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Rosenthal Fibers Contain Ubiquitinated αB-Crystallin

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The vertebrate lens protein, αB-crystallin, is a major component of Rosenthal fibers (RFs) inclusion in astrocytes in Alexander's disease. Antibodies to ubiquitin bind to RFs, but it is not known whether the ubiquitin associated with RFs is bound to a specific protein or proteins, and if so, what the identity of the conjugates is. The authors have analyzed the proteins of RFs from Alexander's disease and have found mono- and polyubiquitinated conjugates of αB-crystallin. (Am J Pathol 1991, 139:933–938)

Rosenthal fibers (RFs) are inclusions that accumulate in massive quantities within astrocytes in the brains of children with Alexander's disease, a rare CNS degenerative disorder,[1] and in some gliomas and glial scars.[2,3] RFs are composed of a granular, osmiophilic matrix intimately associated with intermediate filaments (IF).[4] Biochemical analysis of RF-enriched fractions has shown that major protein components are αB-crystallin and the IF protein, glial fibrillary acidic protein (GFAP).[5,6] Immunocytochemical observations are consistent with biochemical findings in that the RF matrix reacts with antibodies to αB-crystallin[7] and GFAP.[7–10] In addition, antibodies to ubiquitin also react with RFs.[7,11,12]

Ubiquitin is a small protein of about 8500 kDa, widely distributed among many organisms.[13] It can be conjugated enzymatically to other cellular proteins, forming isopeptide bonds between the ubiquitin carboxyterminus and ε-amino groups of lysyl residues. In some cases, this conjugation is the first step in a series of reactions that lead to the intracellular degradation of many proteins. In other cases, apparently stable ubiquitin conjugates can be formed,[14–16] suggesting that proteolysis is not the only function of ubiquitin conjugation.

Ubiquitin has been found associated with a variety of abnormal filamentous accumulations seen in pathologic states. These include the neurofibrillary tangles of Alzheimer's disease, Lewy bodies of Parkinson's disease, Pick bodies of Pick's disease, Mallory bodies seen in alcoholic cirrhosis, and RFs.[7,10–12,17] The presence of ubiquitin and of ubiquitin carboxy-terminal hydrolase in these inclusions could represent an abortive or only partially successful attempt to degrade proteins that accumulate in pathological states.[18] Sequestration of altered and ubiquitinated GFAP filaments or generation of intermediate filament bundles as part of protein sequestration in cells have also been raised as possible factors in the genesis of RFs.[19] Although ubiquitin has been presumed to be bound to protein components of these inclusions, conjugates between ubiquitin and specific proteins have not been identified. In this report, we provide evidence that RFs contain mono- and polyubiquitinated conjugates of αB-crystallin.

Materials and Methods

Antisera

A rabbit antiserum against rat cardiac muscle αB-crystallin was raised, characterized, and affinity purified as described.[6,20] A rabbit antiserum to red cell ubiquitin was raised to a ubiquitin-hemocyanin conjugate and affinity purified as described.[21]

Preparation of RFs

Frozen (−80°C) CNS tissue from a patient with Alexander's disease was extracted with a buffer that contained 50 mM Tris HCl, pH 6.8, 0.5% Triton X-100, 5 mM EDTA, and 2 mM PMSF.[5] The homogenate was centrifuged at 13,000 xg for 20 minutes at 4°C, and the supernate was separated from pellets. Residual myelin was removed by resuspending the pellets in the same buffer made 30% (w/v) in sucrose.[22] Suspensions were centrifuged at 13,000 xg for 30 minutes at 4°C, the top (myelin) material and supernates were discarded and the pellets

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were washed with extraction buffer. RFs are not soluble in Triton X-100.[5] Pellets were solubilized in 2% SDS, 50 mM Tris HCl, pH 6.8, 5 mM EDTA, and protein determination was done.[23] All proteins were dissolved; there was no residual, high-speed centrifugatable material and all protein entered the running gel during electrophoresis.

PAGE and NEPHGE

Proteins were separated by PAGE on 12% acrylamide slab gels using the Laemmli buffer system.[24] Prestained markers (BioRad, Richmond, CA) were included. For two-dimensional gel electrophoresis, proteins were first dissolved in a urea lysis buffer and separated by nonequilibrium pH gradient electrophoresis (NEPHGE,[25]) in a cylindrical gel system and then electrophoresed on a 12% slab gel; 300 μg of protein was typically loaded onto the NEPHGE gel and 100 μg were placed onto slab gels for Western blotting.

Western Blotting

Proteins were transferred from slab gels to nitrocellulose paper.[26] After blocking nonspecific protein binding with 5% nonfat dry milk in PBS, the papers were incubated with the following reagents: primary antiserum (affinity-purified rabbit anti-rat αB-crystallin, 1:500 or rabbit anti-ubiquitin, 1:100) overnight at 4°C; PBS X2; biotinylated horse anti-rabbit IgG (Vector Labs, Burlingame, CA) 1:400; PBS X2; avidin-biotin-peroxidase, 1:100; PBS X2; and the reactions visualized with diaminobenzidine.

For amino-acid sequencing, proteins were first separated by two-dimensional gel electrophoresis, transferred to PVDF paper,[27] and stained with Coomassie blue. Spots were cut out from the paper and amino-acid sequence determinations were performed on a gas-phase sequencer/PTH analyzer (Applied Biosystems 470A).

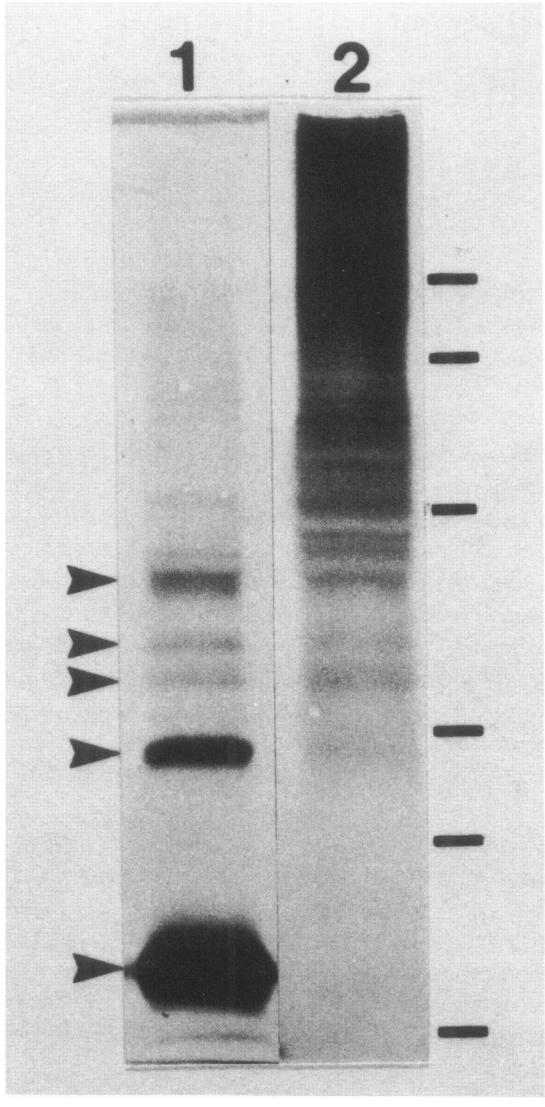

Figure 1. *Western blots of Triton-insoluble fraction from Alexander's disease CNS tissue. Proteins (100 μg/lane) were separated on a 12% acrylamide gel, transferred to nitrocellulose paper and incubated with antisera to αB-crystallin (lane 1) and ubiquitin (lane 2). αB-crystallin (lowest component in left lane, 22kDa) and larger αB-crystallin-reactive bands are marked by arrowheads. Each of the upper 4 bands corresponds to a band on the ubiquitin blot. MW markers (right) are 106,000; 80,000; 49,500; 32,500; 27,500; and 18,500.*

Results

Proteins from a RF-enriched fraction from Alexander's disease CNS were separated by PAGE, transferred to nitrocellulose paper, and examined with antisera to αB-crystallin and ubiquitin. Western blots with an affinity-purified anti-αB-crystallin antiserum showed, in addition to the major band of crystallin itself (22kDa), a series of higher MW bands (Figure 1, Lane 1). Estimations using the pre-stained MW standards gave approximate MWs of 32, 40, 43, and 51kDa, with the 32kDa component the most prominent. A number of faint, higher MW bands are also present. Western blotting of the same fraction with an anti-ubiquitin antiserum showed the same series of bands, except for that of αB-crystallin, which was not labeled with the ubiquitin antiserum (Figure 1, Lane 2).

To examine the immunoreactive components further, proteins were separated by nonequilibrium pH gradient electrophoresis (NEPHGE) followed by PAGE and transferred to nitrocellulose paper. The αB-crystallin band was separated into a series of spots, with pIs ranging from about 7.1 to 6.1 (Figure 2). Between five and seven spots were visible on each of several runs, with three major components invariably present and three to four minor ones seen in variable amounts. This pattern was similar in

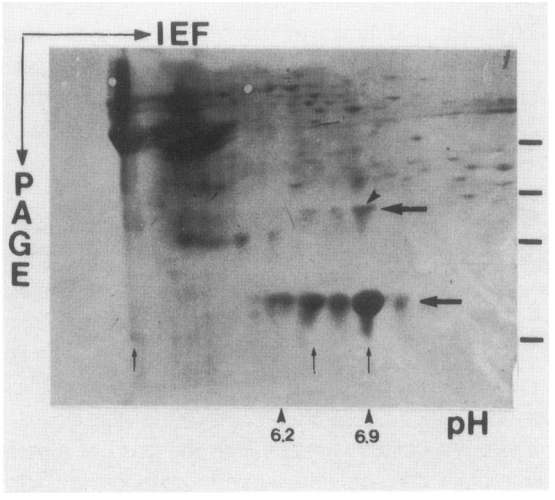

Figure 2. *Two-dimensional gel electrophoresis of a Triton-insoluble fraction from Alexander's disease CNS. Proteins (300 μg) were separated by NEPHGE and then 12% PAGE and transferred to PVDF paper and stained with Coomassie blue. Seven αB-crystallin forms are visible (lower horizontal arrow), as well as several spots directly above the αB-crystallin (upper horizontal arrow). The approximate pI's of the three major spots are 6.9, 6.7, and 6.4. Vertical arrows mark top of IEF gel (left) and two major crystallin spots (see text). The spot marked with the small arrowhead was cut out for N-terminal sequencing. MW markers (right) are 49,500; 32,500; 27,500; and 18,500.*

samples from each of several different Alexander's disease brains (Goldman and Corbin, unpublished observations). The most basic major spot (right vertical arrow, Figure 2) corresponds to the native αB-crystallin translation product, and one of the more acidic spots (middle vertical arrow, Figure 2) to a phosphorylated αB-crystallin.[28] The types of modifications of αB-crystallin that result in the other forms are not known. The higher MW components were also separated into a series of spots, some of which were situated directly above the αB-crystallin (Figure 2). To discover which of these higher MW spots corresponded to the bands seen on PAGE and 2D-gels (Figs. 1 and 2), Western blotting was performed on nitrocellulose replicas of two-dimensional gels. A series of higher MW components were visualized clearly with both the anti-αB-crystallin and the anti-ubiquitin antisera and both antisera gave reactions with the same patterns (Figure 3). The anti-ubiquitin antiserum also stained the more acidic part of the gel in a smear. To confirm that both antisera bound to the same set of proteins, we incubated a nitrocellulose paper first with the anti-ubiquitin antiserum, visualized the immunoreactive components, and then exposed the same blot to the anti-αB-crystallin antiserum (Figure 3). No additional components were visualized, except for αB-crystallin itself. The higher MW proteins reactive with the ubiquitin antiserum were distributed in the same positions relative to the crystallin bands as the spots recognized by the αB-crystallin antiserum.

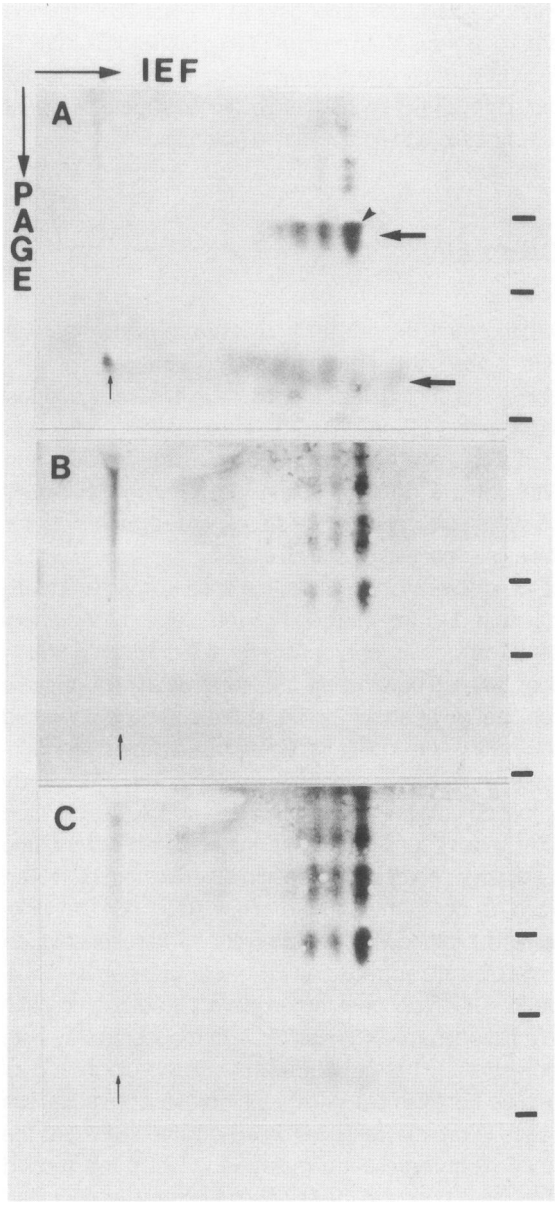

Figure 3. *Two dimensional Western blots of Triton-insoluble fraction from Alexander's disease CNS. Proteins (300 μg/gel) were separated by NEPHGE and then by 12% PAGE and transferred to nitocellulose paper.* **A**: *Reactivity with anti-αB-crystallin antiserum. The αB-crystallin and the higher MW bands are seen as several variants. Crystallin reactive spots and 32kDa reactive spots are marked with horizontal arrows and correspond in apparent MW and pI to those in Figure 2. The small arrowhead marks the position of the spot taken for sequencing (Figure 2).* **B**: *Reactivity with the ubiquitin antiserum. The same pattern of spots is observed as in* A., *except that there is no reactivity with αB-crystallin and there is a smear on the acidic side of the blot. In* (**C**, *the blot shown in* (**B**) *was restained lightly with the αB-crystallin antibody to provide internal standards. In* (**A**), (**B**), *and* (**C**), *only the part of the blots that contained immunoreactive spots is shown. Vertical arrows mark the tops of the IEF gels, acidic side to the left, as in Figure 2. MW standards are 32,500, 27,500, and 18,500.*

To confirm the presence of ubiquitin directly, the largest (most basic) of the 32kDa components resolved on the 2D gel (Figure 2) was transferred to PVDF paper for amino-acid sequencing. Only one N-terminal sequence

was detected, corresponding to the first 20 N-terminal amino acids of ubiquitin: NH_2-MQIFVKTLTGKTI-TLEVEPS-. Only one N-terminal sequence would be predicted in an αB-crystallin-ubiquitin conjugate, because αB-crystallin has an acetylated N-terminus.[29]

Discussion

Although anti-ubiquitin antibodies are known to react with RFs, it has not been determined to what protein(s) the ubiquitin is conjugated. The results in this article suggest strongly that αB-crystallin, the major protein component of RFs, is present in mono- and polyubiquitinated forms. Although we do not present direct evidence for αB-crystallin sequences in the set of ubiquitinated proteins, the affinity-purified crystallin antibody that reacted with all of the discrete, ubiquitinated proteins is specific for αB-crystallin and the migration of the higher MW components on gels is appropriate for αB-crystallin-ubiquitin conjugates. The smallest and most prominent of these conjugates, at about 32kDa, is the component we had originally seen in RF fractions that reacted with anti-crystallin antisera,[5] although we did not know its identity at that time. We infer the presence of polyubiquitinated αB-crystallin conjugates from the apparent MWs of the set of larger components seen on the Western blots. The sizes do not correspond to αB-crystallin (22kDa) polymers, but rather what one predicts if additional ubiquitin molecules are added to an αB-crystallin molecule. Thus, the 32kDa component represents a 1:1 ubiquitin:crystallin molar ratio. The 40 and 43kDa components may both represent a 2:1 ratio, but may be different ubiquitin conjugates. For example, one might represent two ubiquitins bound to two different sites on αB-crystallin, whereas the other might represent a molecule in which the second ubiquitin is bound to the first one. Alternatively, microheterogeneity in ubiquitin conjugates could be due to denaturation and then refolding of a proportion of the ubiquitin during electrophoresis, as suggested to explain heterogeneity in two-dimensional gels.[16] Similarly, the higher MW forms could represent varieties of multiubiquitinated αB-crystallins. Polyubiquitinated molecules that occur normally, such as histone H2A[16] display chains of ubiquitin attached to the conjugate at one lysyl residue. αB-crystallin contains a number of lysines, potential substrates for isopeptide bond formation with ubiquitin. A direct chemical analysis of these conjugates must be performed to clarify their exact structural nature.

Others who have tried to find discrete ubiquitin-protein conjugates in pathologic tissues have not been successful, but have only observed smears of proteins on gels or Western blots.[11,17] Several reasons contributed to our ability to visualize discrete bands. First, we began the analysis with a RF-enriched fraction. Second, in this fraction, αB-crystallin and the ubiquitin-crystallin conjugates represented major protein components. Third, crystallin and the conjugates are small proteins and migrated away from the higher MW smear (see Figure 1) that is typical of ubiquitin Western blots.

Finding ubiquitin-αB-crystallin conjugates in a pathologic inclusion raises a number of questions. One is whether αB-crystallin normally becomes ubiquitinated, possibly as an intermediate in the turnover of the protein, or whether the conjugates in RFs are only seen as part of a pathologic process. Two observations are consistent with the idea that αB-crystallin may be normally degraded by a ubiquitin system. First, the lens has a ubiquitin-protein conjugation system,[30] although the substrates for ubiquitination were not identified in that report. Second, αB-crystallin (A chain and B chain) can be degraded by a ubiquitin-dependent system *in vitro*,[31] but it is not known whether such a system operates *in vivo*. We have looked for the 32kDa αB-crystallin-ubiquitin conjugate in normal CNS tissue and in the astrocytoma cell line U373MG, which expresses αB-crystallin,[6] but have not yet visualized a band of that size. Possibly under normal conditions, too little of the ubiquitin-crystallin conjugate accumulates to be detected.

If ubiquitin conjugation plays a role in the metabolism of αB-crystallin, then it seems paradoxical that the conjugates accumulate. There could be several explanations for the accumulation. The αB-crystallin might be altered, by post-translational modifications, in such a way that it becomes a poor substrate for proteolytic enzymes. The αB-crystallin in RFs could be more stable than soluble αB-crystallin, for example, if the proteins in the matrix of the inclusions are not accessible to enzymes. Or, there could be alterations in proteolytic systems in astrocytes in Alexander's disease.

Most of the αB-crystallin in the RF fractions is not ubiquitinated, judging by the relative sizes and staining intensities of αB-crystallin versus the crystallin-ubiquitin conjugates seen on Western blots. The amount of αB-crystallin may overload the ubiquitin conjugating system in astrocytes, or some of the αB-crystallin may not be available for conjugation, as suggested by EM immunocytochemistry, which shows ubiquitin epitopes in peripheral areas of large RFs but absent from the centers.[7] In any case, these observations suggest that ubiquitination of αB-crystallin in RFs is not a primary phenomenon in the generation of RFs, but probably secondary. Firm conclusions about the role of ubiquitin in the generation of cytoskeletal inclusions are difficult to draw from autopsy tissues and require the use of dynamic systems.

AlphaB-crystallin is a self-aggregating protein[32] with an affinity for intermediate filaments,[32,33] both of which

features are probably critical for the formation of RFs. By definition, RFs and other abnormal inclusions accumulate within cells because they are not degraded efficiently. In the case of RFs, accumulation could be due to modifications of αB-crystallin or to abnormalities in the proteolytic systems of the astrocyte. Ubiquitin has been implicated in the breakdown of proteins,[13] but despite the ubiquitination of αB-crystallin in RFs, the affected astrocytes still accumulate both αB-crystallin and its ubiquitinated conjugates. Further biochemical characterization of both the ubiquitin conjugates and of the αB-crystallin itself may show reasons for this unusual accumulation.

Note Added in Proof

The 32kDa band contains both ubiquitin and αB-crystallin peptides, determined by protein cleavage and sequencing (V. Fried, unpublished observations).

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