αB-Crystallin-coated MAP microtubule resists nocodazole and calcium-induced disassembly

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Summary

αB-Crystallin, one of the small heat-shock proteins, is constitutively expressed in various tissues including the lens of the eye. It has been suggested that αB-crystallin provides lens transparency but its function in nonlenticular tissues is unknown. It has been reported that αB-crystallin is involved in the stabilization and the regulation of cytoskeleton, such as intermediate filaments and actin. In this study, we investigate the possibility whether αB-crystallin interacts with the third cytoskeleton component, microtubules (MTs). First, we precisely observed the cellular localization of αB-crystallin and MT networks in L6E9 myoblast cells and found a striking coincidence between them. MTs reconstituted from cell lysate contained αB-crystallin. Electron micrographs clearly showed direct interactions of purified αB-crystallin with the surface of microtubule-associated proteins (MAPs) attached to MTs. Purified αB-crystallin bound to MAP-MTs in a concentration-dependent manner. However, αB-crystallin did not bind MTs reconstituted from purified tubulin. Finally, we observed that αB-crystallin increased the resistance of MTs to depolymerization in cells and in vitro. Taken together, these results suggest that one of the functions of αB-crystallin is to bind MTs via MAP(s) and to give the MTs resistance to disassembly.

Key words: Tubulin, sHSP, Cytoskeleton, Chaperone

Introduction

αB-Crystallin, one of the small heat-shock proteins (sHSPs), is constitutively expressed in heart, skeletal muscle, kidney and brain (Dubin et al., 1989), as well as in lens. It has an approximate molecular mass of 22 kDa and exists as a large, oligomeric complex of approximately 200-800 kDa in the native state (Bloemendal, 1977). The complex is composed of a globular oligomer, and denatured proteins bind to the molecular surface (Haley et al., 1998) and the central region of the complex (Boyle and Takemoto, 1994). The structure of αB-crystallin is thought to have three domains – the N-terminal hydrophobic domain, the conserved C-terminal 'α-crystallin domain' and an exposed, flexible C-terminal extension (Quax-Jeuken et al., 1985).

In lens, αB-crystallin prevents other lens crystallins from ultraviolet-induced aggregation (Lee et al., 1998), suggesting that αB-crystallin might provide lens transparency. However, the function of αB-crystallin in nonlenticular tissues is unknown. There are some tissues in which the expression level of αB-crystallin is higher in unstressed conditions than those of heat-shocked NIH3T3 cells (Klemenz et al., 1993), so αB-crystallin might have a special role in these tissues. It has been reported that αB-crystallin is involved in the stabilization and the regulation of cytoskeletal proteins, such as actin in C6 glioma cells (Iwaki et al., 1994) and in vitro (Wang and Spector, 1996), vimentin in NIH3T3 fibroblast cells and lens tissue (Djabali et al., 1997; Nicholl and Quinlan, 1994), glial fiber acidic protein in lens and U373MG astrocytoma cells (Nicholl and Quinlan, 1994; Perng et al., 1999), desmin in skeletal muscle (Vicart et al., 1998), and tubulin in L6E9 myoblast cells and in vitro (Arai and Atomi, 1997). Previously, we have identified αB-crystallin as a protein that specifically decreased in atrophied soleus (slow tonic muscle) (Atomi et al., 1991a). It localized around Z-bands (Atomi et al., 1991b), which consist of cytoskeletal proteins receiving tension against muscle contraction (Danowski et al., 1992).

The three cytoskeleton components, fibrous actin, microtubules (MTs) and intermediate filaments (IFs), are basically independently regulated, but affect each other with different tensile and dynamic properties via interacting proteins. Co-localization of IF and MT networks has been reported in various cells (Gyoeva and Gelfand, 1991). In fact, some specific proteins have been identified that connect actin and MTs (Karakesisoglou et al., 2000; Leung et al., 1999), or MTs and IFs (Liao and Gundersen, 1998). It has been suggested that MT depolymerization is induced by actomyosin contraction of stress fibers in cultured cells (Waterman-Storer and Salmon, 1997). This might happen in a similar way to tonic contraction in heart and slow muscle.

MTs have various functions, such as keeping the shape of cell and supplying tracts for translocation of various molecules and organelles in cells, including subunits of the tubulin dimer itself (Terada et al., 2000). MTs have intrinsic dynamic properties, called dynamic instability (Kirschner, 1980; Mitchison and Kirschner, 1984), and de novo tubulin synthesis is autoregulated by free tubulin dimer concentrations (Pachter et al., 1987; Yen et al., 1988). Unassembled tubulin denatures relatively easily and the denatured tubulin can even inhibit the

assembly itself (Maccioni, 1983). αB-Crystallin can suppress the aggregation and precipitation of denatured tubulin in vitro (Arai and Atomi, 1997). Interestingly, the levels of both mRNA and protein of αB-crystallin increased upon treatment with MT-destabilizing drugs such as colchicine, colcemid, vinblastine and nocodazole, whereas they decreased with the MT-stabilizing drug Taxol (Kato et al., 1996). These results suggest that increased free tubulin dimers might be a substrate of αB-crystallin. Tailless complex polypeptide 1 (TCP1) ring complex (TRiC) is a chaperone system for newly synthesized actin and tubulin (Yaffe et al., 1992). However, the cytoskeleton might need another chaperone system for refolding denatured monomers, helping to degrade them and to maintain their intrinsic dynamic properties (Quinlan, 2002). This last possibility might lead to a specific interaction of αB-crystallin with proteasome machinery (Boelens et al., 2001).

In this study, we aimed to investigate the possibility whether αB-crystallin interacts with MT. We demonstrate biochemical and cytological associations of αB-crystallin with MTs mediated by MT-associated protein(s) [MAP(s)], and this interaction gives MTs resistance to disassembly, both in cells and in vitro. This is the first demonstration of the colocalization of αB-crystallin with MTs in unstressed cells. These results demonstrate how to study the function of sHSPs in nonlenticular tissues that express sHSPs constitutively.

Materials and Methods

Materials

Bovine serum albumin (BSA), EGTA, PIPES, GTP and anti-α-tubulin monoclonal antibodies were purchased from Sigma Chemical (St Louis, MO). Fetal bovine serum (FBS) was purchased from Dainippon Pharmaceutical (Osaka, Japan). Porcine brain and bovine lens were from a local slaughterhouse. All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). All reagents were of the highest grade.

Preparation of antibodies

Antibodies C1 and N1 were raised against the C-terminal [(SH)KPAVTAAPKK] and N-terminal [Ac-MDIAIHHPWIC(SH)] peptides of rat αB-crystallin (provided by S. Aimoto, Osaka University, Japan) and conjugated to BSA with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Arai and Atomi, 1997). The immunoglobulins were purified by C- or N-terminal peptide-conjugated affinity chromatography using an MBS linker (Pierce) and EAH Sepharose-4B (Pharmacia Biotech, Shinagawa, Japan).

Cell culture

L6E9 rat myoblast cells (generous gift from T. Endo, Chiba University, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. C6 normal, αB-crystallin sense cDNA transformant (C6SE) and antisense cDNA transformant (C6AS) rat glioma cells (Iwaki et al., 1994), a generous gift from A. Iwaki (Kyushu University, Japan), were cultured in F10 medium supplemented with 10% FBS. For microscopic assays, the cells were cultured on glass cover slips in culture dishes.

Protein purification

αB-Crystallin was purified from bovine lens using ion-exchange chromatography in the presence of urea, as previously described (de Jong et al., 1976). Tubulin was purified by the method of Shelanski et al. (Shelanski et al., 1973), with slight modifications. Porcine brain was homogenized with an equal volume of PME (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8). The polymerization-depolymerization cycle was repeated twice. At this step, the obtained material (MT proteins; MTP) contained MAPs. To eliminate MAPs from the MTP, further purification was performed using phosphocellulose column chromatography (Weingarten et al., 1975). Protein concentration was determined with Bradford Protein Assay kit (Bio-Rad, Hercules, CA) using BSA as the standard.

SDS-PAGE and immunoblotting

Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as previously described (Arai and Atomi, 1997; Atomi et al., 1991b). The C1 and N1 antibodies were generally used at 1:5000 and 1:1000 dilutions, respectively. Immunoblots were visualized by the use of an Enhanced Chemiluminescence (ECL) kit (Amersham International, Amersham, UK). Developed immunoblots and Coomassie Brilliant Blue (CBB) stained gel bands were scanned into a computer using a CCD camera (ATTO Corporation, Tokyo, Japan). Obtained band intensity was quantified using NIH Image.

Immunofluorescence study

Cells were briefly washed with warmed PBS at 37°C and fixed either by incubation at room temperature in pre-warmed (at 37°C) FME (4% formaldehyde, 2 mM $MgCl_2$ and 5 mM EGTA in PBS) or by dipping in methanol at –20°C for 10 minutes. In the case of FME fixation, cells were washed several times with PBS and permeabilized with FME containing 0.3% Triton X-100 (FMET) for 10 minutes at room temperature. Both FMET- and methanol-fixed cells were washed several times with PBS and kept in PBS containing 1% BSA and 0.02% sodium azide. Cells were immunohistochemically stained with anti-α-tubulin monoclonal antibodies. Subsequently, cells were stained with rhodamine-conjugated anti-mouse IgG as a secondary antibody. Subsequently, cells were stained with C1 antibody as a primary antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG as a secondary antibody. All photographs, including color images of double-labeled specimens, were taken using an LSM410 confocal microscope (Carl Zeiss, Oberkochen, Germany). Adobe Photoshop 7.0 was used for pseudocoloration and merging of the images.

MT assembly

The isolation of MTs from the cell lysate was carried out as previously described (Vallee, 1982). Briefly, L6E9 cells were homogenized with PME buffer at 4°C, followed by centrifugation at 30,000 ***g*** for 15 minutes and 100,000 ***g*** for 60 minutes at 4°C. Taxol (20 μM) and GTP (1 mM) were added to the supernatants. After incubation for 15 minutes at 37°C, the supernatant was centrifuged at 37°C for 15 minutes to pellet MTs. Fractions were analysed by immunoblotting with C1 antibody.

Co-precipitation assay

MTP (1 mg ml^{-1}) was polymerized at 37°C for 10 minutes in PME containing 1 mM GTP and incubated with 10 μM Taxol for another 5 minutes. Phosphocellulose column purified tubulin (PCT) (10 μM) was polymerized in the same manner except that 10% DMSO was used to initiate polymerization. Then, polymerized MTs were added to various concentrations of αB-crystallin and allowed to incubate for another 15 minutes at 37°C. The solution was overlaid onto 10% sucrose in PME and centrifuged at 30,000 ***g*** for 15 minutes at 37°C. The precipitate was subjected to SDS-PAGE. Controls containing MTs or αB-crystallin alone were centrifuged under the same conditions.

Electron microscopy

MAP-MTs polymerized with or without αB-crystallin as described above were incubated and then diluted 20-fold with phosphate buffer (5 mM sodium phosphate, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8) containing Taxol (10 μM) and GTP (1 mM). Those samples were placed on uncoated copper grids and stained in 1% uranyl acetate containing bacitracin (Katayama, 1989). Samples were observed with a JEOL 2000ES electron microscope at an accelerating voltage of 80 keV.

Effects of nocodazole on MTs remaining in cells

C6 normal, C6SE and C6AS glioma cells were used in these experiments. In order to examine the suppressive effects of αB-crystallin on nocodazole-induced MT depolymerization, C6 normal, C6SE and C6AS cells were treated with nocodazole (33 μM) at various times. At a given time, the cells were processed by the extraction of free tubulin with MT-stabilizing buffer (MSB) (0.1 M PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 2 M glycerol) supplemented with 0.5% Triton X-100 for 1 minute at 37°C. After that, the cells were fixed with FMET for immunofluorescent study or otherwise analysed biochemically as described below. MTs remaining with the experimental cells were compared by means of immunostaining using anti-α-tubulin antibody as described below.

Dimer and polymer fractions of tubulin were prepared from nocodazole (33 μM) treated cells, according to a previous report (Gundersen et al., 1987) with slight modifications. C6 normal and C6AS cells on plastic Petri dishes were rapidly rinsed twice in MSB containing 4 μg ml^{-1} aprotinin and 0.2 mM phenylmethylsulfonyl fluoride, then extracted in MSB containing 0.5% (vol/vol) Triton X-100 for 2 minutes. The Triton X-100 extract (dimer fraction) was gently transferred to a tube and the volume was measured. The cytoskeleton polymers in those cells remaining on the plate were carefully rinsed once with MSB, solubilized into SDS sample buffer and then the volume was measured. All procedures were performed at 37°C. Samples stored at –80°C were analysed by immunoblotting using anti-α-tubulin antibody. Obtained band intensity was corrected by measured volume.

Effects of αB-crystallin on depolymerization of MTs in vitro

Assembly of MTP, with or without αB-crystallin, in PME containing 1 mM GTP at 37°C was monitored by recording turbidity at 350 nm, using Beckman DU-65 spectrophotometer. After a 15 minute polymerization, 10 μM podophyllotoxin or $CaCl_2$ (113 μM or 1 mM) was added. The final concentration of free calcium ions was calculated using an apparent binding constant of EGTA for calcium. The amount of the remaining MTs was estimated by turbidity and calculated as the percentage of the mean value of the last 1 minute to that of 14-15 minutes.

Results

Association of αB-crystallin with MT in vivo

To observe cellular localization of αB-crystallin precisely, specific antibodies were produced. Polyclonal antibodies (C1 and N1) against the C- and N-terminal 10 amino acid peptides of rat αB-crystallin specifically reacted to the 22 kDa protein (αB-crystallin) of rat skeletal muscle (Fig. 1A). The C1 antibody was mainly used for immunofluorescence studies because of its clear labeling of distinct localization patterns of αB-crystallin associated with cytoskeletal networks. C1 antibody preadsorbed to the C-terminal peptide did not react with αB-crystallin in L6E9 cells (Fig. 1B).

We examined the co-localization of αB-crystallin and MT networks in widely extended L6E9 myoblast cells during interphase. After FMET fixation, cells were stained using anti-α-tubulin antibody for tubulin and MT (Fig. 2A, middle) and anti-αB-crystallin C1 antibody for αB-crystallin (Fig. 2A, left). Intriguingly, from the merged image (Fig. 2A, right), αB-crystallin and MTs appeared to co-localize in L6E9 cells, even in fine filaments (Fig. 2A, inset). However, it seemed that αB-crystallin and free tubulin remained slightly in FME fixed cells. To eliminate these proteins completely, a methanol fixation method was used that is known to extract cytoplasmic proteins more appropriately. After methanol fixation, cells were stained in the same manner (Fig. 2B). The merged image clearly showed that αB-crystallin co-localized with the MT network with complete agreement, even on fine filaments (Fig. 2B, inset). The pretreatment of cells with Taxol resulted in a complete consistent merged network (data not shown). Even pre-extraction of the Taxol-treated cells with Triton X-100 did not abolish the association of αB-crystallin with MTs (data not shown). This is the first demonstration of the colocalization of αB-crystallin with MTs in unstressed cells.

The biochemical association of αB-crystallin with the MTs was examined by the employment of a Taxol-dependent MT

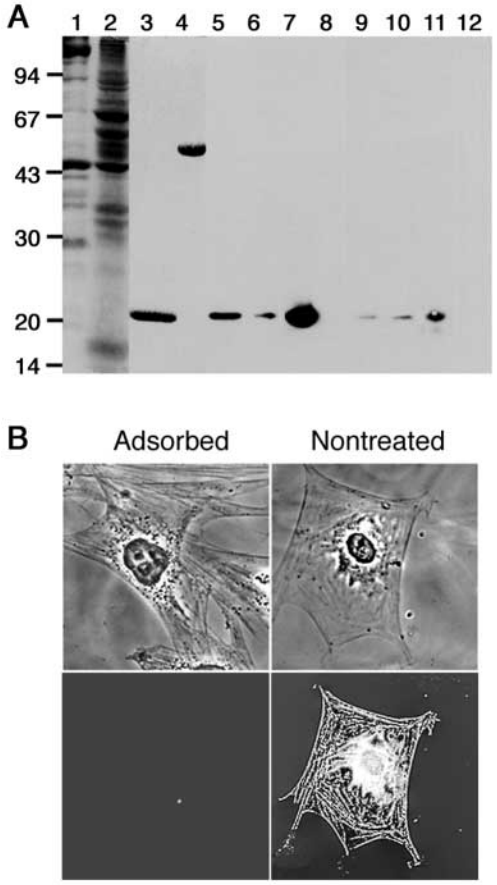

Fig. 1. Characterization of anti-αB-crystallin C-terminal (C1) and N-terminal (N1) peptide antibodies. (A) Coomassie-blue-stained gels of rat soleus muscle homogenate (lane 1), total L6E9 cell lysate (lane 2), purified αB-crystallin (lane 3) and tubulin (lane 4), and immunoblots with C1 antibody (lanes 5-8; corresponding to lanes 1-4) and N1 antibody (lanes 9-12; corresponding to lanes 1-4). (B) C1 antibody recognizes αB-crystallin in L6E9 cells (bottom right) as confirmed by a loss of reaction it after preadsorption of the antibody with the C-terminal peptide (bottom left). Scale bar, 25 μm.

assembly procedure (Vallee, 1982). Taxol and GTP were added to L6E9 cell lysates and the MTs were pelleted by centrifugation (Fig. 2CI, lane 3). Without Taxol, MT did not polymerize and αB-crystallin was not precipitated in those fractions (data not shown). Immunoblotting using anti-αB-crystallin C1 antibody indicated the presence of αB-crystallin in the MT fraction (Fig. 2CII, lane 3). Together with the immunofluorescence study, it is clear that αB-crystallin is associated with MTs in L6E9 cells.

Association of αB-crystallin with MAP-MTs but hardly with PCT-MTs in vitro

To investigate this interaction more precisely, we employed electron microscopic observation using purified bovine αB-crystallin and MTs reconstituted from porcine MTP. After mixing, αB-crystallin and MTP polymerized at the ratio of 4:1 calculated as αB-crystallin monomer and tubulin dimer, respectively. The electron micrograph clearly showed αB-crystallin-bound MAPs-MTs (Fig. 3). However, αB-crystallin hardly bound to PCT-MTs (data not shown). αB-Crystallin exists as assembled oligomers (Haley et al., 1998; Nicholl and Quinlan, 1994) (Fig. 3A, arrowheads), which binds to MAP-MTs. αB-crystallin with a 12 nm diameter bound to 8-10 nm spaced MAP-MTs (Fig. 3B, top).

To investigate this interaction more stoichiometrically, we added various concentrations (2.5-80 μM) of αB-crystallin to Taxol-stabilized MTP-MTs and incubated them at 37°C for 15 minutes. Reaction mixtures were centrifuged and the pellets were electrophoresed and CBB stained. αB-Crystallin precipitated with the MTs (Fig. 4A, lanes 2-7) in a dose-dependent manner. Next, we investigated whether this interaction of αB-crystallin with MTs was direct to the tubulin component of MTs. We reconstituted MTs from PCT which eliminated MAPs from MTP. Various concentrations (0.5-80 μM) of αB-crystallin were added to PCT-MTs and incubated at 37°C for 15 minutes. Reaction mixtures were centrifuged, the pellets electrophoresed and the gel CBB stained (Fig. 4B, lanes 2-6). Under the same buffer conditions containing αB-crystallin alone, only the small amount was precipitated (data not shown). The band intensity of αB-crystallin alone was subtracted from that of αB-crystallin with MAP-MTs or PCT-MTs. The amount of bound αB-crystallin was calculated using purified αB-crystallin as standards and plotted on a graph (Fig. 4C). The correlation coefficient with natural logarithmic curve fitting was 0.995 for MTP-MTs and 0.091 for PCT-MTs. We concluded that αB-crystallin did not bind directly to tubulin in MTs.

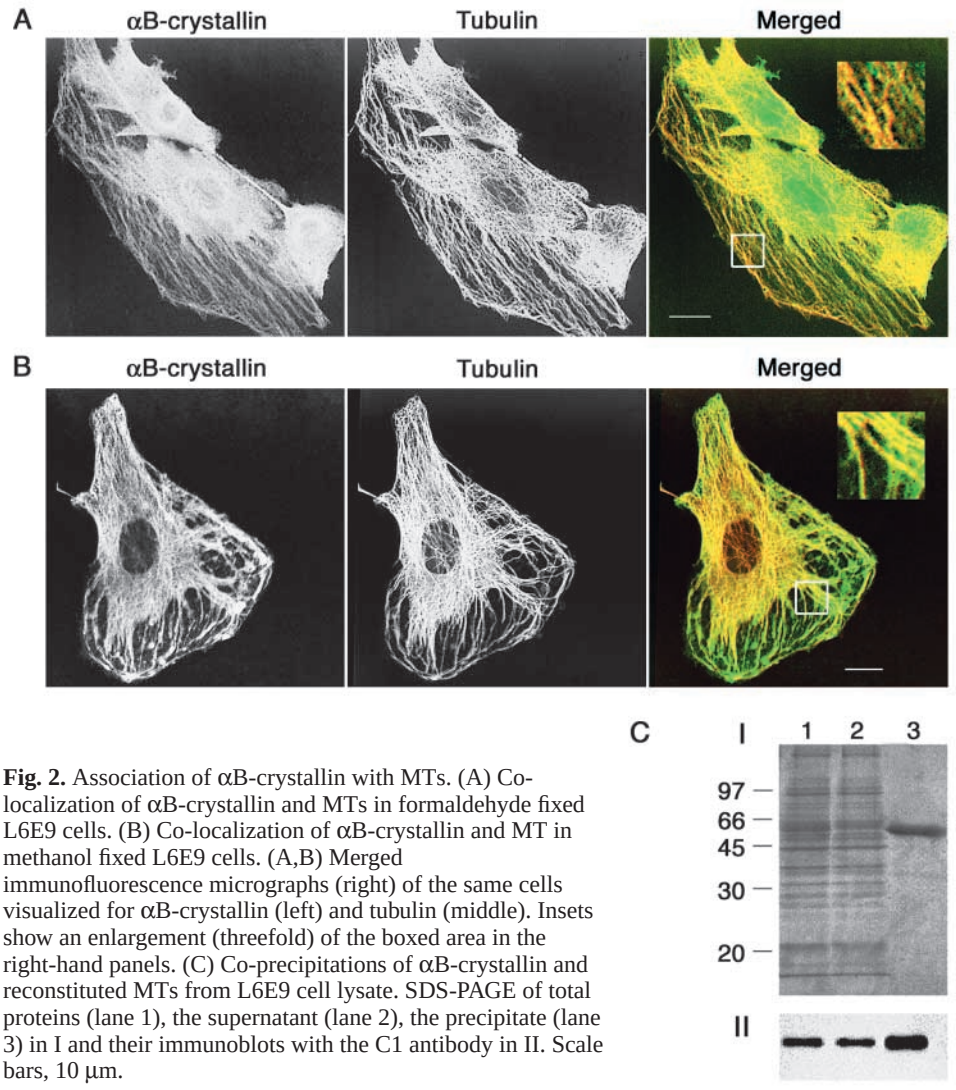

Fig. 2. Association of αB-crystallin with MTs. (A) Co-localization of αB-crystallin and MTs in formaldehyde fixed L6E9 cells. (B) Co-localization of αB-crystallin and MT in methanol fixed L6E9 cells. (A,B) Merged immunofluorescence micrographs (right) of the same cells visualized for αB-crystallin (left) and tubulin (middle). Insets show an enlargement (threefold) of the boxed area in the right-hand panels. (C) Co-precipitations of αB-crystallin and reconstituted MTs from L6E9 cell lysate. SDS-PAGE of total proteins (lane 1), the supernatant (lane 2), the precipitate (lane 3) in I and their immunoblots with the C1 antibody in II. Scale bars, 10 μm.

Instability of MTs in C6AS cells

The next question was whether this interaction influences MT polymerization and/or depolymerization. To examine this, C6 glioma cells transformed with a sense or an antisense strand of αB-crystallin cDNA were used (Iwaki et al., 1994), denoted as C6SE and C6AS cells, respectively. We observed the colocalization of αB-crystallin with MT networks in C6 glioma cells (Fig. 5A), even after a brief treatment with Triton X-100 in MSB followed by FMET fixation, which results in the extraction of free tubulin dimer. αB-Crystallin localized with the MTs in C6 normal cells, as in L6 cells, and also in C6SE and C6AS cells (data not shown). αB-Crystallin production in those cells was compared by western blotting using C1 antibody (Fig. 5B). C6SE and C6 normal cells expressed about five- to tenfold and two- to threefold more αB-crystallin, respectively, than C6AS cells. Next, we examined the effect of the difference in cellular amount of αB-crystallin on MT depolymerization by nocodazole. MT stability against (33 μM) nocodazole-induced depolymerization was clearly maintained at 2 minutes and 10 minutes in C6SE cells, and at 2

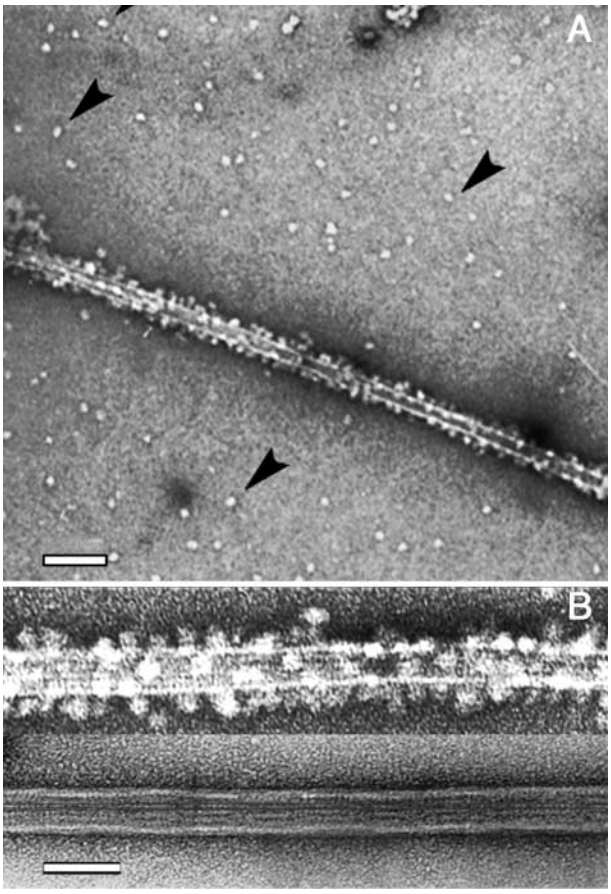

Fig. 3. Electron micrographs of αB-crystallin bound MAP-MTs. MTs polymerized in the presence (A; B, top) or absence of αB-crystallin (B, bottom) as observed by electron microscopy. MTP (1 mg ml^{-1}) was polymerized in the presence of αB-crystallin (40 μM as monomer). Arrowheads indicate unbound αB-crystallin. Scale bar, 100 nm (A), 50 nm (B).

minutes in C6 normal cells. By contrast, MT depolymerization was observed in C6AS cells at 2 minutes (Fig. 5C). Moreover, at 2 minutes and 10 minutes in C6AS cells, and at 10 minutes in C6 normal cells, unextracted tubulin was observed as white background, which was stained with anti-α-tubulin antibody, even after the treatment of Triton X-100.

To investigate this MT-stabilizing effect of αB-crystallin more quantitatively, dimer and polymer fractions of tubulin were separated from (33 μM) nocodazole-treated cells and evaluated by immunoblotting (Fig. 5D). To separate the dimer and polymer, we followed a protocol described by Gundersen et al. (Gundersen et al., 1987), in which cells were extracted with Triton X-100 in a MT-stabilization buffer. Polymer ratios of C6AS cells after nocodazole treatment for 15 minutes (31.8±6.4%: mean±s.d.) and 30 minutes (18.6±9.8%) were significantly lower than those of C6 normal cells (48.9±11.5% and 36.9±9.1%, respectively) (Fig. 5E).

Resistance to MT disassembly in vitro

We also examined the effects of αB-crystallin on assembly modifications by podophyllotoxin (another MT-depolymerizing drug) or calcium [which is reported to increase dynamic instability in vitro (O'Brien et al., 1997) and is thought to induce depolymerization in vivo]. MTP, together with various concentrations of αB-crystallin, was allowed to polymerize for 15 minutes and then 10 μM podophyllotoxin was added to the solution. The changes in turbidity at 350 nm were monitored (Fig. 6A). The amount of MT remaining was estimated from the ratio of the mean turbidity at 44-45 minutes to that at 14-15 minutes (Fig. 6B). Without αB-crystallin, the MT fraction was 71.6±6.6% (mean±s.d.); with 2.5 μM and 10 μM αB-crystallin, the MT fractions were 89.4±8.4% and 93.4±6.6%, respectively. The stabilizing effect of αB-crystallin inhibiting depolymerization of MTs by podophyllotoxin was concentration dependent, and the ratio in the presence of 10 μM αB-crystallin was significantly higher than that in the absence of αB-crystallin (n=3, $^{*}P<0.05$).

The MT-resisting effect of αB-crystallin against calcium was examined. MTP was polymerized in PME as described above. αB-Crystallin suppressed 1 mM free-calcium-induced MT depolymerization in a concentration-dependent manner (Fig. 6C). The polymerized level attained after the addition of 113 μM free calcium with αB-crystallin (10 μM) was significantly higher than that without αB-crystallin (Fig. 6D, n=6, $^{**}P<0.01$). The effects of αB-crystallin on the properties of MAPs, such as MT nucleation activity, were examined and αB-crystallin found to have no assembly-promoting activity (data not shown). However, the plateau level of polymerization of MTs with αB-crystallin was slightly higher in any concentration of αB-crystallin tested (see also Fig. 6A).

Discussion

The present study is the first demonstration of the interaction of αB-crystallin with MTs, both in vivo and in vitro in the absence of stress or normal condition. αB-Crystallin localized with MT networks in myoblast and glioma cells, and precipitated with MTs polymerized from myoblast cell extracts. Electron micrographs presented in this report clearly show this interaction. Co-precipitation assay revealed that the amount of bound αB-crystallin with reconstituted MTs from MTP was dose dependent. However, this interaction was not observed using MTs reconstituted from PCT. It was suggested that this interaction was mediated by MAP(s), because MTP contained MAPs. MAPs are proteins that bind along the side of MTs and can stabilize them against disassembly (Maccioni and Cambiazo, 1995). Recently, it was suggested that HSP70 and HSP90, both of which have been well characterized as molecular chaperones, can bind tau protein, one of the MAPs, and refold it (Dou et al., 2003). It has been reported that αB-crystallin localized with neurofibrillary tangles, which contain amyloid β and tau in Alzheimer's-disease-patient brain (Cooper et al., 1995; Lowe et al., 1990; Lowe et al., 1992). Together, these results and this study suggest that there is a possibility that αB-crystallin also binds tau. However, what component in MTP might interact with αB-crystallin is to be elucidated in future.

These results remind us of a hypothesis (Carver et al., 2002) to explain the ability of sHSPs to protect other proteins from denaturation: that the oligomer form of sHSP can supply interactive sites for the substrates on the surface of the spheres of sHSPs. It might be that the surface of αB-crystallin oligomers, when attached to MTs by MAPs, is accessible as a

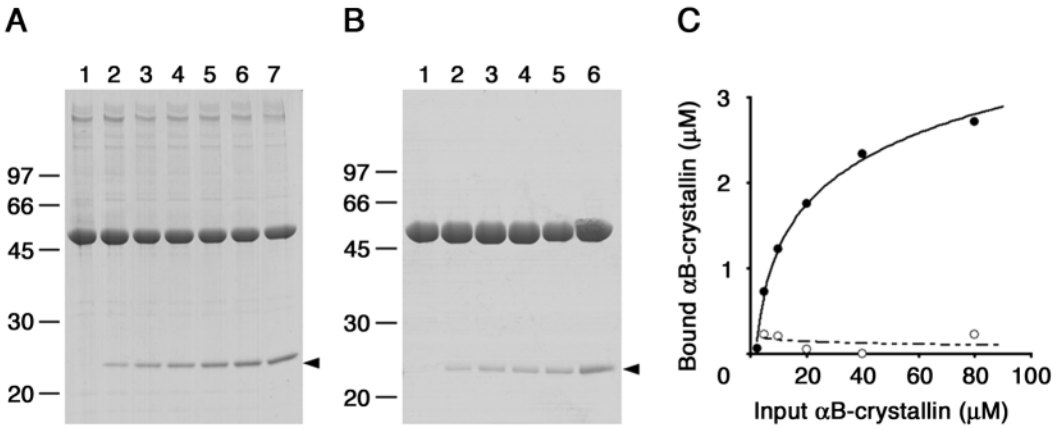

Fig. 4. Binding of αB-crystallin to reconstituted MTs. (A) CBB-stained gel of co-precipitated αB-crystallin and MAP-MTs. All lanes contain 1 mg ml^{-1} MAP-MTs. The other lanes also contain: 2.5 μM αB-crystallin (lane 2); 5 μM αB-crystallin (lane 3); 10 μM αB-crystallin (lane 4); 20 μM αB-crystallin (lane 5); 40 μM αB-crystallin (lane 6); 80 μM αB-crystallin (lane 7). Arrowhead indicates αB-crystallin band. (B) CBB-stained gel of co-precipitated αB-crystallin and PCT-MT. All lanes contain 10 μM PCT-MT. The other lanes also contain: 5 μM αB-crystallin (lane 2); 10 μM αB-crystallin (lane 3); 20 μM αB-crystallin (lane 4); 40 μM αB-crystallin (lane 5) and 80 μM αB-crystallin (lane 6). (C) The amount of co-precipitated αB-crystallin was plotted for input αB-crystallin. Closed circle represented for MTP-MTs and open circle for PCT-MTs.

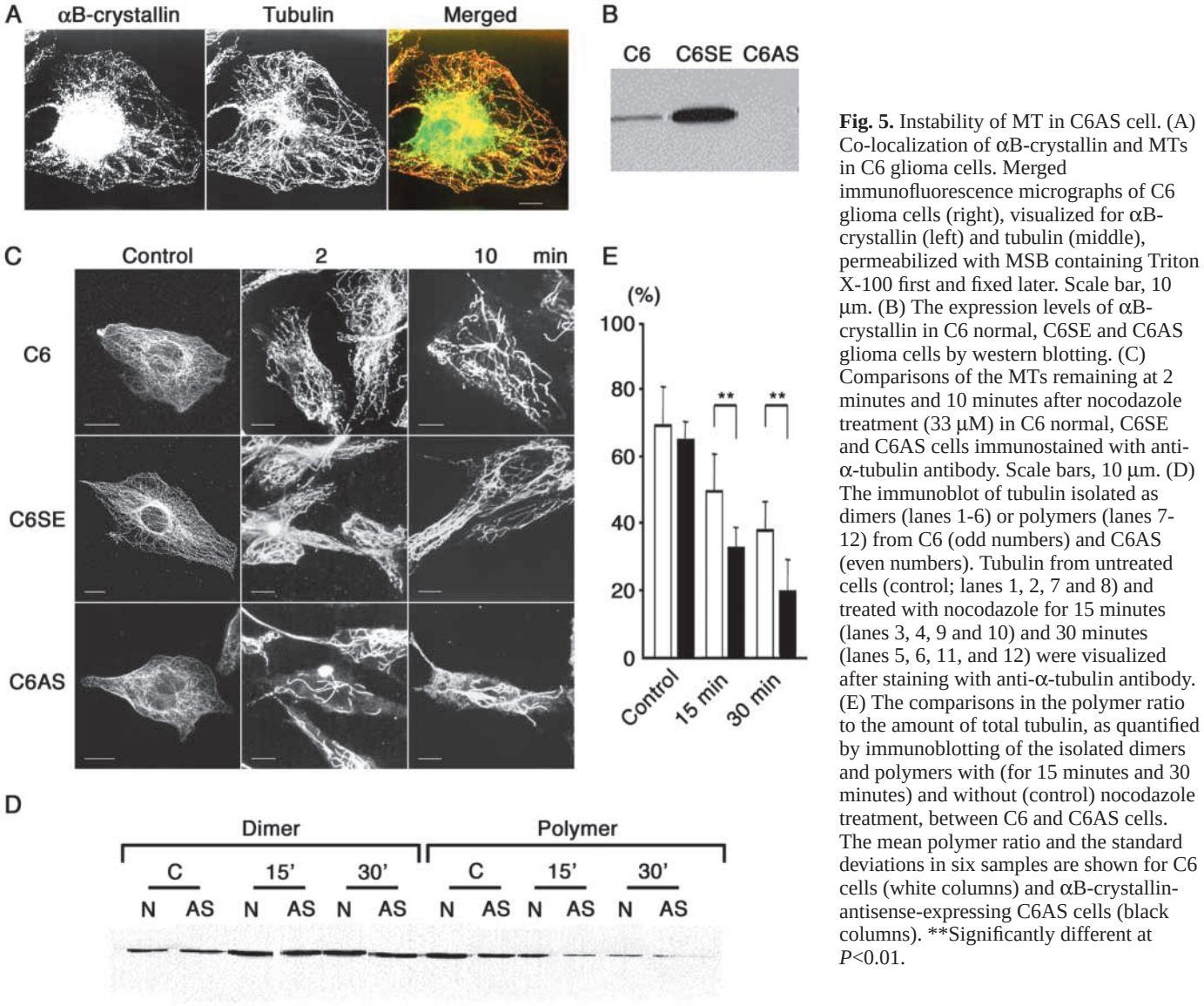

Fig. 5. Instability of MT in C6AS cell. (A) Co-localization of αB-crystallin and MTs in C6 glioma cells. Merged immunofluorescence micrographs of C6 glioma cells (right), visualized for αB-crystallin (left) and tubulin (middle), permeabilized with MSB containing Triton X-100 first and fixed later. Scale bar, 10 μm. (B) The expression levels of αB-crystallin in C6 normal, C6SE and C6AS glioma cells by western blotting. (C) Comparisons of the MTs remaining at 2 minutes and 10 minutes after nocodazole treatment (33 μM) in C6 normal, C6SE and C6AS cells immunostained with anti-α-tubulin antibody. Scale bars, 10 μm. (D) The immunoblot of tubulin isolated as dimers (lanes 1-6) or polymers (lanes 7-12) from C6 (odd numbers) and C6AS (even numbers). Tubulin from untreated cells (control; lanes 1, 2, 7 and 8) and treated with nocodazole for 15 minutes (lanes 3, 4, 9 and 10) and 30 minutes (lanes 5, 6, 11, and 12) were visualized after staining with anti-α-tubulin antibody. (E) The comparisons in the polymer ratio to the amount of total tubulin, as quantified by immunoblotting of the isolated dimers and polymers with (for 15 minutes and 30 minutes) and without (control) nocodazole treatment, between C6 and C6AS cells. The mean polymer ratio and the standard deviations in six samples are shown for C6 cells (white columns) and αB-crystallin-antisense-expressing C6AS cells (black columns). **Significantly different at $P<0.01$.

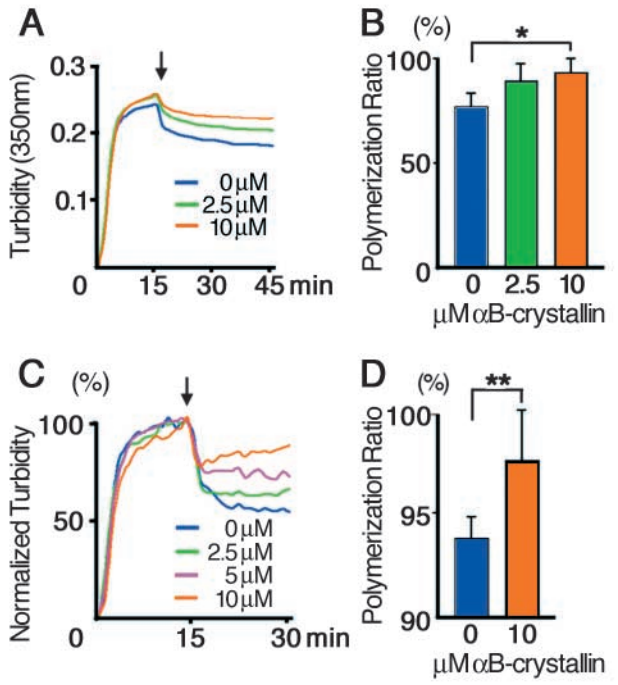

Fig. 6. αB-Crystallin increases MT resistance to disassembly in vitro. (A) Changes of turbidity in assembly of MT proteins, over 15 minutes and disassembly after the addition of 10 μM podophyllotoxin (indicated by arrow) with (2.5 μM, 10 μM) or without αB-crystallin. (B) Mean levels of remaining MTs and standard deviations for each of three trials calculated from the ratio of the absorbance at 44-45 minutes to the level at 14-15 minutes (at polymerized peak) with different levels of αB-crystallin. αB-Crystallin (10 μM) significantly increased the amount of remaining MT compared with that in the absence of αB-crystallin. Values are means±s.d. (n=3, *significantly different at P<0.05). (C) Changes of turbidity in assembly of MT proteins over 15 minutes and disassembly after the addition of 1 mM calcium (indicated by arrow) with (2.5 μM, 5.0 μM, 10.0 μM) or without αB-crystallin. (D) The ratio of the attained polymerization as a mean value at 14-15 minutes after 113 μM calcium addition with αB-crystallin (10 μM) was significantly higher than that without αB-crystallin. Values are means±s.d. (n=6, **significantly different at P<0.01).

chaperone bed for denatured tubulin dimers and/or other substrates. Taken together, there is a possibility that one important role of αB-crystallin is to protect cytoskeletal proteins from denaturation while keeping monomers close to the polymeric cytoskeletons, with the transformation between the two forms being dynamically regulated (Goldman et al., 1999; Kirschner, 1980; Mitchison and Kirschner, 1984) under both normal and stressed conditions.

Otherwise, it seems that the possible function additive above discussion might relate closely to a maintenance of tensegrity of cytoskeletons (Ingber, 1993) against long-lasting tonic contraction. Because all sHSPs are highly expressed in muscle cells, especially in heart (Dubin et al., 1989) and in slow skeletal muscle (fiber-type-dependent expression) (Atomi et al., 2000), the function of these tissues might be related to tonic contraction, to be maintained with endlessly energy-consuming fashion. In addition, a close correlation has been reported between oxidative metabolism and the expression level of αB-crystallin in various cells in rat tissues (Iwaki et al., 1990).

Three cytoskeleton components (fibrous actin, MT and IF) are basically independently regulated but affect each other with different tensile and dynamic properties. Vimentin co-localization with MT networks has been reported in most cultured cells (Gyoeva and Gelfand, 1991). In our study, co-localization of MTs and vimentin was ascertained in L6E9 and C6 cells (data not shown). Recently, some specific proteins were identified that connect actin and MTs (Karakesisoglou et al., 2000; Leung et al., 1999) or MTs and IFs (Liao and Gundersen, 1998). Thus, two or three cytoskeletal systems might work together in living cells. αB-Crystallin might work as a chaperone for both the IF (Nicholl and Quinlan, 1994; Perng et al., 1999; Vicart et al., 1998) and the MT systems, because they align laterally in most cells. The maintenance of cytoskeleton dynamics, as well as the tensegrity model of Ingber (Ingber, 1993), might need sHSPs.

After nocodazole treatment, it was clearly observed that more MTs remained in both C6 normal and C6SE cells than in C6AS cells. However, after 2 hours, MTs in both C6SE and C6AS cells completely depolymerized. In other words, αB-crystallin does not completely inhibit MT depolymerization but it seems to promote resistance to MT depolymerization in cells. We used podophyllotoxin for in vitro MT depolymerization because nocodazole had measurable absorbance in our condition. Both nocodazole and podophyllotoxin are vinca alkaloids and bind the same site of tubulin (Hoebeke et al., 1976; Luduena and Roach, 1981) and depolymerize MTs in the same manner. αB-Crystallin also had significant protective effects on MT disassembly by not only podophyllotoxin but also Ca^{2+} (at 113 μM) in vitro in this study. MTs are stabilized with MAPs and this stabilization resulted in the terminal amino acid modification to Glu (Cook et al., 1998) in some cell types (Gundersen et al., 1989). However, it has been reported that various factors, such as divalent cations, pH, magnesium, phosphorylation and kinase activation, influence tubulin and MT conditions. Further precise mechanisms of αB-crystallin for stabilizing MT need to be resolved in future.

The present study has shown a close relationship between αB-crystallin and MTs, in cells and in vitro, and a resistant effect of αB-crystallin on nocodazole- and calcium-induced depolymerization of MT, both in cells and in vitro. This suggests that the role of αB-crystallin for MTs might be that of a possible modulator for disassembly of MTs mediated by MAPs.

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