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αB SUBUNIT OF LENS-SPECIFIC PROTEIN α-CRYSTALLIN IS PRESENT IN OTHER OCULAR AND NON-OCULAR TISSUES

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α-Crystallin, a tissue specific structural protein of the ocular lens, is known to be composed of two subunits, αA and αB. By using a specific antibody in an immunoblotting procedure we have found that one of the subunits, αB is present in a number of non-lenticular tissues including the retina, heart, skeletal muscle, skin, brain, spinal cord and lungs. Interestingly, in the rat, this protein is present in significantly higher concentrations in adult than in fetal tissues and, with the exception of the lens, fetal and adult heart has the highest concentration among the tissues examined. That the protein in question is, in fact, αB, was confirmed a) by the remarkable similarity of *Staphylococcus aureus* protease peptide maps of the protein in the heart and purified α-crystallin and b) by the sequence analysis of a rat heart cDNA clone identified by the αB antibody. Based on these observations we conclude that while αA has a tissue-specific role, αB is a polypeptide of independent function not restricted to the ocular lens.

Up to 90% of the soluble protein in the ocular lens is represented by crystallins, which are known to be tissue specific (1-3). α-crystallin which represents about 20-30% of the total protein, is one of the earliest proteins to appear during the development of the lens (4,5). It is a polymeric protein, thought to be composed of monomeric subunits: acidic αA (173 amino acids) and basic αB (175 amino acid). There is about a 57% similarity between the amino acid sequences of these two subunits (6). An interesting feature of α-crystallins is their extensive sequence homology to small heat shock proteins (7-9) and with the egg protein of the parasite *Schistosoma mansoni* (10). This suggests that these proteins belong to a family of stress proteins which may have specialized as structural proteins within the lens. Based on these seemingly unifying characteristics, αA and αB have been historically grouped together as the two subunits that make the structural protein, α-crystallin. There are however, very important temporal and spatial differences between αA and αB with respect to their synthesis within the developmental framework of the ocular lens. αB is predominantly a lens epithelial/cortical protein (1,11,12) while αA is synthesised only in the differentiating fibers. In the present investigation, we show that αB is present in a number of non-lenticular tissues, suggesting a functional role for this polypeptide independent of αA.

EXPERIMENTAL PROCEDURES

Protein extraction and immunoblotting: Cells in culture were incubated with ^{35}S-methionine (Trans-Label, ICN, Irvine, CA) and processed for protein extraction and immunoblotting (12,13).

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Tissues from the Sprague Dawley rats (Charles River Co., Philadelphia, PA) were homogenized (12) in presence of 0.2 mM phenyl-methyl sulfonyl fluoride, and a mixture of protease inhibitors (Sigma Chemical Co., St. Louis, MO.) The 17,000xg supernatant fraction was used for analysis. Immunodetection was done using anti-αB and anti-rabbit IgG conjugated to horseradish peroxidase (Bio-rad Inc.).

Peptide mapping: Authentic bovine α-crystallin (2 μg) or soluble protein extract of the rat heart (180 μg) and 0.4 μg of the protease (Millipore Corp., Freehold, NJ) were used for each reaction. The reaction was done in the presence of 0.1% SDS (14). The reactions were terminated at different times by adding sample loading buffer (which contained SDS and β-mercaptoethanol and the tracking dye) and by heating for 1-2 min. The reaction contents were electrophoresed on a 15% gel, and immunoblotted (15) against anti-αB.

Characterization of cDNA clones: A commecrially available (Clontech, Palo Alto, CA) λgt11 expression cDNA library prepared from the rat heart was screened according to the manufacturer's instructions, using the protoblot immunoscreening system (Promega Corp., Madison, WI), and the same αB antibody (diluted 300X), used in Figures 1, 2 and 3. Using standard methodology (16), the cloned fragment from one of the positive clones (named as λαH1) was isolated by EcoRI digestion and subcloned into a pGEM3Z vector (Promega) and sequenced using Sequenase kit (USB Corp., Cleveland, OH).

RESULTS AND DISCUSSION

We were lead to examine the extralenticular presence of αB while characterizing the expression of the human lens epithelial cell specific proteins in comparision with two other epithelial cell lines, one derived from an ocular tissue and the other from a non-ocular source (human retinal pigment epithelial cells and mouse kidney epithelial cells respectively). Polyacrylamide gel electrophoresis of the proteins synthesised in these cells revealed a definite similarity among the three patterns (unpublished), including the presence of a protein comparable in its molecular mass to αB, a polypeptide considered to be a specific marker of lens epithelial cells (1,12). The protein in question was identified by immunoblotting (Fig. 1), using a specific antibody directed against αB. The antibody was raised against the C-terminal decapeptide of αB which is conserved across the species (1). This peptide antibody reacts specifically with αB and not αA (12). The data presented here further attests to the excellent specificity of this antibody. Also, it is important to note that the C-terminal decapeptide of αB (to which the antibody was raised) falls outside of the homologous region that this protein shares with small heat shock proteins (8,9).

The data presented in Figure 1 shows the presence of an immunoreactive band of molecular mass identical to that of αB (22K) in the retinal pigment epithelial cells (Fig. 1, lane 2). Although radioactive bands of variable intensity close to that molecular mass can be seen in all the lanes (Fig. 2, right panel), there is no detectable immunoreaction in the lane 3 (Fig. 1). The radioactive band in this lane is stronger suggesting that either this band represents more than one protein or the turnover rate of presumtive αB may be different in mouse kidney epithelial cells from that in the retinal pigment epithelial cells which show a weaker radioactive band but positive immunoreactivity (Fig. 1, lane 2). We believe that the concentration of αB in mouse kidney epithelial cells may be very low (see below).

The positive data in Fig. 1 prompted us to examine the putative presence of αB in different tissues. We selected a rodent model. Figure 2 presents data obtained with some of the fetal and adult tissues of the rat. In the fetal tissues, the protein is easily detectable in the heart, skeletal muscle, and skin (Fig. 2, upper panel). Positive but weaker reactions (not clearly seen in the

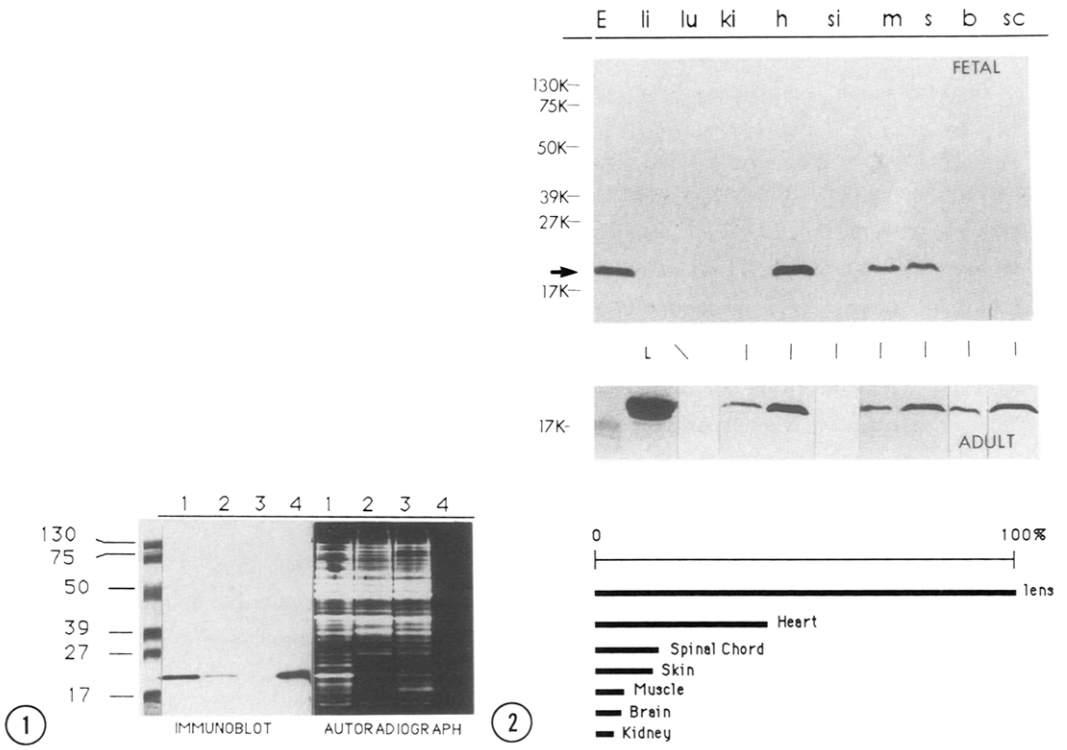

Figure 1. Immunoblot analyses of ^{35}S-labeled proteins isolated from cultured epithelial cell (left panel). On the right is the autoradiograph of this immunoblot. 1) human fetal lens epithelial cells 2) human retinal pigment epithelial cells. 3) mouse kidney epithelial cells Lane 4 contains the unlabelled human fetal lens homogenate. Prestained protein markers (Bio-rad, Richmond, CA) with the indicated molecular masses in kilodaltons (K) are on the left side. 5 to 6 μg of protein per each lane were analyzed.

Figure 2. Presence of αB in non-ocular tissues. Shown in the upper panel is an immunoblot of various fetal rat tissues (20 day gestational age) E = eye, li = liver, lu = lungs, ki = kidney, h = heart, si = small intestine, m = skeletal muscle, s = skin, b = brain, sc = spinal cord. The arrow indicates αB (22K). The middle panel shows an immunoblot done with adult tissues from the rat from whom the fetal tissues were analyzed. Only the region of interest is shown. L= lens. The first lane in this panel shows the 17K marker of the standard proteins. One hundred μg of protein were used per lane for all tissues (fetal and adult) except the fetal eye. Lane E contained 25 μg of protein. The corresponding tissues in the fetal and adult panels are indicated by short lines. Lung is not included in the analysis of the adult tissues. The numbers on the left represent molecular mass standards (K). All tissues were analyzed simultaneously in the same blot. The lower panel schematically represents the relative intensities of the immunoreactive bands as determined by the LKB ultroscan analyses (Pharmacia LKB, Piscataway, NJ) of the immunoblot shown in the middle (adult) panel. Three other determinations were also done with this preparation They fit the general pattern presented above.

reproduction in Fig.2) were detected in the lungs, brain, spinal cord, and kidneys. However , when adult tissues were examined, immunoreactive bands were clearly seen in the heart, skeletal muscle, skin, brain, spinal cord, and kidney (Fig. 2, middle panel). Interestingly, the liver and the small intestine were negative in both the fetal and adult rats, suggesting that αB may not be present in endodermally derived tissues. The bottom panel in Fig. 2 represents the relative intensities of the immuno-reactive bands obtained in different tissues of the adult rat. The relative estimate in the heart could be somewhat exaggerated considering that the parameters such as transfer efficiency of very high and very low concentrations of the proteins may not be the same.

The concentrations of the proteins used in the experiment presented in Fig. 1 are fifteen to twenty times lower than those used in the immunoblots presented in Fig. 2. This is the reason for the lack of reaction in the kidney cells (Fig. 1, lane 3).

The above data shows that with the exception of the heart, αB seems to increase significantly in concentrations from fetal to adult life in non-eye tissues where it is present. Similar results were obtained when fetal and adult human eye tissues such as the retinal pigment epithelium and neuroretina were examined (data not shown). Immunofluorescence in Muller glia cells in neuroretina has been reported in a study (17), which employed an antiserum raised against a ckicken lens protein fraction enriched in α-crystallin. In view of our findings it will be interesting to ascertain if, in fact, the antigen responsible for these results was αB. We were unable to detect αA in the tissues examined above using an antibody directed against αA (data not shown). This has also been confirmed by RNA blot analysis (Dubin and Piatigorsky, personal communication).

That the immunoreactive bands in Figures 1 and 2 were indeed due to αB is strongly suggested by the lack of non-specific reaction with other proteins (Fig. 2, upper panel) present in far greater concentrations than the putative αB. In fact, very little Coomassie blue staining can be seen in the low molecular mass (20-22 K) region of this minigel (not shown). To further corroborate the presence of αB in a non-lenticular tissue we compared a peptide map of the authentic total α-crystallin isolated from the lens with a peptide map of the protein in heart. Total protein extracts were subjected to controlled digestion with *Staphylococcus aureus* protease (14), and the peptides were detected by immunoblotting, using the same αB antibody. Only those peptides which contain within them the C-terminal decapeptide of αB will be immunoreactive. Fig. 3 shows remarkable similarities in the number and size of predominant peptides generated in the known α-crystallin sample and protein from the heart (the two predominant peptides with apparent mo-

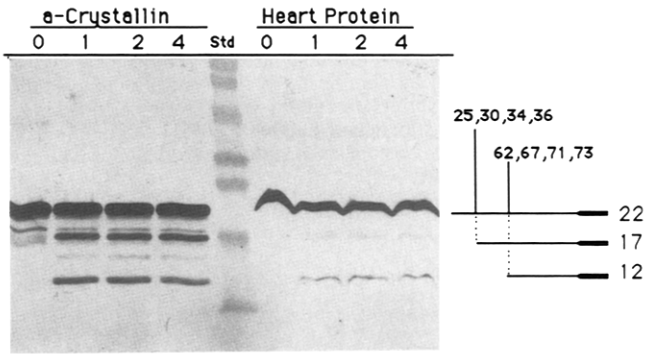

Figure 3. Comparison of peptide maps generated by *Staphylococcus aureus* protease. The predicted digestion products based on the specificity of the enzyme (28) are shown schematically on the right side of the blot by the horizontal lines. The dotted and vertical lines with numbers (amino acid residues) indicate possible cleavage points in the N-terminal half of the protein which would generate a 17K polypeptide and a 12K polypeptide containing the C-terminal decapeptide (represented by a shaded bar) against which the antiserum was raised. The middle lane contains the same standards as in Fig. 1. The numbers on the right indicate the molecular mass (K). The numbers on the top indicate time of digestion with protease in minutes.

Figure 4. Sequence (upper case letters) of an αB cDNA fragment isolated from a rat heart cDNA clone (λαH1) and its alignment with the published hamster lens αB gene sequence (lower case letters). The coding sequence was extracted from the published hamster αB gene sequence (18). The base number 435 of this extracted coding sequence represents the base number 234 of the third exon of the hamster αB gene (18). Identity is represented by vertical bars between the two sequences. Gaps are denoted by a dot between the bases. The deduced protein sequence (36 amino acid residues, indicated by single letter codes) represent part of the C-terminal region of αB and is 100% homologous with the known sequence (1,18). The thick underlines indicate the termination codon and the polyadenylation signal. Interestingly the untranslated 3'sequences also show a very high degree of similarity. The alignment was done by UWGCG programmes.

lecular masses around 17K and 12K are fainter (than the control) but clearly present in the digested total heart protein extract). This data suggests that the protein detected by the αB antibody in the heart is αB or an extremely close homologue. In order to further prove the identity of αB in heart, using this very antibody we screened a λgt11 expression cDNA library. A number of positive clones were identified. Since the selection was done by immunoscreening, the cloned DNA fragments would be expected to contain the C-terminal decapeptide against which this antiserum was raised. Analysis of these clones revealed that, in fact was the case. The DNA sequence in Fig. 4 shows the coding sequence for 36 C-terminal amino acid residues and the untranslated sequence upto the polyadenylation signal. Both the coding and the untranslated sequences show almost perfect identity with the published (18) sequence of the hamster αB (Fig. 4). This data confirms the immunoreactivity of the antibody used and proves unequivocally that the protein in question is in fact αB.

Extralenticular presence of αB draws attention to the reported presence of avian and reptile specific δ-crystallin RNA transcripts in non-lenticular tissues (19,20) and has been discussed in the light of trans-differentiation. These observations need re-evaluation in light of recently discovered sequence homologies of taxon-specific crystallins to some metabolic enzymes such as lactate dehydrogenase, argininosuccinate lyase and enolase in reptiles and birds (21-24) and the proposed recruitment of enzymes as structural proteins in the amphibian and bird lenses (21). None of these crystallins are found in mammalian lenses

The function of αB in the lens has been considered to be structural (1-3) In the light of the data presented, function(s) of αB can be only speculated. The fact that only one subunit, (αB and

not αA), of the structural protein α-crystallin is found in non-ocular tissues points to the possible dichotomy in the biological functions of these two polypeptides. This is also suggested by the reported qualitative and quantitative differences in the phosphorylation of these two subunits (25,26). Considering the sequence relationships between αA and αB and the small heat shock proteins, it is possible to concieve that after an ancestral gene duplication that gave rise to these two related subunits, αA was adapted to fit an important specific structural role in the terminally differentiated and metabolically less active lens fiber cells, while αB may have retained its functional role similar to heat shock proteins in the metabolically and developmentally active lens epithelium and other tissues. Finally, it is worthy of note that both the fetal and the adult rat hearts seemed to contain similar amounts of αB (Fig. 2). This may be related to the early functional maturation of this tissue (27).

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