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Phosphorylation of α-crystallin B in Alexander's disease brain*

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The phosphorylation of α-crystallin B was studied in homogenates of autopsy samples of brain tissue from patients with Alexander's disease, a condition characterized by over-expression of this protein. After incubation in the presence of [γ-^{32}P]ATP and cAMP the homogenates were analyzed by two-dimensional electrophoresis, (isoelectric focusing followed by SDS-PAGE). Three major polypeptides having the same molecular weight as bovine lens α-crystallin B and pIs 7.1, 6.9 and 6.7 were detected in the Coomassie blue stained gels. These three polypeptides were recognized by an α-crystallin B-specific antiserum in Western blots. The polypeptides with pIs 7.1 and 6.7 co-migrated in isoelectric focusing gels with bovine lens αB and its phosphorylated form αBp, respectively. Radioautography of the two-dimensional gels demonstrated the presence of ^{32}P in the most acidic polypeptide. The results demonstrate the occurrence of αB phosphorylation in Alexander's disease brain tissue.

Lens protein; Protein phosphorylation; Alpha crystallin; Alexander's disease

1. INTRODUCTION

The crystallins are a family of proteins present at very high concentrations in the eye lens where they account for 20–60% of the wet weight of the tissue. Whereas some crystallins are specialized lens proteins, other appear to be cellular enzymes which are expressed specifically at very high levels in the lens but are also present in other tissues [1,2]. α-Crystallin, a major crystallin found in the lens of all vertebrates, is a highly conserved protein related to the small heat shock proteins [3–5]. It is composed of 2 polypeptide chains, αA and αB, of approximately 20 000 Da, each of which is the product of a different gene and which share 57% amino acid homology [1,4]. Whereas αA is exclusively expressed in the lens, αB is also expressed in a variety of other tissues [6–13].

The normal functions of α-crystallin B are poorly understood. In the lens, α-crystallin B is believed to be present in large aggregates of 800 000 Da composed of both A and B polypeptides [14,15]. These aggregates, ordered and densely packed in the cytoplasm of the lens fiber cells, are thought to be responsible for the transparency and refractive power of the tisse [16–18]. In non-lenticular tissues, such as brain and cardiac muscle, αB also appears to occur in aggregates similar in size to those in the lens [19]. In cardiac myocytes, these aggregates appear to be localized specifically at the myofibrilar z-lines, where they are thought to play a structural or protective role [20].

Though little is understood about α-crystallin function, it is now clear that phosphorylation at specific serine residues accounts for the major post-translational modification of both αA and αB polypeptides in the lens in vivo [21–24]. These phosphorylations are apparently regulated by cAMP and seem to be reversible since phosphorylated αA can be dephosphorylated in lens epithelial cell extracts by a endogenous calcium/calmodulin-dependent phosphoprotein phosphatase [25].

Compared to the lens, normal non-lenticular tissues express very low levels of αB. However, under certain pathologic conditions, the expression of this protein can be enhanced. Increased αB expression occurs particularly in the brain of patients with Alexander's disease [9,26], but also in some glial tumors and scars, and in scrapie-infected hamsters [2]. Alexander's disease is a degenerative disorder primarily affecting children, and characterized by loss of (or lack of) myelin in the CNS and the presence of large numbers of inclusions known as Rosenthal fibers [27]. These inclusions, which occur in astrocytes throughout the brain, are composed of granular deposits associated with intermediate filaments. αB represents the major component of Rosenthal fibers [9,28].

Although αB phosphorylation in the lens has been studied to a certain extent, the phosphorylation of this polypeptide in non-lenticular tissues has not been examined. Since Alexander's disease brain tissue expresses high levels of αB, experiments were conducted to determine whether this protein undergoes phosphorylation in this tissue as it occurs in the normal lens.

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2. MATERIALS AND METHODS

Alexander's disease brain tissue was obtained postmortem and stored at −70°C. Approximately 50 mg of tissue was minced and then disrupted gently in a ground glass homogenizer in 0.5 ml of a buffer solution containing 20 mM MOPS, pH 7.6, 20 mM ATP, 20 mM $MgCl_2$, 20 μM cAMP and 4 mM NaF. This reaction mixture containing Alexander's disease brain homogenate, cAMP and ATP was incubated at 37°C. The reaction was started by adding 10 μl of 50 μCi/ml [γ-^{32}P]ATP to the mixture and stopped after 0 and 2 h by precipitation with 10% trichloroacetic acid at 0°C. The protein precipitates were collected by centrifugation at 12 000 × g for 15 min. The pellets were washed three times with 95% ethanol and once with anhydrous diethyl ether, dried and then analyzed by two-dimensional gel electrophoresis (2-D gels) i.e. isoelectric focusing under denaturing conditions (IEF) followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). IEF gels were performed as previously described [29] and contained 2.4% Ampholine pH 5–8, 0.6% Ampholine pH 3.5–10 (Pharmacia-LKB) and 6 M urea. Samples were applied in a solution containing 6 M urea and 0.1% 2-mercaptoethanol. For the second dimension, SDS-PAGE was performed according to the procedure described by Laemmli [30] in an 8-18% polyacrylamide gradient gel. The gels were stained with Coomassie blue, dried onto filter paper and exposed for autoradiography. Western blots were performed by transferring protein from IEF gels onto PVDF membrane (Immobilon P, Millipore). After the blocking with a 0.05% solution of Tween 20 in phosphate buffered saline (TPBS) containing 3% bovine serum albumin (BSA) for 30 min, the membrane was incubated with an anti α-crystallin antiserum at a 1:500 dilution in BSA/TPBS for 60 min. This polyclonal antiserum, #23.1, raised in rabbits against total bovine α-crystallin (0.8 × 10^6 Da aggregate) isolated from lens fiber cells is specific for α-crystallin and recognizes both the αA and the αB polypeptides of the bovine and rat lens protein [31]. The blots were visualized with horseradish peroxidase conjugated goat anti-rabbit IgG using a kit from Bio-Rad.

3. RESULTS AND DISCUSSION

Fig. 1 illustrates a Coomassie blue stained gel of Alexander's disease brain homogenate fractionated by 2-D gel electrophoresis. Three major polypeptides having the same apparent molecular mass (M_r) as bovine lens α-crystallin B (i.e., approximately 22 000) and pIs of 7.1, 6.9, and 6.7 were resolved. These 3 polypeptides were recognized in Western blots by a rabbit antiserum against total bovine α-crystallin (Fig. 2), and by a rabbit antiserum against α-crystallin B from rat cardiac muscle [11]. The polypeptides present in the Alexander's disease brain homogenate with pIs 7.1 and 6.7 co-migrated with bovine αB and its phosphorylated form, αB_p, respectively (Fig. 2). Similar Western blot analysis of normal human brain homogenates using the same antisera demonstrated the same polypeptide pattern, i.e. 3 major 22 000 Da polypeptides with pIs of 7.1, 6.9, and 6.7 (not shown). However, given the very low levels of αB in normal brain, it was necessary to increase the amount of protein loaded into the gels approximately 20-fold, and to use a more sensitive alkaline phosphatase system to detect the polypeptides.

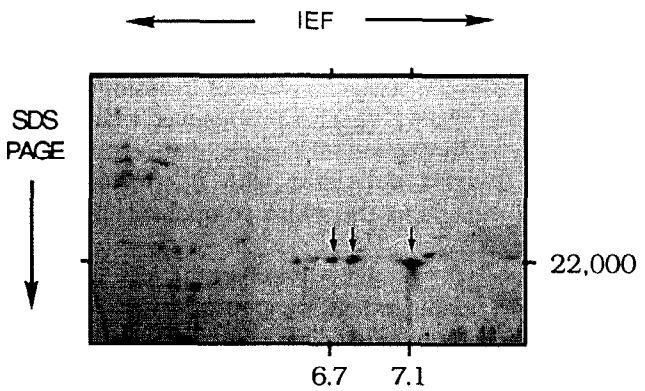

Fig. 1. Two-dimensional gel electrophoresis analysis of Alexander's disease brain homogenate. A Coomassie blue stained gel is shown demonstrating 3 major polypeptide species (arrows) with M_r of approximately 22 000 and pIs of 7.1, 6.9 and 6.7.

Coomassie blue-stained gels of a phosphorylation reaction mixture containing Alexander's disease brain homogenate, [γ-^{32}P]ATP and cAMP, incubated for 0 and 2 h, both showed the 2 species (pIs 6.9 and 6.7) more acidic than the unphosphorylated αB (pI 7.1), (Fig. 3A and B). However, autoradiography of the 2-D gels (Fig. 3C) demonstrated that after 2 h incubation only the most acidic polypeptide with a pI of 6.7 was labelled with ^{32}P. This polypeptide with pI 6.7 co-migrated with the in vivo phosphorylated bovine α-crystallin B, αB_p (Fig. 2) and corresponds to the form of αB which is phosphorylated at a single site [23–32]. There is no evidence, however, that the polypeptide with pI 6.9 is a phosphorylated form of the αB. Thie polypeptide must, therefore, be a product of another type of post-translational modification.

The finding in Alexander's disease brain tissue (and in normal human brain tissue as well) of a polypeptide with M_r and pI indistinguishable from those of lens αBp, which is recognized by an antibody specific for bovine lens α-crystallin B, indicates that the phosphorylated form of αB indeed occurs in human brain in vivo. In addition, the results of the in vitro ^{32}P incorporation experiments demonstrate the presence of αB phosphor-

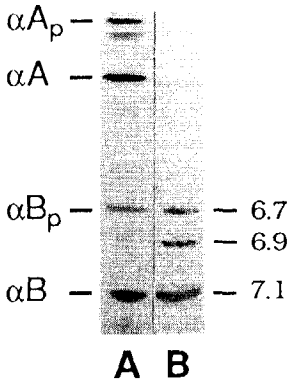

Fig. 2. Western blot analysis of the Alexander's disease brain homogenate after separation by flat bed IEF. A rabbit serum anti-bovine lens α-crystallin was used. (A) α-crystallin from bovine lens fiber cells (approximately 0.2 μg of total protein). (B) Alexander's disease brain homogenate (approximately 10 μg of total protein). Three species with pIs 7.1, 6.9 and 6.7 in the homogenate were recognized by the antiserum. The species with pIs of 7.1 and 6.7 co-migrate with bovine αB and its phosphorylated form αBp, respectively.

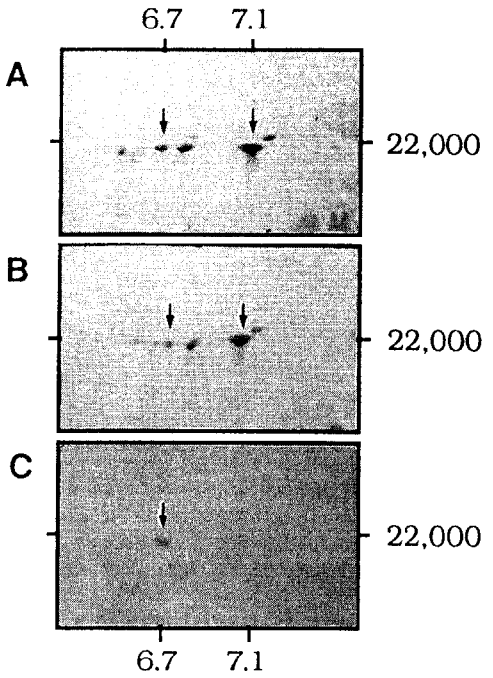

Fig. 3. Two-dimensional gel electrophoresis analysis of a reaction mixture containing Alexander's disease brain homogenate, ATP and cAMP, incubated for 0 h (A) and 2 h (B and C). The relevant areas of the Coomassie blue stained gels are shown in A and B. The autoradiogram of the gel in B is shown in C. The arrows in A and B indicate αB (pI 7.1) and its phosphorylated form αBp (pI 6.7). The arrow in panel C indicates αBp (pI 6.7). Incubation for 2 h in the presence of [γ^{32}P]ATP and cAMP results in ^{32}P-labelling of αBp, the most acidic polypeptide.

ylation pathway(s) in this tissue. A more acidic form of αB, comparable to the lens αB_p, has been observed in mouse and bovine heart extracts [32]. Thus, the phosphorylation of αB appears to be a normal feature of non-lenticular tissues, including the normal human brain.

In normal tissues, protein phosphorylation generally serves the purpose of regulating the function of proteins [33]. However, the role of α-crystallin B phosphorylation in normal tissues including the lens remains unknown. In normal rat tissues such as brain and cardiac muscle, the native αB is present in large aggregates (800 000 Da) similar in size to those of lens α-crystallin [19]. In cardiac myocytes, these aggregates appear to interact with the cytoskeletal filaments of the myofibrilar z-lines [20]. Perhaps phosphorylation modulates the normal interactions of αB polypeptides among themselves or with cytoskeletal structures in the brain tissue.

The significance of α-crystallin B phosphorylation in the pathogenesis of Alexander's disease is uncertain. Disturbance of the phosphorylation of tau proteins, a family of developmentally regulated proteins that stabilize the microtubule network, has been demonstrated in the brain of heat-shocked rats [35], and appears to be a contributing factor in the formation of neurofibrillary tangles, a characteristic neuropathological lesion of Alzheimer's disease [34]. It is then possible that abnormalities in αB phosphorylation may affect the interactions of these polypeptides with cytoskeletal structures in the brain, and in this manner contribute to the formation of the Rosenthal fibers. However, the αB polypeptide pattern observed in Western blots of Alexander's disease brain was not significantly different from that of the normal human brain. The 3 major αB polypeptides of pIs 7.1, 6.9 and 6.7 were present in both pathological and normal tissue. Therefore, qualitative abnormalities in αB phosphorylation apparently are not related to the formation of Rosenthal fibers.

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