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# The Molecular Chaperone αB-crystallin Enhances Amyloid β Neurotoxicity

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**Amyloid β (Aβ) is a 40- to 42-residue peptide that is implicated in the pathogenesis of Alzheimer's Disease (AD). As a result of conformational changes, Aβ assembles into neurotoxic fibrils deposited as 'plaques' in the diseased brain. In AD brains, the small heat shock proteins (sHsps) αB-crystallin and Hsp27 occur at increased levels and colocalize with these plaques. *In vitro,* sHsps act as molecular chaperones that recognize unfolding peptides and prevent their aggregation. The presence of sHsps in AD brains may thus reflect an attempt to prevent amyloid fibril formation and toxicity. Here we report that αB-crystallin does indeed prevent *in vitro* fibril formation of $A\beta_{1-40}$. However, rather than protecting cultured neurons against $A\beta_{1-40}$ toxicity, αB-crystallin actually increases the toxic effect. This indicates that the interaction of αB-crystallin with conformationally altering $A\beta_{1-40}$ may keep the latter in a nonfibrillar, yet highly toxic form.** 

The main histological characteristic of Alzheimer's disease (AD) is the deposition of amyloid β peptides (Aβ or A4) in extracellular plaques in the central nervous system and in the walls of cerebral blood vessels (1, 2). The Aβ peptide is derived by proteolytic processing of the amyloid precursor protein (APP), a transmembrane protein of unknown function. The biological activity of Aβ correlates with its conformational state. Monomeric $A\beta_{1-40}$ or $A\beta_{1-42}$ is in a random coil or α-helical conformation and stimulates neuronal outgrowth *in vitro* (3). A change into β-sheet conformation leads to the multi-step assembly of fibrils (4), and a concomitant toxic effect towards neurons *in vitro* (3, 5). The plaques contain besides Aβ also several other proteins, including the small heat shock proteins Hsp27 and αB-crystallin (6, 7). Hsp27 and αB-crystallin are molecular chaperones, able to prevent aggregation of other proteins (8, 9). The expression of sHsps in AD might be a defensive response to diminish amyloid fibril formation and subsequent toxicity. We therefore investigated whether αB-crystallin has chaperone activity towards Aβ and thereby protects cells against amyloid toxicity. In this article we report that αB-crystallin does prevent the fibrillization of $A\beta_{1-40}$. However, it induces αB-crystallin/Aβ complexes that are highly neurotoxic. This suggests that αB-crystallin is involved in the pathogenesis of AD by influencing the process of amyloid toxicity.

## MATERIALS AND METHODS

*Cell culture and toxicity assay.* Neurons were isolated form the cerebral cortex or hippocampus of newborn rats as described (10), plated in 96-well plates and cultured at 37°C and 5% $CO_2$ in DMEM (glucose 4.5 g/liter) (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies). After 1 week, the neuron cultures were incubated with various amounts of $A\beta_{1-40}$ (Anaspec, San Jose, CA) with or without αB-crystallin or bovine serum albumin (BSA) (Boehringer Mannheim) in 100 μl serum-free DMEM for 72h. Neurotoxicity was determined by the MTT assay (11), using the tetrazolium salt WST-1 (Boehringer Mannheim) as substrate. $A\beta_{1-40}$ was solubilized in demineralized water at a concentration of 1 mg/ml. αB-crystallin was isolated from bovine eye lenses or as a recombinant rat protein expressed in *E. coli* (12, 13). No differences were observed between the effects of bovine and recombinant rat αB-crystallin.

*Light scattering.* Aggregation was measured by right angle scattering at 405 nm using a Perkin-Elmer 650-40 spectrofluorometer as described (14). Aliquots of $A\beta_{1-40}$ (10 μg) were incubated in phosphate buffer (PB; 10 mM phosphate, 20 mM NaCl), pH 6.0, in the presence and absence of αB-crystallin for 30 minutes at room temperature.

*Thioflavin T fluorescence.* Aliquots of $A\beta_{1-40}$ were incubated in PB, pH 6.0 or 7.4, in the presence and absence of αB-crystallin or BSA as a control protein. Fluorescence assays were performed in 50 mM glycine (pH 9.0) and 1 μM thioflavin T in a final volume of 1 ml (15, 16). Thioflavin T fluorescence was measured in a Perkin-Elmer 650-40 spectrofluorometer at room temperature with excitation and emission wavelengths of 450 nm (slit 10 nm) and 482 nm (slit 5 nm), respectively.

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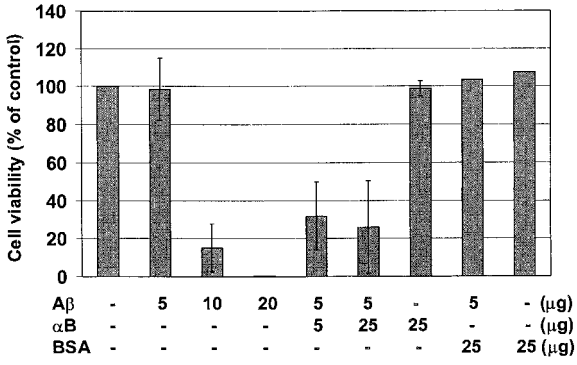


**FIG. 1.** Effect of αB-crystallin on the toxicity of $A\beta_{1-40}$ towards rat neuron cultures. Neuronal viability was assessed by measuring the cleavage of the tetrazolium salt WST-1, 72 hr after the addition of $A\beta_{1-40}$, αB-crystallin and/or BSA to the neuron cultures. Data presented are means of four independent experiments (± SD).

*Electron microscopy.* Protein sample solutions for electron microscopy were spread on carbon-coated grids, stained with 2% uranyl-succinate for 2 minutes, and rinsed with water. Specimens were examined with a JEOL 1210 electron microscope with an acceleration voltage of 80 kV.

## RESULTS AND DISCUSSION

To test the assumption that sHsps constitute a defensive response to diminish amyloid fibril formation and subsequent toxicity in AD, neuron cultures were isolated from cerebral cortex or hippocampus of newborn rats, and exposed to $A\beta_{1-40}$ in the absence and presence of αB-crystallin. $A\beta_{1-40}$ neurotoxicity appeared to be dose-dependent, being undetectable at 5 μg/100 μl, and increasing to 100% at 20 μg/100 μl (Fig. 1). When neurons were exposed to 10 or 20 μg $A\beta_{1-40}$ in the presence of 5 μg αB-crystallin, no significantly diminishing effect on Aβ toxicity was observed (data not shown). Surprisingly however, when neurons were exposed to 5 μg $A\beta_{1-40}$ in the presence of 5 μg αB-crystallin (molar ratio ± 5:1), neuronal viability became severely decreased in comparison with a treatment with $A\beta_{1-40}$ alone (Fig. 1). The neurotoxicity is probably mediated via apoptosis, as αB-crystallin increased the Aβ-induced activation of caspase-3 (data not shown). Additional αB-crystallin (25 μg; molar ratio ± 1:1) did not further increase neurotoxicity. The

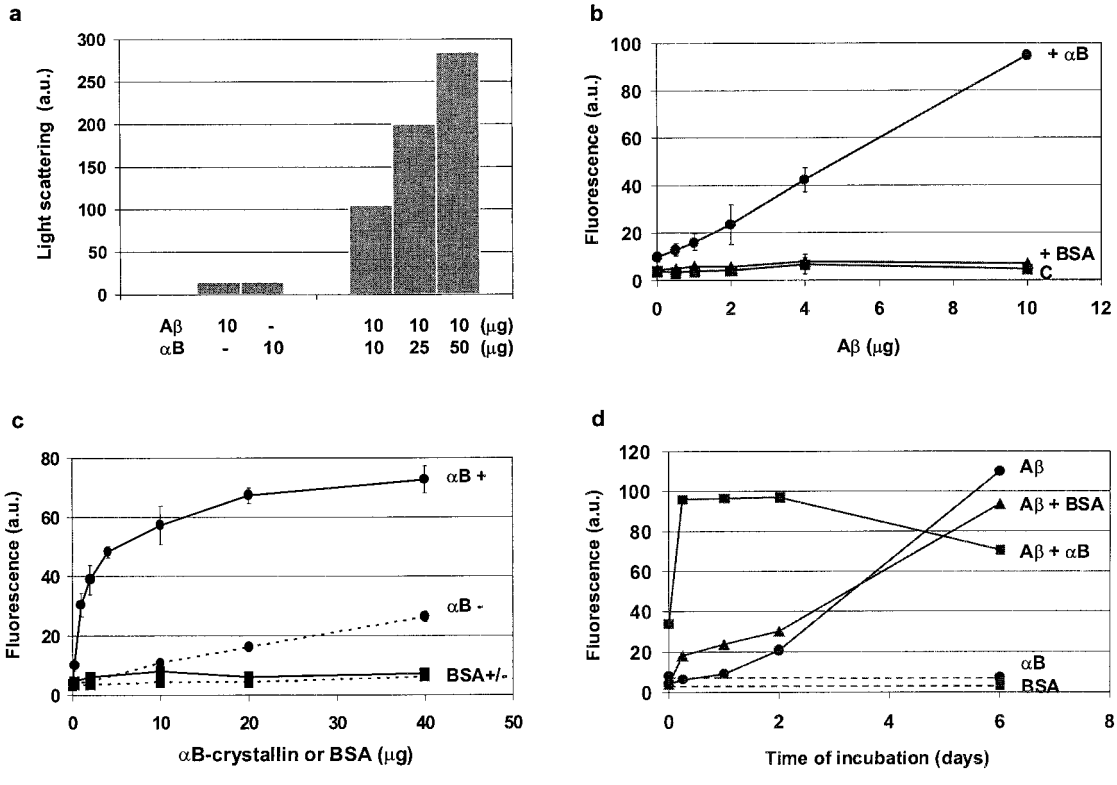


**FIG. 2.** Effect of αB-crystallin on $A\beta_{1-40}$ aggregation as measured by right angle light scattering (a), and on β-sheet formation of $A\beta_{1-40}$ as measured by thioflavin T fluorescence (b–d). (a) Induction of $A\beta_{1-40}$ aggregation by αB-crystallin in phosphate buffer (PB), pH 6.0, at room temperature. (b) Increasing amounts of $A\beta_{1-40}$ were incubated for 30 minutes without (■; C) or with αB-crystallin (10 μg) (●; + αB) or with BSA (10 μg) (▲; + BSA) in 40 μl PB, pH 6.0 at room temperature. Data presented are means of four independent experiments (± SD). (c) Increasing amounts of αB-crystallin (●) or BSA (■) were incubated with (—) or without (···) $A\beta_{1-40}$ (4 μg) in 40 μl PB, pH 6.0, for 30 minutes at room temperature. Data presented are means of four independent experiments (± SD). (d) $A\beta_{1-40}$ (10 μg) was incubated for up to 6 days in the absence (●) or presence of αB-crystallin (10 μg) (■) or BSA (10 μg) (▲) in 100 μl PB, pH 7.4 at 37°C. Incubations of αB-crystallin (10 μg) and BSA (10 μg) alone are indicated by the dashed lines.

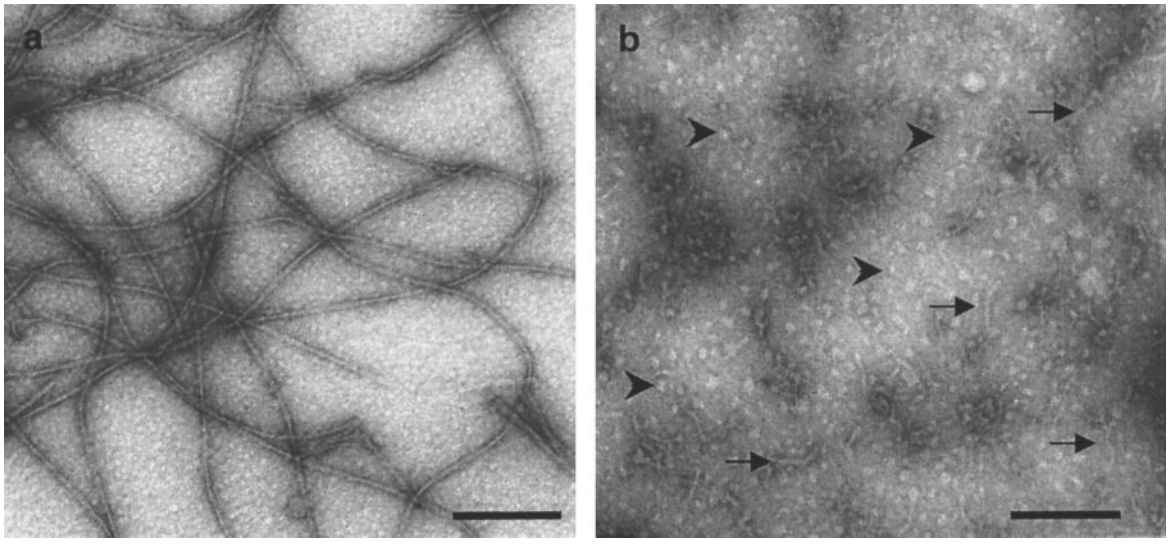


**FIG. 3.** Effect of αB-crystallin on amyloid fibril formation by $A\beta_{1-40}$ as observed by electron microscopy. $A\beta_{1-40}$ (10 μg) was incubated for 5 days in the absence (a) or presence (b) of bovine αB-crystallin (10 μg) in 100 μl PB, pH 7.4 at 37°C. Arrows, protofiber structures; arrowheads, αB-crystallin complexes. Scale bar is 200 nm.

presence of a control protein, bovine serum albumin (BSA), did not affect $A\beta_{1-40}$ toxicity, nor did αB-crystallin or BSA alone influence cell viability (Fig. 1). Thus, the extracellular presence of αB-crystallin did not protect neurons from $A\beta_{1-40}$ toxicity; rather, it dramatically increased the toxicity of $A\beta_{1-40}$ *in vitro*.

This result resembles the observation that proteins such as α1-antichymotrypsin and acetylcholinesterase enhance the toxicity of Aβ (17, 18). These proteins also stimulate the fibril formation of Aβ *in vitro* (17, 19). To investigate whether αB-crystallin similarly enhances Aβ fibril formation, we incubated $A\beta_{1-40}$ with increasing amounts of αB-crystallin at pH 6.0, where fibrillogenesis is optimal (20,21). Fibril formation was measured after 30 minutes by right angle scattering at 405 nm (14). As obvious from Fig. 2a, addition of αB-crystallin to $A\beta_{1-40}$ resulted in a significant and dose-dependent increase in light scattering, indicating that αB-crystallin stimulated protein aggregation. The aggregates, collected by centrifugation and analyzed by SDS-PAGE, contained both $A\beta_{1-40}$ and αB-crystallin (data not shown).

To examine whether these aggregates indeed consisted of amyloid fibrils, the thioflavin T fluorescence assay was used (15, 16). Thioflavin binds specifically to β-pleated sheet structures, as present in the amyloid fibril, resulting in an enhanced fluorescence at emission wavelength of 485 nm that is directly proportional to the amount of amyloid fibrils formed (16). Increasing amounts of $A\beta_{1-40}$, incubated at room temperature for 30 min at pH 6.0, gave almost no fluorescence during this time period (Fig. 2b). Addition of the control protein BSA did not affect the fluorescence intensity. However, in the presence of αB-crystallin a clear increase in the fluorescence intensity was observed, indicating that β-pleated sheet structures had formed. The fluorescence increased proportionally with the amount of $A\beta_{1-40}$ used, indicating that $A\beta_{1-40}$ was the limiting component, at least up to a 5-fold molar excess over αB-crystallin. To determine the effect of the αB-crystallin ratio on the increased fluorescence of $A\beta_{1-40}$, we performed similar experiments with 4 μg of $A\beta_{1-40}$ and increasing amounts of αB-crystallin or BSA (Fig. 2c). Addition of up to 40 μg of BSA had no effect, whereas 1 μg of αB-crystallin already highly increased the fluorescence intensity, which corresponds with a 20-fold molar excess of Aβ over αB-crystallin. The increase in fluorescence with increasing amounts of αB-crystallin above 10 μg was similar to the increase in fluorescence of αB-crystallin alone, which is most likely caused by the binding of thioflavin T to the β-pleated sheets present in sHsps (22).

Next we tested whether the stimulation of the conformational change of $A\beta_{1-40}$ into β-pleated sheets by αB-crystallin can also be detected at 37°C and pH 7.4, which is more comparable to the conditions used in the *in vitro* toxicity assay. The reaction at pH 7.4 is much slower than at pH 6.0, and therefore incubations were performed for up to six days. Addition of BSA had, again, little effect on thioflavin T fluorescence of $A\beta_{1-40}$ (Fig. 2d). Addition of αB-crystallin already resulted in an increase in thioflavin T fluorescence after 5 hrs of incubation, showing that αB-crystallin greatly accelerated the β-sheet formation of $A\beta_{1-40}$. Under these conditions the fluorescence of αB-crystallin or BSA itself did not change during the time of incubation (Fig. 2d).

It thus appears that the αB-crystallin-induced increase of $A\beta_{1-40}$ toxicity is associated with aggregate

formation and enhanced β-pleated sheet conformation of $A\beta_{1-40}$. The size and shape of the aggregates of $A\beta_{1-40}$ and αB-crystallin were analyzed by electron microscopy. Interestingly, this revealed that the effect of αB-crystallin on Aβ fibril formation was different from that of apolipoprotein E, acetylcholinesterase and α1-antichymotrypsin (17,18). Incubation of $A\beta_{1-40}$ for one day at 37°C (pH 7.4) did not result in amyloid fibrils (data not shown), as was also indicated by the thioflavin fluorescence (Fig. 2d). After five days, $A\beta_{1-40}$ had formed fibrils displaying typical amyloid morphology (Fig. 3a) (cf. e.g. 23). In the presence of αB-crystallin, that itself exists as 8–18 nm hollow spherical complexes (24), no fibers could be detected (Fig. 3b). Instead, shorter and less regular structures (up to ±100 nm in length) were observed, indicating that $A\beta_{1-40}$ fibrillization was prevented by αB-crystallin. Therefore, the effect of αB-crystallin on Aβ toxicity was, unlike that of acetylcholinesterase and α1-antichymotrypsin, not based on stimulation of $A\beta_{1-40}$ fibril formation.

From our results we conclude that αB-crystallin acts as a molecular chaperone by preventing proper fibril formation of $A\beta_{1-40}$. However, by doing so, it induces the formation of non-fibrillar, β-sheet-rich Aβ/αB-crystallin aggregates that are highly toxic to cells *in vitro*. This means that $A\beta_{1-40}$ toxicity can not solely be ascribed to its fibrillar form, but other aggregates or small (soluble) $A\beta_{1-40}$ complexes must be toxic as well. Recently, it has indeed been reported that small soluble oligomeric species of Aβ are neurotoxic *in vitro* (25, 26). These approximately 200-nm oligomeric structures, also called protofibrils or protofilaments, are formed as intermediates during fibrillogenesis of Aβ (27,28). αB-crystallin may associate with these conformationally altering structures, thereby stabilizing toxic Aβ. It may well be that other sHsps share this feature with αB-crystallin since mouse Hsp25—the orthologue of human Hsp27—also increased the thioflavin T fluorescence of $A\beta_{1-40}$ (data not shown). This seems at variance with the report that Hsp25 and Hsp27, but not eye lens α-crystallin (composed of αA- and αB-crystallin), inhibit *in vitro* the initiation of $A\beta_{1-42}$ fibril formation as determined by thioflavin T fluorescence (29). Also the finding that the electron microscopic appearance of $A\beta_{1-42}$ fibrils formed in the presence or absence of Hsp27 was similar (29), differs from the present results with αB-crystallin (Fig. 3). However, considering the extreme complexity of amyloidogenesis and sensitivity for experimental variables (4), these disparate results, though difficult to reconcile at present, may not be too surprising.

The present data suggest that αB-crystallin and possibly Hsp27 as well may be involved in the etiology of AD by stimulating the formation or stabilization of toxic Aβ species. Since it is likely that this interaction occurs extracellularly, it is important to notice that the normally cytoplasmic sHsps have been found extracellularly (30, 31), expressed on the cell surface (32), and colocalizing with the core of plaques in AD brain (7). These considerations may have implications for the development of therapeutic strategies, which currently are often aimed at prevention of fibril formation of Aβ (33, 34). If αB-crystallin indeed can stabilize non-fibrillar neurotoxic species of Aβ in the brain, the prevention of fibril formation may enlarge the pool of smaller toxic species, thereby actually enhancing neuronal damage.

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