens fiber cells supports this hypothesis (22).

In conclusion, analysis of α-crystallin truncation suggested that the recently discovered lens-specific member of the calpain family, Lp82, was more active in young rat lens than m-calpain and was responsible for the majority of crystallin fragmentation during maturation and cataract formation. The regulated activity of Lp82 by calcium may allow an ordered truncation and insolubilization of crystallins in rat lenses during normal maturation. Loss of calcium homeostasis in young rats may cause cataract by overactivation of Lp82.

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