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An Atypical Form of αB-crystallin Is Present in High Concentration in Some Human Cataractous Lenses

IDENTIFICATION AND CHARACTERIZATION OF ABERRANT N- AND C-TERMINAL PROCESSING*

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Two unique polypeptides, 22.4 and 16.4 kDa, were prominent in some human cataracts. Both proteins were identified as modified forms of the small heat shock protein, αB-crystallin. The concentration of total αB-crystallin in most of these cataracts was significantly increased. The 22.4-kDa protein was subsequently designated as αB_g. Mass spectrometric analyses of tryptic and Asp-N digests showed αB_g is αB-crystallin minus the C-terminal lysine. αB_g constituted 10–90% of the total αB-crystallin in these cataracts and was preferentially phosphorylated over the typical form of αB-crystallin. Human αB_g and αB-crystallin were cloned and expressed in *Escherichia coli*. The differences in electrophoretic mobility and the large difference in native pI values suggest some structural differences exist. The chaperone-like activity of recombinant human αB_g was comparable to that of recombinant human αB-crystallin in preventing the aggregation of lactalbumin induced by dithiothreitol. The mechanism involved in generating αB_g is not known, but a premature termination of the αB-crystallin gene was ruled out by sequencing the polymerase chain reaction products of the last exon for the αB-crystallin gene from lenses containing αB_g. The 16.4-kDa protein was an N-terminally truncated fragment of αB_g. The high concentration of αB-crystallin in these cataracts is the first observation of this kind in human lenses.

The three major classes of mammalian crystallins, α-, β-, and γ-crystallins, constitute about 90% of the total protein in the eye lens and are considered to determine the refractive properties of the lens. Post-translational modification of the crystallins has been a major focus of the research in trying to elucidate causes for the loss of lens transparency or cataract development (1–4). α-Crystallins have received the most attention in this case. The two homologous subunits, αA- and αB-crystallin, make up about 30% of the proteins in young human lenses and in the outer cortex of the adult human lens. αB-crystallin is a normal constituent of most mammalian tissues but is present in the highest concentrations in lens (5, 6). In addition to the lens, αA-crystallin is found in spleen and thymus (7). α-Crystallins are present in fiber cell extracts as large heteroaggregates with apparent molecular masses reported from 300 to 1000 kDa (8, 9). In cataracts the sizes of the aggregates reportedly increase and these aggregates are thought to be responsible for the light scattering (2).

On the other hand, α-crystallins are members of the family of small heat shock proteins and are thought to provide protection against cellular stresses (10–12). Members of this family are structurally related via the α-crystallin domain, they form large aggregates, are phospho-proteins, and have chaperone-like activity (12–19). α- Crystallins prevent the aggregation of proteins induced by heat, oxidation, or chemicals. In cultured cells, expression of αB-crystallin is induced by heat shock, oxidative stress, osmotic stress, arsenite, phorbol 12-myristate 13-acetate, and hormones such as estrogen and dexamethasone (20–23). Thus, the role of αB-crystallin in many systems is considered to be that of a stress protein. In the lens, however, it is not known whether the primary function of αB-crystallin is that of a stress protein and related to its chaperone-like function or if its major role is structural. It is possible its role in the lens may change depending on the stage of development and location in the lens.

Both α-crystallins undergo post-translational modifications including truncation of both N and C termini, deamidation, racemization, phosphorylation, methionine oxidation, glycation, disulfide formation, addition of *O*-GlcNAc, and the addition of 72 mass units to the C-terminal lysine of αB-crystallin (4, 17–19, 24, 25). Some of these such as phosphorylation and specific cleavage may be important functionally, others are likely the result of aging and detrimental stresses. Any of these modifications are likely to alter the protein conformation which in turn could alter the aggregate size and/or the function of α-crystallin in the cell.

Two-dimensional electrophoresis of tissue proteins is the major technique used to detect post-translationally modified proteins in cell and tissue extracts. We have used this technique extensively in an attempt to identify changes in the concentration or modification of lens proteins that could be unique to developmental, aging, and cataractogenic processes.

In this study we report the identification and characterization of two unique polypeptides observed on two-dimensional gel electrophoresis of human cataracts. One of the proteins was present in high concentration comparable to the concentration of a crystallin. Both are likely the result of stress-induced processes on the path to cataract formation.

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MATERIALS AND METHODS

Normal Lenses and Cataracts—Normal human lenses were obtained from the National Disease Research Interchange, Philadelphia, PA. Human cataractous material was obtained from intracapsular cataract surgery done in India and from extracapsular extractions done at the National Eye Institute. Tenets of the Declaration of Helsinki for dealing with human samples were strictly followed. The lens capsule epithelia were removed, and the lenses were separated into the lens cortical and nuclear regions as described previously (26).

Two-dimensional Gel Electrophoresis—A proteinase inhibitor mixture containing 4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), bestatin, leupeptin, and aprotinin (Sigma) was added to frozen lens cortex samples. The samples were thawed and homogenized in 9 M urea, 2 or 4% Nonidet P-40, 10 mM DTT,[1] 2% Resolyte 3.5–10. Two-dimensional gel electrophoresis was done according to previously described procedures (27, 28). Non-linear, pH 3–10, 18-cm dry strips (Amersham Pharmacia Biotech) were used. Samples were loaded onto the acidic end and focused for 32,000 V-h. The strips were then frozen at least 1 h and equilibrated twice, first in 50 mM Tris, pH 6.8, containing 6 M urea, 1% SDS, with 1% DTT and then in the same buffer mix but with 4.5% iodoacetamide. The second dimension was done in the Iso-Dalt Gel Electrophoresis System using 15–18% gradient gels. Molecular weight markers (Bio-Rad) and pI markers (carbamylated CPK, BDH Laboratory Supplies) were included. Proteins were stained with colloidal Coomassie Blue G-250 or with PhastGel BlueR (Amersham Pharmacia Biotech) when protein spots were analyzed by mass spectrometry. Gels were scanned using a Molecular Dynamics Personal Densitometer, and protein spots were quantified using ImageQuant software.

Protein Sequencing and Immunoreactivity—Proteins were electrophoretically transferred from the two-dimensional gels to polyvinylidene difluoride membranes (Millipore). Tryptic peptide maps and Edman sequencing were done by Harvard MicroChem. Immunoreactivity was determined using Tropix Western-Light detection kit (PE Applied BioSystems).

Mass Spectrometry—Proteins were subjected to in-gel digestion with either trypsin (Promega) or Asp-N endoproteinase (Wako) as described previously (29). Extracted peptides were spotted with α-cyano-4-hydroxycinnamic acid and analyzed using a PE Biosystems Voyager DE STR matrix-assisted laser desorption-time of flight (MALDI-TOF) mass spectrometer. Data were processed using GRAMS/386 software.

Cloning of αB-crystallin and αB_g—RNA was obtained from a 16-year-old human lens. The isolation was done by combining the guanidine isothiocyanate lysis with the silica gel membrane technology using the Qiagen RNeasy kit. Oligo(dT)-primed cDNA was prepared from total RNA using the Amersham Pharmacia Biotech T-primed first strand kit. The coding regions of human αB-crystallin and αB_g were amplified by PCR using primers containing *Nco*I or *Hin*dIII sites and were inserted into the cloning vector pCR2.1-TOPO (Invitrogen, San Diego, CA). In the PCR reactions the following primer (forward), which corresponds to the 5′ end of the coding region of human αB-crystallin, was used for both αB-crystallin and αB_g: 5′-TAAGAAGGAGATATACCATGGACATCGCCA-3′. The reverse primers used for human αB-crystallin and αB_g were 5′-CAAAAGCTTATTACTATTTCTTGGGGGCTG-3′ and 5′-GGCCGCAAGCTTTCACTTGGGGGCTG-3′, respectively. The latter will terminate the coding sequence after Lys^{174}. The underlined nucleotides indicate the restriction sites. The clones were propagated in *Escherichia coli* DH5 α^{TM} (Life Technologies Inc.). The coding region of these constructs were confirmed by DNA sequence analysis using the dye terminator cycle sequencing method (PE Applied Biosystems, Warrington, UK).

Construction of Expression Vectors—The coding region of human αB-crystallin and αB_g were removed from the cloning vector by double digestion with *Nco*I and *Hin*dIII restriction enzymes and ligated into *Nco*I-*Hin*dIII-cut pET-21d(+) (Novagen, Madison, WI). Recombinant plasmids were identified by *Nco*I-*Hin*dIII digestion and amplification of human αB-crystallin by PCR using internal primers.

Expression and Purification of Human αB-crystallin and αB_g—Recombinant pET-21d(+)-HαBC and pET-21d(+)-HαB_gC expression plasmids were used to transform competent *E. coli* BL21(DE3) cells (Stratagene, San Diego, CA). Transformants were grown at 37 °C in 1 liter of Super Broth to A_{600} = 0.8. αB-crystallin and αB_g expression were then induced by addition of isopropyl-β-D-galactospyranoside to a final concentration of 1 mM and then the culture was incubated at 37 °C for 5 h. Cells were collected by centrifugation at 3,000 × *g* for 10 min at 4 °C and resuspended in 25 ml of lysis buffer, 50 mM sodium phosphate buffer, pH 7, containing 150 mM NaCl, 0.02% sodium azide, and a protease inhibitor mix (Roche Molecular Biochemicals). Cells were disrupted by sonication on ice. The bacterial lysates were then centrifuged at 10,000 × *g* for 30 min at 4 °C. The αB-crystallin and αB_g, which were primarily soluble, were purified by gel filtration and ion exchange chromatography. The sequences of αB-crystallin and αB_g were confirmed by sequencing the plasmids used for the expression and by mass spectral fingerprinting of the expressed proteins. The sequences of the recombinant proteins were identical to those of the proteins in the cataractous lenses with the exception that the recombinant proteins were not acetylated on the N termini.

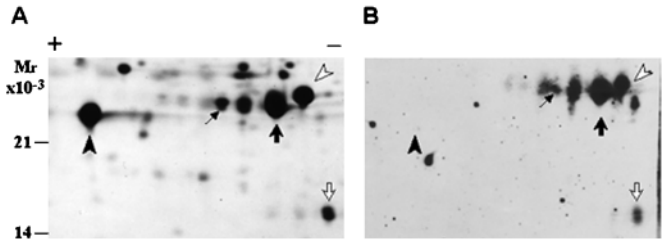

FIG. 1. **Two-dimensional gel electrophoresis of a cataractous human lenses.** A portion of the two-dimensional gel protein pattern of cortical fiber cell protein was Coomassie stained (*A*) and immunostained (*B*) with antibodies against recombinant human αB-crystallin (a gift of Dr. J. Horwitz, UCLA). αB_g, *thick black arrow*; typical form of αB, *white arrowhead*; 16.4-kDa fragment, *thick white arrow*; phosphorylated αB_g, *thin black arrow*; and, typical form of αA-crystallin, *black arrowhead.*

Isolation of Genomic DNA, αB-crystallin Exon 3 Amplification, and Sequencing—Genomic DNAs were isolated from small pieces of lens tissue (5–10 mg) by a rapid desalting process using a DNA purification kit (Epicentre Technologies) and following the recommendations of the manufacturer. These DNAs (200 ng) served as templates for PCR amplification of exon 3 and flanking regions of human αB-crystallin gene (nucleotide identifier (NID) g181075). Primers were 24-mers in length and localized at positions 3722 (upper) and 4172 (lower). PCR cycling conditions were 40 cycles of 94 °C for 5 s, 50 °C for 30 s, and 72 °C for 1 min, using Pfu polymerase (Promega). PCR products were sequenced by the BigDye Terminator method (PE Applied Biosystems) using the same primers.

Chaperone-like Activity—Chaperone-like activity was measured as the ability to protect against the DTT-induced aggregation of lactalbumin (30). The reaction was done at 23 °C in 50 mM sodium phosphate buffer, pH 6.9, containing 0.1 M NaCl and 2 mM EDTA. Lactalbumin was at 1 mg/ml, and crystallin was at 0.2, 0.5, and 1 mg/ml. Turbidity was measured at 360 nm.

RESULTS

Two unique protein spots were striking on two-dimensional electrophoresis gels of the cortical fiber cell protein from some human cataractous lenses (Fig. 1*A*). The positions of these proteins are indicated in Fig. 1*A*. The spot indicated by the *thick black arrow* migrated at a position one charge more acidic than αB-crystallin and at a M_r of 22,400 which is approximately 600 less than αB-crystallin (Fig. 1*A*). The second spot, indicated by the *white arrow* migrated at a M_r of 16,400. Both of these proteins reacted with antibodies made against recombinant human αB-crystallin (Fig. 1*B*) suggesting that the proteins were related to αB-crystallin. Data that will subsequently be presented in this report show that the M_r 22,400 protein is a modified form of αB-crystallin. To simplify discussion, this protein will henceforth be referred to as αB_g. Neither αB_g nor the 16.4-kDa protein has been observed on two-dimensional gels of total fiber cell protein from any normal lens examined (Fig. 2 (*a*—*c*)) or in all cataracts. The cataracts shown in Fig. 2 (*d*—*f*) that contain αB_g were not clinically classified before extraction but were determined to be mixed cataracts by visual examination after intracapsular extraction. The concentration

[1] The abbreviations used are: DTT, dithiothreitol; PCR, polymerase chain reaction; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

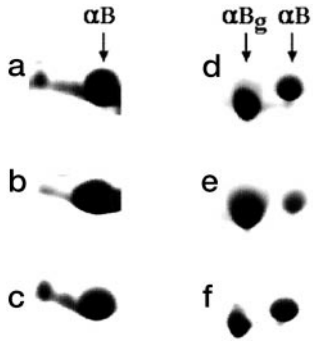

FIG. 2. **Composite of the αB-crystallin and αB$_g$ from two-dimensional electrophoresis gels of cataracts and normal human lenses.** Indicated is the position of αB-crystallin and αB$_g$. The Coomassie-stained gels were scanned and these spots quantified to determine the ratios of the species of αB-crystallins present in these cataracts. Normal lenses: *a,* 57 years; *b,* 17 years; and *c,* newborn. Cataracts with αB$_g$: *d, e,* and *f,* each were 60–70 years.

TABLE I
Ratio of αB/αA-crystallin
Cataracts are from donors between 60 and 70 years of age.

Lenses	αB/αA
Normal	0.3–0.5
Cataract A	1.5
Cataract B	0.6
Cataract C	0.9

of αB$_g$ varied from 10 to 90% of the total αB-crystallin (αB$_g$ plus αB-crystallin) in those cataracts in which it was present. In addition, the total content of αB-crystallin (αB$_g$ plus αB-crystallin) was significantly increased in most of these cataracts. Representative data on three cataracts that contain high concentrations of αB$_g$ are shown in Table I. Up to 5 times the normal amount of αB-crystallin was seen. This was determined by calculating the ratio of αB-crystallin to αA-crystallin. The amount of αA-crystallin in these cataracts was present at normal concentrations relative to β-crystallins. To our knowledge, an increase in the concentration of αB-crystallin in lenses has not been previously reported.

α-Crystallins are phosphorylated *in vivo*; however, in the lens the function of the phosphorylation is not known. As can be seen in Fig. 1*A* (*thin black arrow*), αB$_g$ was the only form of αB-crystallin for which a phosphorylated species was obvious; phosphorylated species of the typical form of αB-crystallin were not observed. Three phosphorylation sites have been identified for αB-crystallin**,** Ser^{19}, Ser^{45}, and Ser^{59}. Mass spectral data indicated the presence of a phosphate in the peptide corresponding to residues 2–24 in αB$_g$.

Characterization of αB$_g$—The HPLC chromatograms of the tryptic digests of αB$_g$ and αB-crystallin from cataracts of two different individuals are presented in Fig. 3. There were no consistent differences between the chromatograms of αB$_g$ and αB-crystallin that were common to the respective samples from both individuals. Edman sequencing of several tryptic peptides of αB$_g$ was performed. For each peptide sequenced there was 100% agreement with the sequence of human αB-crystallin (data not shown). These results confirm that the protein is an αB-crystallin, albeit, based on the electrophoretic migration, a modified form of αB-crystallin. Direct Edman sequencing of αB$_g$, electroblotted onto polyvinylidene difluoride, gave no sequence, suggesting that the N terminus of the protein was blocked. In all αB$_g$ samples, a peptide eluting at 40 min corresponded to the C-terminal residues 164–174. Edman sequencing verified the identity of the peptide and the mass of the peptide agreed with its theoretical mass. The presence of C-terminal Lys^{175} in αB$_g$, however, could not be verified by this experiment because trypsin would cleave between Lys^{174} and Lys^{175}.

Mass fingerprinting was done on trypsin and Asp-N protein digests of αB-crystallin and αB$_g$ using MALDI-TOF mass spectrometry. The results are illustrated in Tables II and III. In Table II, the protonated molecular weights for those peptides derived by trypsin digestion of αB-crystallin and αB$_g$, as well as the theoretical protonated molecular weights, are listed. The presence of peptides with *m/z* values of 1430.7 and 1431.0 in the tryptic digests of αB$_g$ from both samples indicate that the N terminus is present and acetylated. In the trypsin digests there were a few masses that could not be assigned, but there were no masses consistently present in αB$_g$ that were missing in αB-crystallin or vice versa.

In Table III, the masses for the peptides derived by Asp-N endoproteinase digestion are listed. αB-crystallin samples contained peptides with *m/z* 3842.9 and 3842.0. These molecular weights are consistent with the C-terminal peptide, residues 140–175, that has a theoretical *m/z* value of 3842.4. A mass that corresponded to this peptide was absent in both αB$_g$ samples, but in both cataracts αB$_g$ contained a peptide absent in either αB-crystallin spot, with *m/z* values of 3715.0 and 3714.4 (Fig. 4). These masses are consistent with the theoretical *m/z* value of the C-terminal peptide minus Lys^{175}, 3714.3. Asp-N endoproteinase does not remove Lys^{175}; therefore, the digest product of residues 140–174 from αB$_g$ confirms that Lys^{175} is not present on αB$_g$. In the Asp-N digests there were very few masses that could not be assigned, and other than the differences just discussed there were no other peptide masses that were present in αB$_g$ that were missing in αB or vice versa. Only one peak corresponding to residues 129–139 (DPLTITSSLSS) was consistently absent in Asp-N spectra, and it was not present in either crystallin. In cataract B, there was evidence for Met^{68} being present as methionine sulfoxide in both αB-crystallin and αB$_g$. The *m/z* value of 1294.9 is consistent with the expected molecular weight of residues 62–72 plus a mass of 16 mass units. The theoretical *m/z* value of the same peptide with Met^{68} oxidized is 1294.61. Between the two enzymatic digestions spectra were obtained for peptides that covered 100% of the polypeptide sequence. The regions of the polypeptide that could not be accounted for by tryptic peptides were accounted for with the peptides from Asp-N digests and the reverse was also true, making it possible to detect a difference in any residue of αB-crystallin and αB$_g$. There were no unique modifications, amino acid substitutions, or internal deletions detected in αB$_g$. The excellent agreement of the masses obtained on the peptide digests of αB$_g$ with the respective theoretical masses of the same peptides in αB-crystallin indicate that there is only one difference between αB$_g$ and αB-crystallin. The terminal Lys^{175} is missing in αB$_g$.

The loss of the C-terminal lysine could readily explain the migration of αB$_g$ on two-dimensional gel electrophoresis at a position 1 charge more acidic than the typical form of αB-crystallin. However, it did not seem likely that the loss of one lysine (128 mass units) could explain the migration of αB$_g$ at the lower molecular weight. The only reasonable explanations were that αB$_g$ had region(s) of structure different from αB-crystallin that remained throughout the electrophoresis even in the presence of 9 M urea, 2% Nonidet P-40 or 1% SDS and affected its electrophoretic migration, or that an adduct was lost during the subsequent analyses.

Recombinant Human αB-crystallin and αB$_g$—To test whether the removal of the C-terminal lysine was sufficient to induce the altered migration on electrophoresis as was observed in the second dimension of two-dimensional electro-

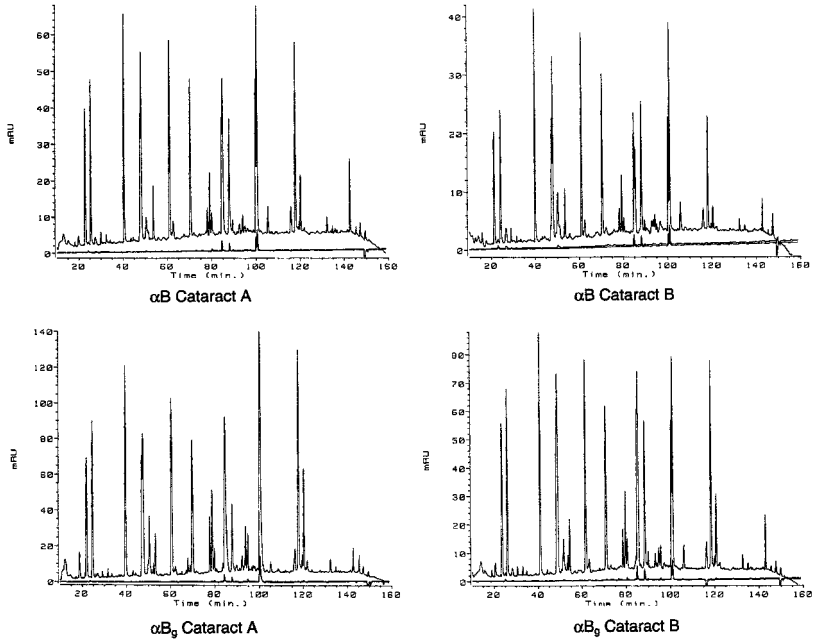

FIG. 3. **HPLC chromatograms of tryptic digests of αB-crystallin and αB$_g$ from two cataracts.** Reverse phase HPLC of native αB-crystallin and αB$_g$. Tryptic peptides were monitored at A_{205}.

TABLE II
αB-Crystallin tryptic digest products determined by MALDI-TOF mass spectrometry

Residues	Theoretical m/z[a]	Cataract A		Cataract B	
		αB	αB$_g$	αB	αB$_g$
1–11/Ac	1430.7	1430.9	1430.7		1431.0
12–22	1374.7	1374.8	1374.8	1374.9	1374.9
23–56	4006.5				4006.7
57–69	1496.7	1496.9	1496.9		1497.0
75–82	921.5	921.5	921.5		921.7
83–92	1213.7	1213.8	1213.6	1213.8	1213.9
93–116	2788.1	2787.8	2787.8	2787.9	2787.0
117–120	588.3	588.3	588.3	588.3	588.4
121–149[b]	3073.5	3073.2	3074.2	3073.6	3073.9
150–157	900.5		900.6	900.6	900.7
158–174	1822.0	1822.0	1822.5	1822.4	1822.4

[a] Theoretical monoisotopic *m/z* values are listed for *m/z* less than 2000, and theoretical average *m/z* values are listed for *m/z* greater than 2000.
[b] The identical mass also corresponds to residues 122–150.

TABLE III
αB-crystallin Asp-N endoproteinase digest products determined by MALDI-TOF mass spectrometry

Residues	Theoretical m/z[a]	Cataract A		Cataract B	
		αB	αB$_g$	αB	αB$_g$
2–24	2875.4	2875.1	2875.5	2875.1	2875.5
25–35	1321.6	1321.8	1321.8	1321.8	1321.8
36–61	3031.5	3031.1	3031.5	3031.2	3031.0
62–72	1278.6	1278.8			1278.8
62–72/mso[b]	1294.9			1294.8	1294.8
73–79	850.4	850.5	850.5	850.5	850.6
80–95	1825.0	1826.3	1826.3	1826.3	1825.3
96–108	1575.8	1576.0	1576.0	1576.0	1576.0
109–126	2259.5	2259.6	2259.5	2259.7	2259.5
129–139	1121.2				
140–175	3842.4	3842.0		3842.9	
140–174	3714.3		3715.0		3714.4
		αB PAVTAAPKK[175]			
		αBg PAVTAAPK[174]			

[a] Theoretical monoisotopic *m/z* values are listed for *m/z* less than 2000, and theoretical average *m/z* values are listed for *m/z* greater than 2000.
[b] The expected mass for peptide 62–72 upon oxidation of Met[68].

phoresis, both human αB-crystallin and human αB-crystallin minus the C-terminal lysine (αB$_g$) were cloned and expressed in *E. coli*. Both were expressed as soluble proteins in this

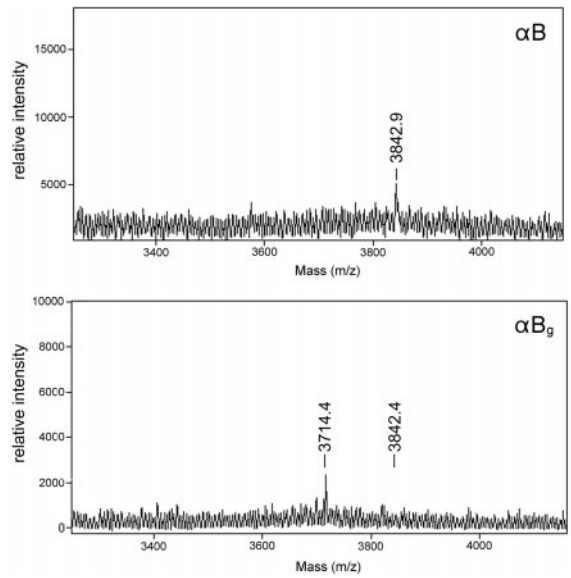

FIG. 4. **Mass spectra of C-terminal, Asp-N endoproteinase digest peptides of native αB-crystallin and αB$_g$.** Protein spots of αB-crystallin and αB$_g$ were cut out of a two-dimensional gel and subjected to in-gel digestion by Asp-N endoproteinase. Resultant peptides were extracted and analyzed by MALDI-TOF mass spectrometry. The portions of the spectra showing the difference between αB-crystallin and αB$_g$ are shown. The *m/z* value of 3842.9 corresponds to the singly charged peptide of residues 140–175, which has a theoretical average *m/z* of 3842.4. The *m/z* 3714.4 corresponds to the singly charged peptide of residues 140–174, which has a theoretical average *m/z* of 3714.3. The absence of a peak at *m/z* 3842.4 and the coincident mass at *m/z* 3714.4 in the spectrum of αB$_g$ indicates that Lys[175] is not present in αB$_g$.

system. Neither protein was acetylated on the N-terminal methionine. The recombinant human αB-crystallin and the recombinant human αB$_g$ eluted from a Superose 6 gel filtration column as aggregates with apparent molecular weights of 570,000 and 510,000, respectively (Fig. 5). Significantly smaller species were also observed for αB$_g$ (data not shown). As shown in Fig. 6*A*, even after boiling in SDS, the recombinant αB$_g$ migrated slightly faster than recombinant αB-crystallin on SDS-PAGE. The calculated difference in the molecular weight between the recombinant αB-crystallin and recombinant αB$_g$ was approximately 700, similar to the difference observed on

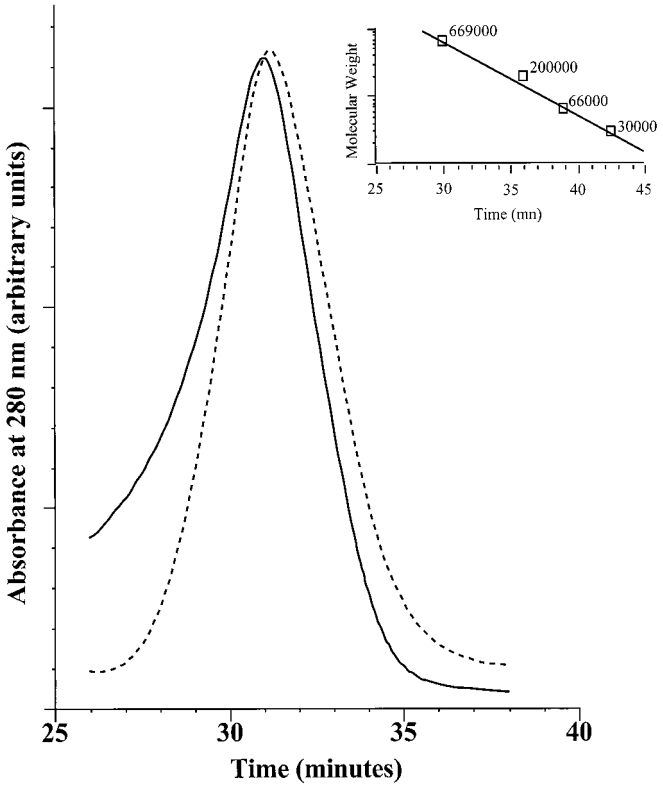

FIG. 5. **Oligomeric sizes of recombinant human αB crystallin and αB_g.** Recombinant human αB-crystallin and αB_g were purified by gel filtration and ion exchange chromatography. The figure is an overlay of the final purification chromatograms of both recombinant proteins from a Superose 6 HR 10/30 gel filtration column. Recombinant human αB-crystallin peak (*solid line*) eluted at ~570 kDa (90% of the peak area was in the range of 270–970 kDa) and recombinant human αB_g peak (*dotted line*) eluted at ~510 kDa (90% of the peak area was in the range of 260–940 kDa). The column was calibrated immediately before purification of both recombinants using thyroglobulin (669 kDa), β-amylase (200 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (30 kDa) (*inset*).

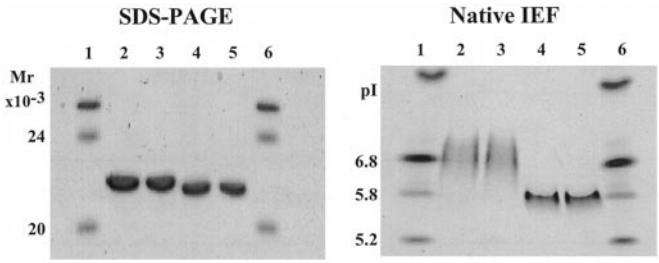

FIG. 6. **SDS-PAGE and native isoelectric focusing of recombinant human αB-crystallin and αB_g.** The relative sizes of the subunits of purified rhαB-crystallin and rhαB_g were calculated from their migration on 14% polyacrylamide gels. The relative isoelectric points of the two recombinants were calculated from their migration under native conditions on 5% polyacrylamide isoelectric focusing gel. SDS-PAGE: *lanes 1* and *6*, molecular weight markers; *lanes 2* and *3*, rhαB-crystallin (21.5 kDa); *lanes 4* and *5*, rhαB_g (20.7 kDa). Native isoelectric focusing: *lanes 1* and *6*, isoelectric point markers; *lanes 2* and *3*, rhαB-crystallin (pI 6.8); *lanes 4* and *5*, rhαB_g (pI 5.8).

two-dimensional gels of lens proteins. These results show that the removal of the C-terminal lysine is sufficient to cause the migration of αB_g at a significantly lower molecular weight than αB-crystallin. The pI values determined by isoelectric focusing under non-denaturing conditions for both proteins are shown in Fig. 6*B*. The recombinant αB-crystallin had a native pI of 6.8, and recombinant αB_g had a native pI of 5.8. The theoretical pI values are 6.76 and 6.50, respectively.

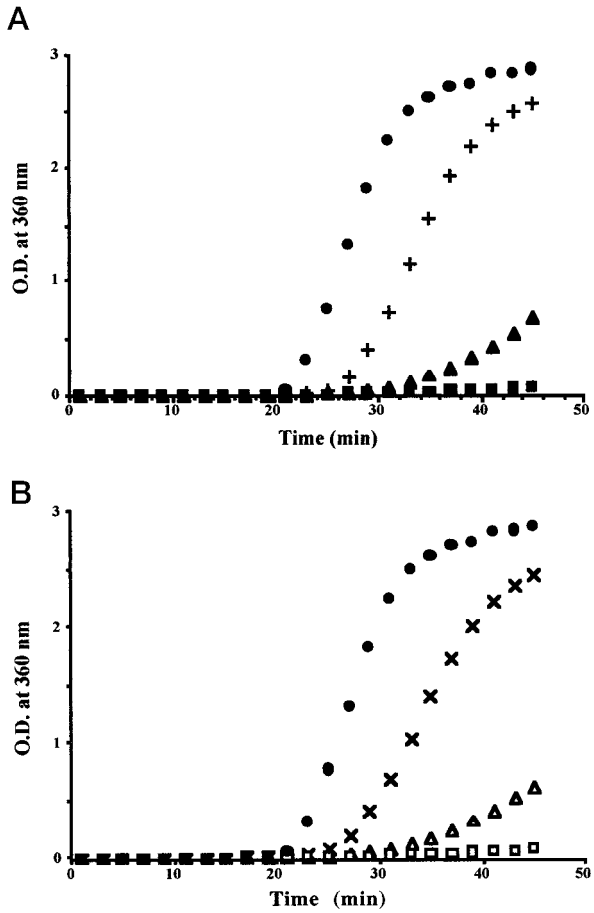

FIG. 7. **Chaperone-like activity of recombinant human αB-crystallin and αB_g.** The incubation mixture contained 50 mM sodium phosphate, 0.1 M NaCl, 2 mM EDTA, pH 6.9, 50 mM DTT, and 1 mg/ml α-lactalbumin, in the absence (●) and presence of rhαB-crystallin, 1 mg/ml (■), 0.5 mg/ml (▲), and 0.2 mg/ml (+) (*A*); 1 mg/ml α-lactalbumin in the absence (●) and presence of rhαB_g 1 mg/ml (□), 0.5 mg/ml (△), and 0.2 mg/ml (×) (*B*).

Chaperone-like Activity—α-Crystallins exhibit a chaperone-like activity, protecting against aggregation of proteins that is induced by heat, oxidation, and reduction by DTT (30). The effect of the removal of the C-terminal lysine on the chaperone-like activity of αB-crystallin was assessed by determining the ability of the recombinant forms of human αB-crystallin and αB_g to prevent the DTT-induced aggregation of lactalbumin. As shown in Fig. 7, the removal of the C-terminal lysine had essentially no effect on the chaperone-like activity of this protein under these conditions.

16.4-kDa Protein—The 16.4-kDa protein was present at a concentration about one-tenth that of αB_g and has only been observed in samples that contain αB_g. Direct Edman sequencing was performed on the 16.4-kDa protein from cataracts of two different individuals. These data are shown in Fig. 8. The 16.4-kDa protein in lens 1 yielded multiple N-terminal sequences, Ser^{43}, Ser^{41}, Ser^{45}, and Phe^{47}. All sequences were identical to αB-crystallin. MALDI-TOF derived data on the 16.4-kDa protein from a third individual indicated the N termini were Ser^{43} and Ser^{41}. In a fourth individual the N terminus was Pro^{39} (Table IV). Two of the cleavages were between Thr and Ser, one between Leu and Ser, one between Pro and Phe, and the fourth was between Phe and Pro. The significance of these data is that multiple N termini have been identified, each different by two or multiples of two residues.

Tryptic digests of this spot were similar but not identical to that of αB-crystallin. A peptide with a mass of 1140 Da that

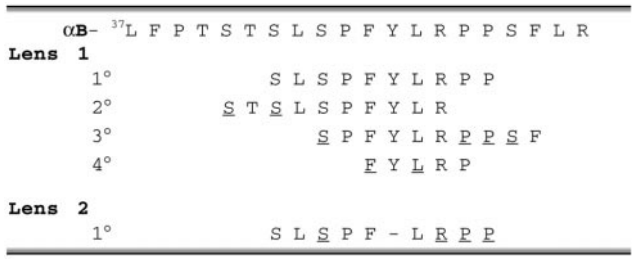

FIG. 8. **N-terminal sequences of αB-crystallin 16-kDa fragments.** N-terminal sequences of the 16.4 kDa are compared with the αB-crystallin sequence. Sequencing was performed by Harvard MicroChem. *Underlined* residues are lower confidence. For lens 1, determination of the predominance of sequences was done by Harvard Microchem using the relative concentrations of amino acids in each cycle and the typical yields of each amino acid.

TABLE IV
16.4-kDa protein Asp-N digest products determined by MALDI-TOF mass spectrometry

Residues[a]	Theoretical *m/z*[b]	Measured 16.4 kDa
39–66	3129.8	3130.5
62–72	1278.6	1278.7
73–79	850.4	850.4
80–95	1825.0	1825.2
96–108	1575.8	1575.9
109–126	2259.5	2259.4
127–139		
140–174	3714.3	3714.5

[a] Residue numbers correspond to those of αB-crystallin.
[b] Theoretical monoisotopic *m/z* values are listed for *m/z* less than 2000, and theoretical average *m/z* values are listed for *m/z* greater than 2000.

eluted at the same time as a peptide identified by sequencing as residues 164–174 was present. MALDI-TOF mass spectral analysis of Asp-N endoproteinase digests showed the absence of a peak at *m/z* 3842.4 which would correspond to residues 140–175 of αB-crystallin (Table IV). There was, however, a peak at *m/z* 3714.5 which corresponds to residues 140–174. As shown in Table IV, experimental *m/z* values were matched to theoretical *m/z* values from Asp-N cleavage that covered most of the polypeptide. Aside from the N-terminal peptides, the only mass missing was the same one not observed in αB and αB_g. A unique peak at *m/z* 3130.5 was observed. This mass may correspond to residues 39–66 (3129.8). These results show that the 16.4-kDa protein is derived from αB_g.

Genomic Analysis—The mechanism by which αB_g is generated in these cataractous lenses is not known. One possibility was that αB_g is the product of a mutated gene for αB-crystallin since a single nucleotide change could convert the codon for lysine to a stop codon. To test this possibility PCR products were obtained for the last exon of the αB-crystallin gene using genomic DNA prepared from lenses containing αB_g. The sequences of the PCR products indicated that there was no mutation in the gene for αB-crystallin (data not shown).

DISCUSSION

We have demonstrated that in some human cataracts the concentration of the small heat shock protein, αB-crystallin, is significantly increased. This could be the result of an up-regulation of its expression and/or a diminution of its degradation in the lens. Regardless, it represents a significant deviation from the normal protein composition of the lens and is the first time such an observation has been made in a human cataract. We have also provided evidence for aberrant N- and C-terminal processing of αB-crystallin. One atypical form of αB-crystallin found in high concentrations in these cataracts has been designated αB_g. This species is αB-crystallin minus the C-terminal lysine. A second atypical form of αB-crystallin, found at about 10% the concentration of αB_g, is a 16.4-kDa fragment of αB_g. In this protein 38–46 N-terminal residues have also been removed.

The removal of the C-terminal lysine does not diminish the chaperone-like activity of αB-crystallin under the conditions used for these studies. As reported here (Fig. 7) the chaperone-like activity of the recombinant human αB_g is comparable to that of the recombinant human αB-crystallin in protecting against the DTT-induced aggregation of lactalbumin at room temperature. αA- and αB-crystallin have polar, flexible C-terminal extensions that are thought to contribute to the solubility of these crystallins and have been implicated in their chaperone-like activity (31). Substitution of Lys^{174}-Lys^{175} of αB-crystallin with Leu-Leu significantly diminished chaperone-like activity; however, removal of the last 5 residues had little effect on chaperone-like activity (32). Likewise, in αA-crystallin the introduction of tryptophan at the C terminus and removal of 17 C-terminal residues diminished chaperone activity (33–35). Maintenance of a polar, flexible C-terminal extension appears to be an important factor for maintaining chaperone-like activity (33). Thus, it is not surprising that αB_g has full activity in protecting against the DTT-induced aggregation of lactalbumin.

The only modification identified for αB_g was the loss of Lys^{175}. There was nothing in the mass spectral, HPLC, and protein sequence data that suggested an additional modification. The N and C termini of the protein were otherwise intact. The N-terminal methionine was acetylated, and every peptide sequence examined matched αB-crystallin exactly. With the exception of the mass corresponding to the loss of the C-terminal lysine, there were no masses found by MALDI-TOF mass spectrometry that were consistently present in αB_g that were not present in αB-crystallin from the same cataract and *vice versa*. This held for both Asp-N and trypsin digests of the proteins, and, combining data from both digests, the entire sequences of both αB_g and αB-crystallin were analyzed. These data rule out splice variants and other mutations unless replacements have the same mass and would not be detected in the MALDI-TOF mass spectrometry fingerprint analysis.

It was confirmed that the removal of the carboxyl-terminal lysine (128.09 mass units) was responsible for the faster migration of αB_g on SDS-PAGE relative to αB-crystallin. This was demonstrated using the recombinant forms of these proteins. In addition to showing that a difference of one lysine was sufficient to alter the migration on SDS-PAGE, it also supported the conclusion that the only modification of αB_g was the lack of the terminal lysine. Smulders *et al.* (33) demonstrated that a mutant of αA-crystallin with an extension of ALRKG migrated on SDS-PAGE slightly slower than mutants with ALGKG or ALDKG. Thus, addition of one more positive charge in the C-terminal extension of either αA- or αB-crystallin can slightly retard its electrophoretic mobility.

In this study, αB_g and the 16.4-kDa proteins were only observed in cataracts but not in all human cataracts. Over 70 cataracts from 60 to 90 years have been analyzed. Eight cataracts have the high concentration of αB_g. Many other cataracts have αB_g but at lower concentrations. So far, a correlation cannot be made between the presence of these proteins in cataracts and any cataract etiology. Neither αB_g nor the 16.4-kDa fragment have been observed in our laboratory on two-dimensional electrophoresis of normal human lens total protein, water-soluble or water-insoluble fractions, of about 50 non-cataractous lenses (newborn to 75 years). However, the presence in a normal lenses of a low concentration of an α-crystallin with a mass that corresponded to αB-crystallin minus the C-terminal lysine has been reported using liquid chromatography/mass spectrometry (36–38). The sensitivity of electrospray

ionization mass spectrometry made the detection of very low levels of αB_g in non-cataractous lenses possible, whereas Fig. 2 clearly shows that αB_g is not detected by Coomassie Blue staining of normal lens proteins. The findings in other laboratories suggest that at least very low concentrations of αB_g may exist in normal lenses and that αB_g and possibly the 16.4-kDa fragment may be intermediates in the normal pathway of processing αB-crystallin (36–38). In the cataracts examined in this study, there may be increases and/or decreases in proteinases that result in the accumulation of these species. Interestingly, αA-crystallin which has Ser-Ser as the final C-terminal residues is found with only the final Ser removed (60). This form of αA-crystallin has been observed in normal human and bovine lenses. The functional significance of the modified form is not known. Furthermore, the significance of the processing of α-crystallins to cataractogenesis is not known.

Multiple proteinases are certain to be involved in the post-translational modification of αB-crystallin generating αB_g and the 16.4-kDa fragment. The data reported in this study rule out the possibility that αB_g is the result of a mutation in the αB-crystallin gene but cannot rule out a genetic component involving other genes such as proteinases or involving the regulation of proteinases. There was a high incidence of αB_g in cataracts from India. This supports the possibility of a genetic component. Alternatively, the high incidence in cataracts from India could indicate the presence of a particular form of stress on the lens which alters the proteinase activities.

In αB_g the terminal lysine is removed but not the penultimate lysine. The first cleavage would be a Lys-Lys cleavage and the second a Pro-Lys cleavage. Carboxypeptidases have been described that are specific for basic amino acids for which the penultimate amino acid alters the rate of cleavage (39). Peptide carboxypeptidases have been described that will not cleave prolysyl bonds, and endopeptidases with specificity for pairs of basic amino acids that will remove one or both of the basic residues have been described (39). Thus, the specificity for removing one but not both lysine residues is possible; however, whether any of these proteinases are in the lens is not known. If the removal of the C-terminal lysine is related to function, it is possible a specific carboxypeptidase may be induced during stress.

The 16.4-kDa protein is an N-terminally truncated form of αB_g and was only observed in those lenses in which αB_g was in high concentration. Multiple N termini were identified, but the major N terminus was Ser^{43}. Since each N terminus represented the removal of two residues or multiples of two, it is reasonable to suggest that cleavage is catalyzed by a dipeptidyl peptidase. Dipeptidyl peptidases II (lysosomal) and III (cytoplasmic) are present in lens and cataracts (40, 41). However, both of these preferentially use peptides as substrates, not proteins. Dipeptidyl peptidases that utilize proteins as substrates exist but, to our knowledge, have not been described in the lens. The N termini of αB_g and αB-crystallin are blocked, so presumably a different proteinase made the first cleavage. Acylaminohydrolases that could cleave the acetylmethionine are present in lenses (42).

Our hypothesis is that a stress mechanism is involved in generating both the high concentrations of αB-crystallin and the subsequently modified forms, αB_g and the 16.4-kDa protein, and that the increased concentration of αB-crystallin is an effort by the lens to protect against loss of lens function. That a stress mechanism is involved is strongly supported by our observation that αB_g was present in the lens from an infant with microphthalmia, coloboma, and persistent hyperplastic primary vitreous.[2] The latter is a condition where the vessels do not regress at the appropriate time in development and cells invade the back of the lens. Our assumption is that this is perceived by the lens as an extreme stress condition. In addition, two forms of αB-crystallin were induced in hypertonically stressed dog lens epithelial cells in culture (21). Direct evidence is not yet available, but it is highly likely, based on the migration on two-dimensional gels, that the atypical form of αB-crystallin found is αB_g. αB-crystallin is a member of the family of small heat shock proteins and in cultured cells, including lens cells, αB-crystallin is induced by heat shock, oxidative stress, osmotic stress, sodium arsenite, phorbol 12-myristate 13-acetate, and hormones such as estrogen and dexamethasone (20–23, 43, 44). Heat shock proteins are also phosphorylated in response to stress (45). α-Crystallins are phosphorylated *in vivo* and *in vitro* (46–48). The role of phosphorylation of αB-crystallin in lens is not understood, but αB_g was preferentially phosphorylated in the cataracts examined.

This is the first time an increased concentration of αB-crystallin has been described *in vivo* in human lenses. The increased concentration is due to the presence of αB_g not to an increased concentration of the typical form of αB-crystallin. Due to the nature of the lenses, it is difficult to determine whether there is truly an increased expression or a decreased degradation of αB-crystallin. If the increased concentration of αB-crystallin reflects diminished processing, the degradation of this protein must occur at a much greater rate in human lens than previously appreciated. We propose that there is an increased expression due to chronic stress conditions and altered processing of αB-crystallin generating αB_g in these cataracts. αB-crystallin also accumulates in brains of individuals with neurological disorders (49–51). It is unclear in these cases, as well as the cataracts, whether the presence of αB-crystallin is an attempt to rescue the tissue or if it is a contributor to the pathology. We think it is unlikely that the increased concentration of αB-crystallin and the presence of the modified forms cause the loss of lens clarity in the cataracts, but it must be considered.

The functional significance of the post-translational modification of αB-crystallin described here is not known. In the intact lens, it is unclear whether the primary function of αB-crystallin is that of a stress protein and related to its chaperone-like activity or if it is structural. The findings that αB_g is present in a lens with persistent hyperplastic primary vitreous and is induced by hypertonic stress in lens cells suggests a major role is stress-related. Based on a number of reports, αB-crystallin interacts with actin, intermediate filaments, membranes, and components in cell nuclei (52–57). Lys^{175} has been implicated as an amine donor substrate for transglutaminase reactions (58, 59). The data presented suggest αB_g has some difference in structure from the typical form of αB-crystallin. This is likely to alter specific protein-protein interactions. In fact, the larger than expected change in the native pI of αB_g supports the observations that the C-terminal lysine is involved in intramolecular interactions and is likely to affect intermolecular interactions as well. Further evidence for this is the preferential phosphorylation of αB_g over αB-crystallin.

Studies in progress will hopefully elucidate the mechanisms involved in the increased concentration of αB-crystallin and the function of αB_g in lens and in cataracts. They will also determine whether the pathway for modification of αB-crystallin is one specific to lens or is a general pathway induced by stress in other tissues.

[2] D. Garland, unpublished data.

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