Identification of a Major Continuous Epitope of Human Alpha Crystallin

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Human lens proteins were digested with trypsin or V8 protease, and the resulting peptides resolved on a C₁₈ reverse phase column. Fractions from this column were probed with polyclonal antiserum made against the whole alpha crystallin molecule. Peptides in the seropositive fraction were purified to homogeneity, then characterized by mass spectral analysis and partial Edman degradation. The tryptic and V8 digests contained only one seropositive peptide that was derived from the C-terminal region of the alpha-A molecule. To determine the exact boundaries of the epitope, various size analogues of this region were synthesized and probed with anti-alpha serum. Together, these studies demonstrate that the major continuous epitope of the alpha-A chain includes the sequence KPTSAPS, corresponding to residues 166–172 of the human alpha-A crystallin chain. Invest Ophthalmol Vis Sci 33:2024–2028, 1992.

The alpha crystallin fraction from the human lens is made up of two homologous but nonidentical polypeptide chains.¹ In the young human lens, alpha-A crystallin is more abundant than the alpha-B chain.² These chains undergo extensive posttranslational modification, including phosphorylation³ and truncation.⁴ Polyclonal antisera against these polypeptide chains have been used to assess qualitative and quantitative changes in the alpha crystallins during the processes of aging and cataractogenesis of the lens.⁵,⁶ Implicit in these studies is the assumption that polyclonal antiserum recognizes numerous epitopes along the amino acid sequence of these molecules.

Previous “epitope mapping” studies of other proteins have demonstrated that polyclonal antiserum against a specific polypeptide chain may recognize only a few regions of the molecule that encompass only a relatively small part of the amino acid sequence of the protein.⁷,⁸ Therefore, great care must be taken in the interpretation of data that arises from antibody binding studies, which could vary greatly with the covalent modification of only a few amino acids of the protein.

However, once these antibody binding regions have been identified, polyclonal and monoclonal antisera of high titer and good specificity can be obtained by simply immunizing animals with a synthetic peptide corresponding to the region of interest. Immunization with a synthetic peptide instead of an alpha crystallin preparation eliminates the possibility that the animal will make antibody against the other crystallin polypeptides that almost always contaminate an alpha preparation.

For these reasons, it is important to identify the number and location of major epitopes in the alpha crystallins. For this report, we used a sensitive solid phase radioimmunoassay for peptides, plus polyclonal antiserum to human alpha crystallin, to identify seropositive peptides in proteolytic digests of total proteins from the human lens. The results of our analysis suggest that the alpha-A molecule contains a single major epitope located near the C-terminus of the molecule.

Materials and Methods

Human lenses were obtained from various eye banks and stored at −70°C until used. Informed consent was obtained in all cases. Eight lenses from donors of 23–32 yrs of age were used for the preparation of alpha crystallin using G-200 Sephadex chromatography (Pharmacia, Piscataway, NJ). The resulting preparation was used to immunize rabbits as previously described.⁹ All procedures involving the rabbits adhered to the ARVO Resolution on the Use of Animals in Research. We are indebted to Dr. S. Zigler of the National Eye Institute for the gift of this antiserum.
Total protein from individual human lenses were used for the production of proteolytic digests. The lenses were decapsulated, anaerobically solubilized, reduced carboxymethylated, dialyzed, lyophilized, and digested with various proteases as previously described. DPCC-treated trypsin (type 11) was from Sigma Chemical Co. (St. Louis, MO) and V8 protease from Staphylococcus aureus was from Miles Laboratories (Naperville, IL). A constant weight ratio of 1:40 (protease:lens protein) was used in all digestions. The lyophilized digest was resolved by a C18 reverse phase column, using a linear gradient of acetonitrile in 0.1% (volume/volume) heptafluorobutyric acid, and the individual fractions collected from the column were analyzed with a modified nitrocellulose spot blot as previously described.

Fractions containing the seropositive peptides were further purified to homogeneity with a C18 reverse phase column using gradients of acetonitrile or isopropanol in 0.1% (v/v) trifluoroacetic acid (TFA). After each purification step, the fraction containing the seropositive peptide was identified with the modified spot blot procedure. The purified peptide was quantitated by amino acid analysis using the Picotag method, and the molecular weight and partial amino acid sequence were determined using mass spectral analysis and gas phase sequencing, respectively.

Peptides were synthesized using the t-Boc method as described by Merrifield. The cleaved peptides were purified with a C18 reverse phase column and gradient of 0.1% (v/v) TFA in acetonitrile. The purified peptides were quantitated by amino acid analysis using the Picotag method.

Results

To identify amino acid sequences containing the major continuous epitope(s), it is necessary to localize the reactivity of polyclonal antiserum toward specific proteolytic fragments of the alpha crystallin molecule. In Figure 1, a tryptic digest of total lens proteins was resolved on a C18 reverse phase column, followed by probing of each of the fractions with polyclonal antiserum. Of the 40 fractions analyzed, only fraction number 20 contained a seropositive peptide, suggesting that polyclonal antiserum to human alpha crystallin recognizes only one continuous epitope.

To identify the exact sequence of this peptide, fraction number 20 was further resolved on the C18 column with two additional gradients containing 0.1% (v/v) TFA in acetonitrile, followed by 0.1% TFA in isopropanol. Each gradient involved the collection of 1 min fractions, followed by lyophilization and analysis of 5% of each fraction using the polyclonal antiserum to human alpha. Figure 2 shows the results of the last purification step, involving the isopropanol gradient. Analysis of total peptides demonstrated the presence of only one component eluting a 16.6 min. This peak reacts with the antialpha serum, demonstrating that the seropositive peptide has been purified to homogeneity.

After quantitation by amino acid analysis, this peptide was characterized by mass spectral analysis and Edman degradation. In Table 1, mass spectral analysis showed the presence of two peptides with molecular weights of 1032 and 945 daltons, while Edman degradation indicated an N-terminal sequence of EE. Based upon the known sequence of the human alpha crystallins, these peptides were derived from the alpha-A chain and make up the sequences EEKPT-SAPSS and EEKPTSAPS corresponding to residues 164-173 and 164-172 of the C-terminal end of the protein, respectively.

The reason for two, rather than one peptide sequence, is not clear. However, based upon the elution profile in Figure 2, only one peptide sequence would be expected. It is possible that the C-terminal serine might be cleaved during mass spectral analysis to produce the additional 945 dalton component (T. Williams, personal communication).
Fig. 2. Final purification of seropositive peptide from a trypic digest of human lens proteins. The seropositive fraction in Figure 1 was further purified using a C_{18} analytical column (Vydac, 250 mm × 4.6 mm) with a gradient of 10%-40% (v/v) acetonitrile in 0.1% (v/v) TFA. The seropositive fraction from this column was checked for purity by resolution on an analytical C_{18} column (Beckman, 250 mm × 4.6 mm) using a gradient of 0%-40% (v/v) isopropanol in 0.1% (v/v) TFA. Flow rate was 1.0 ml/min. One-minute fractions were collected, and 5.0% of each fraction was lyophilized and probed with the polyclonal antiserum to alpha crystallin. The results of this spot blot are superimposed above the elution profile of total peptides. The gradually increasing base line represents the increasing absorbance of the acetonitrile gradient.

Overall, characterization of the trypic digest of human lens proteins by the polyclonal antiserum suggests that the major epitope is located at the C-terminus of the alpha-A chain. However, it is possible that other continuous epitopes exist that could contain an arginine or lysine residue as part of the antibody binding region. These epitopes would lose the ability to bind antibody when treated with trypsin. To eliminate this possibility, human lens proteins were also digested with V8 protease that cleaves only on the carboxyl side of glutamic acid residues, when the digestion takes place in the presence of NH_{4}HCO_{3}.^{17}

Figure 3 shows the results of probing the C_{18} fractions with the polyclonal anti-alpha serum. Only one seropositive fraction is present (number 21), which was purified to homogeneity using two additional linear gradients of acetonitrile in 0.1% (v/v) TFA, followed by isopropanol in 0.1% (v/v) TFA.

Table 1 shows that characterization of this peptide by mass spectral analysis demonstrates that it is made up of two peptides that have molecular weights of 903 and 816. Partial Edman degradation produced only one sequence corresponding to EK. These peptides correspond to the alpha-A sequences of 165–173 and 165–172, respectively. As in the case of the seropositive peptides from trypic digestion, these peptides represent the C-terminal end of the alpha-A molecule. The 816 dalton peptide apparently has also lost the C-terminal serine.

Based upon the probing of the trypic and V8 digests with polyclonal antiserum, there appears to be only one major continuous epitope, located at the C-terminus of the alpha-A molecule. Because the sequence of the seropositive trypic peptide included the sequence of the V8 seropositive peptide, various size analogues of this trypic peptide were synthesized and probed with the polyclonal antiserum to locate the exact boundaries of the epitope.

Table 2 shows that EEKPTSSAPSS and EEKPT-

<table>
<thead>
<tr>
<th>Protease</th>
<th>MW</th>
<th>Sequence*</th>
<th>Alpha-A location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>1032</td>
<td>EEKPTSSAPSS</td>
<td>164–173</td>
</tr>
<tr>
<td>Trypsin</td>
<td>945</td>
<td>EEKPTSSAP</td>
<td>164–172</td>
</tr>
<tr>
<td>V8</td>
<td>903</td>
<td>EEKPTSSAPSS</td>
<td>165–173</td>
</tr>
<tr>
<td>V8</td>
<td>816</td>
<td>EEKPTSSAP</td>
<td>165–172</td>
</tr>
</tbody>
</table>

* Based on the molecular weight as determined by mass spectrometry, and on the identity of the first two amino acids (underlined) as determined by gas phase Edman degradation.

Table 2. Antiserum binding to size analogues of the major epitope

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Antiserum binding (cpm SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTSAPSS</td>
<td>0</td>
</tr>
<tr>
<td>KPTSSAP</td>
<td>1061 ± 524</td>
</tr>
<tr>
<td>EEKPTSSAPSS</td>
<td>1822 ± 586</td>
</tr>
<tr>
<td>EEKPTSSAPS</td>
<td>4053 ± 477</td>
</tr>
<tr>
<td>EKKPTSSAP</td>
<td>6348 ± 1057</td>
</tr>
<tr>
<td>EEKPTSSAP</td>
<td>426 ± 289</td>
</tr>
</tbody>
</table>

* The mean of five replicate determinations using 250 picomoles of conjugated peptide for each determination.
SAPS reacted with the antiserum, demonstrating that the C-terminal serine number 173 is not part of the antibody binding site. However, removal of serine number 172 to produce the peptide EEEKPTSAP resulted in a dramatic loss in antibody binding, demonstrating that the C-terminal boundary of the epitope occurs between residue numbers 172 and 173 of the protein.

In a similar manner, peptide analogues were synthesized with the progressive loss of residues from the N-terminal side. Removal of glutamic acid residue numbers 164 and 165 to produce the peptides EKPTSAPSS and KPTSAAPSS resulted in reduced but detectable binding, while removal of lysine number 166 resulted in complete loss of binding. Therefore, the minimal size of the epitope from the N-terminal side includes the lysine at residue number 166. The significant but decreased binding when glutamic acid residue numbers 164 and 165 were removed may be the result of multiple clones of antibody species in the polyclonal antiserum, resulting in binding to epitopes of slightly different size.

Although the polyclonal antiserum reacted against overlapping tryptic and V8 peptides present only at the C-terminal part of the human alpha-A molecule, it is still possible that these proteases may have destroyed other continuous epitopes in the alpha-A sequence. More specifically, Puri et al have used hydrophilicity plots to predict the possible presence of four continuous epitopes in the sequence of human alpha-A crystallin. None of these sequences completely overlaps the major continuous epitope, as determined from Table 2. To test the possibility that these sequences may contain continuous epitopes, they were synthesized and probed with the polyclonal antiserum to human alpha crystallin. Table 3 demonstrates that none of these peptides reacts with the polyclonal antiserum. These results strengthen the earlier conclusion that the C-terminal region of the alpha-A molecule represents the major continuous epitope of the molecule.

Table 3. Antiserum binding to synthetic peptides

<table>
<thead>
<tr>
<th>Alpha-A location</th>
<th>Sequence</th>
<th>Antiserum binding (cpm SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>65–70</td>
<td>RSDRDK</td>
<td>0</td>
</tr>
<tr>
<td>101–106</td>
<td>NERQDD</td>
<td>0</td>
</tr>
<tr>
<td>112–117</td>
<td>REFHRR</td>
<td>0</td>
</tr>
<tr>
<td>162–168</td>
<td>SREEKPT</td>
<td>0</td>
</tr>
<tr>
<td>164–173†</td>
<td>EEEKPTSAPSS</td>
<td>6983 ± 1620</td>
</tr>
</tbody>
</table>

* The mean of five replicate determinations using 250 picomoles of conjugated peptide for each determination.
† Positive control that includes the boundaries of the epitope as determined from Table 2.

Discussion

Polyclonal antisera to the lens soluble crystallins have been used in numerous studies to study the composition of the lens proteins. Despite all of these studies, no one has ever determined the number or sequence location of any crystallin epitope. This is surprising, because anticrystallin sera have been used in a wide variety of applications that depend upon the number and location of antibody binding epitopes along the polypeptide sequence. It is assumed that polyclonal antiserum made against an intact protein such as alpha crystallin will react at numerous sites along the polypeptide sequence. However, previous epitope mapping studies of nonlenticular proteins have established that only a few sequences along a polypeptide chain are highly antigenic.

The results of the present study strongly suggest the existence of only one major continuous epitope in the sequence of human alpha-A crystallin. Although this conclusion does not eliminate the possibility that antiserum also binds to discontinuous epitopes, it is obvious from the present study that great care must be taken in the interpretation of data derived from the use of polyclonal antiserum to alpha crystallin.

The major continuous epitope of alpha-A crystallin would appear to include residues 166–172, which are situated almost at the C-terminus of the molecule. Previous studies have established that this region of the molecule often is cleaved during the processes of aging in the human lens. Furthermore, this same region is also covalently modified during the process of cataractogenesis of the human lens. Therefore, great care must be taken when interpreting data with polyclonal antialpha serum, because it is highly possible that reactivity toward the protein could be abolished by a simple covalent modification at the C-terminal region of the human alpha-A molecule.

Identification of the major continuous epitope, however, permits the production of high titered antiserum, with little or no cross reactivity toward the other crystallins. Because many alpha crystallin preparations often are contaminated with small amounts of beta and gamma crystallins, it is more advantageous to immunize an animal with a synthetic peptide corresponding to the major continuous epitope of this crystallin. The small amounts of chemical contaminants in the synthetic peptide preparation should not cross react with any of the other crystallins. By identifying the major continuous epitopes of beta and gamma crystallins, it also should be possible to identify peptide sequences that can be synthesized and used for the production of very specific antisera with a high titer against these other crystallins.

Key words: alpha crystallin, continuous epitope
Acknowledgments

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References