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Small Heat Shock Protein 27 (HSP27) Associates with Tubulin/Microtubules in HeLa Cells

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One of the monoclonal antibodies raised against mitotic HeLa cells (termed as mH3) recognized a 27-kDa protein and stained microtubules in the mitotic spindles of HeLa cells. Immunoscreening of a HeLa cDNA library revealed that mH3 antigen is a small heat shock protein, HSP27. Immunoprecipitation analysis using mH3 suggested that both α- and β-tubulin are associated with HSP27. Further, sucrose-cushioned ultra centrifugation revealed that HSP27 is co-sedimented with taxol-stabilized microtubules. These results indicate that HSP27 associates with tubulin/microtubules in HeLa cells.

Key Words: **small heat shock protein; HSP27; microtubules; tubulin; mitotic spindle; monoclonal antibody.**

The small heat shock proteins (small HSPs), a group of HSPs with molecular masses less than 30-kDa, play an important role not only in the stress response, but also in other physiological activities. It is well known that HSP27 and αB-crystallin, which are members of small HSPs, act as modulators of the cytoskeleton such as microfilaments and intermediate filaments. HSP27 has been characterized as an inhibitor of actin polymerization and this inhibitory activity is suppressed by phosphorylation [1, 2]. Such a phosphorylation-mediated regulation of actin polymerization-inhibitory activity of HSP27 is thought to be important *in vivo* [3–6]. On the other hand, αB-crystallin has a chaperon activity to actin [7, 8].

Furthermore, small HSPs also interact with intermediate filaments. Both HSP27 and αB-crystallin reorganize abnormal intermediate filament aggregates into normal filament network [9, 10].

Thus, many studies have been done on the interaction of small HSPs with actin and intermediate filament proteins. However, much less is known about relationship between small HSPs and microtubules. Kato *et al.* reported that exposure of C6 glioma cells to microtubule depolymerization drugs such as colchicine, colcemid, vinblastine and nocodazole increases the contents of αB-crystallin but not HSP27 [11]. Taxol, a microtubule stabilization drug, does not stimulate the synthesis of αB-crystallin, suggesting that depolymerization of microtubules is required for this increasing of αB-crystallin contents. Bluhm *et al.* reported that αB-crystallin but not HSP27 protects microtubule integrity during simulated ischemia in rat neonatal cardiac myocytes [12]. Further, in other work, it was shown that αB-crystallin recognizes denatured tubulin [13]. Thus, the interaction of αB-crystallin with tubulin has been documented. However, at present, no paper notes the interaction of HSP27 with tubulin/microtubules.

As described in our previous paper [14], we generated several strains of monoclonal antibodies raised against mitotic HeLa cells for studying the molecular identity of substances involved in mitosis. Among these, one monoclonal antibody was selected for its reactivity with mitotic spindles of HeLa cells and was designated as mH3. By using this mH3, we present here histochemical and biochemical evidences showing the association of HSP27 with tubulin/microtubules in HeLa cells.

EXPERIMENTAL PROCEDURES

Materials. Piperazine-N,N′-bis (2-ethanesulfonic acid) (PIPES), and bovine serum albumin (BSA; fraction V) were obtained from Sigma Chem. Co. (St. Louis, MO). Fluorescein-5-isothiocyanate (FITC) -conjugated goat anti-mouse Ig's (G + L) and Rhodamine-conjugated goat $F(ab')_2$ anti-rabbit Ig's (G + L) were from Tago Inc. (Burlingame, CA). A rabbit polyclonal anti-human HSP27 antibody (Lot 14533) was from Upstate Biotechnology Inc. (Lake Placid, NY). Recombinant human HSP27 (Lot 805435) was from StressGen Biotechnologies Corp. (Victoria, Canada). A rabbit polyclonal anti-tubulin antibody was from Polyscience Inc. (Warrington, PA). Mouse monoclonal anti-α-tubulin antibody (N356) and anti-β-tubulin antibody (N357) were from Amersham International Inc. (Buckinghamshire, England). Polyvinylidene difluoride (PVDF) membranes (Immobilon (IPVH304F0) and Immobilon-PSQ (ISEQ26260)) were from Millipore (Bedford, MA). Leupeptin and pepstatin A were from Peptide Inst. Inc. (Osaka, Japan).

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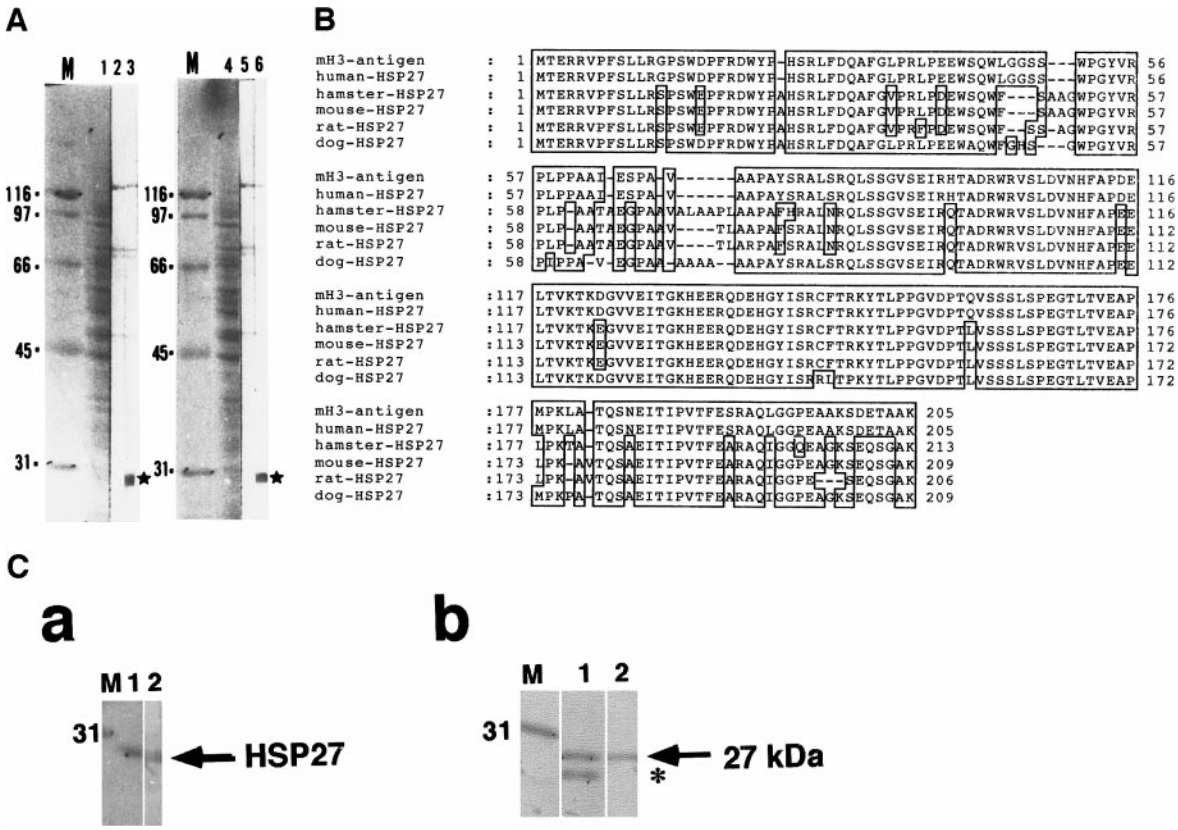

FIG. 1. Characterization of mH3 antigen. (A) Immunoblotting of HeLa cell extract with mH3. Total cell extracts prepared from interphasic (lanes 1–3) and mitotic (lanes 4–6) HeLa cells were separated on SDS–PAGE, transferred to a PVDF membrane, and stained with Coomassie brilliant blue (CBB) (lanes 1 and 4) or immunoblotted with mH3 (lanes 3 and 6). As control experiments, each extract was immunoblotted only with the second antibody (lanes 2 and 5). The 27-kDa component (asterisk) is similarly detected in the interphasic and mitotic cells. Molecular weight markers are shown (M) with molecular mass in kDa. (B) Comparison of amino acid sequence of mH3 antigen and HSP27 from human, hamster, mouse, rat, and dog. The amino acids are indicated by one letter code. Identical amino acids are boxed. The nucleotide sequence and the deduced amino acid sequence of mH3 antigen will appear in the DDBJ, EMBL, and GenBank nucleotide sequence database with the Accession No. AB020027. The sequences of human HSP27 (X54079), hamster HSP27 (P15991), mouse HSP27 (P14602), rat HSP27 (P42930), and dog HSP27 (P42929) were obtained from the DDBJ and SWISS-PROT database using accession number given in parentheses. (C) Confirmation of mH3 antigen as HSP27. (a) Immunoblotting of HSP27 with mH3. Recombinant HSP27 was subjected to SDS–PAGE, transferred to a PVDF membrane and stained with CBB (lane 1) or immunoblotted with mH3 (lane 2). Lane M, carbonic anhydrase as a molecular weight marker (31-kDa). (b) A 27-kDa component of immunoprecipitates of mH3 was probed with polyclonal anti-HSP27 antibody. Lane 1, CBB staining; Lane 2, immunoblotting using polyclonal anti-HSP27 antibody; Lane M, carbonic anhydrase as a molecular weight marker (31-kDa). The light chain of IgG (asterisk) is also shown.

Cell culture and treatment of cells. HeLa cells obtained from Riken Cell Bank (Tsukuba, Japan) were cultured as described previously [15]. Mitotic and interphasic HeLa cells were prepared according to Yamashiro *et al.* [16]. Heat shock treatment was performed by incubating HeLa cells at 42°C for 30 min. Cells were mixed with F-buffer (20 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM EGTA, 200 mM NaCl, 1 mM PMSF, and 10 μg/ml leupeptin) and then sonicated on ice with a Branson Sonifier 450 (Danbury, CT) and centrifuged at 127,000 g for 1 h in a Beckman TLA-100.2 rotor (Beckman Inst. Inc., Palo Alto, CA). The supernatant was collected as the cell extract and stored at −80°C until use.

Production of monoclonal antibodies against the mitotic HeLa cells. Monoclonal antibodies raised against the mitotic HeLa cells were described previously [14]. Among these monoclonal antibodies, one monoclonal antibody reacted with mitotic spindle was designated as mH3. For immunofluorescence staining and immunoblotting, hybridoma culture supernatant was used. Production of ascitic fluid and purification of IgG fraction from the ascitic fluid were described previously [14].

Immunostaining. Indirect immunofluorescence staining was performed as described previously [14]. All immunostained specimens were examined under a Zeiss laser scan microscope LSM410 (Carl Zeiss Co. Ltd., Tokyo, Japan).

Immunoscreening and sequence analysis. The HeLa cell cDNA library in lambda ZAP II was constructed as described previously [17], and was screened with mH3. Recombinant phages were plated with *Escherichia coli* XL1 Blue and blotted onto PVDF membranes previously soaked with 10 mM isopropyl-β-thiogalactoside. The transferred membranes were soaked in PBS containing 3% (w/v) BSA and then reacted with mH3. Immunoreactive plaques were visualized by Vectastain ABC kit (Vector Lab. Inc. Burlingame, CA). Positive phage clones were purified by two additional rounds of screening as described above. The cDNA insert was subcloned into pBluescript SK(−) and sequencing was carried out as described previously [17].

Immunoprecipitation. The cell extracts from HeLa cells prepared as described above were preadsorbed with protein A-Sepharose

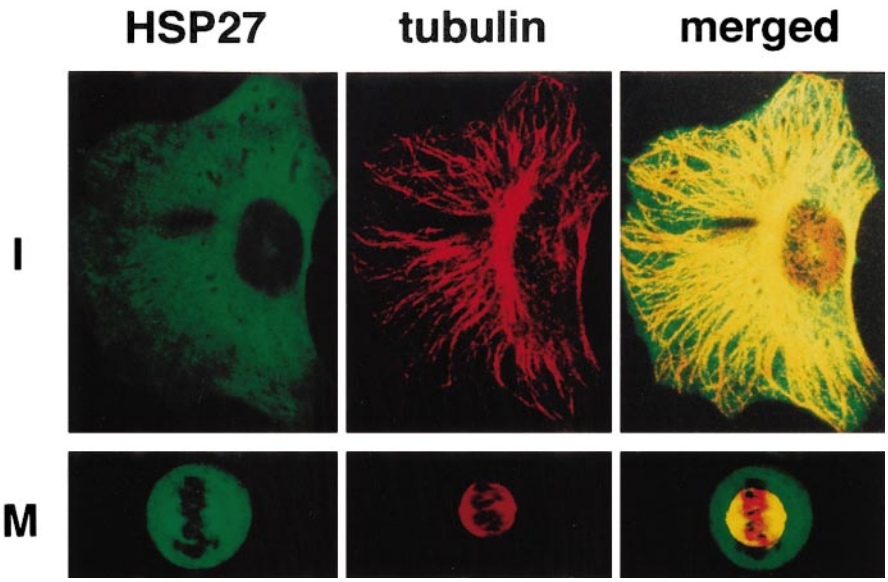

FIG. 2. Colocalization of HSP27 with microtubules in HeLa cells. Confocal immunofluorescence images of interphasic (I) and mitotic (M) HeLa cells that have been stained simultaneously with mH3 (left column) and anti-tubulin antibody (mid column) are shown. Merged images are also shown in right column.

beads (Pharmacia Biotech, Uppsala, Sweden) for 1 h at 4°C. Then the extract was incubated with mH3 from ascitic fluid for 1 h at 4°C. Fresh protein A-Sepharose beads were added to the mixtures, and incubated for an additional 1 h at 4°C. The mixture was centrifuged at 1600 g for 5 min at 4°C and the pellet was washed three times with PBS. The samples were collected and subjected on a SDS–PAGE for immunoblotting.

Microtubules sedimentation assay. Microtubules from HeLa cells were prepared according to the method of Vallee [18] with some modifications. Briefly, cells were mixed with reassembly buffer (100 mM PIPES (pH 6.8), 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, and 5 μg/ml pepstatin A) and then homogenized and centrifuged at 127,000 g for 1 h. Then the supernatant was incubated with 0.5 mM GTP and 40 μM Taxol at 37°C for 20 min. The microtubules formed were centrifuged at 34,000 g for 30 min through a cushion of 15% sucrose in reassembly buffer. The microtubule pellet was washed by suspension in 15% sucrose in reassembly buffer and was further centrifuged. The pellet was collected and then subjected on a SDS–PAGE for immunoblotting.

Other procedures. SDS–PAGE and immunoblotting were carried out in the same manner as described previously [19]. Amino acid sequences were analyzed with an Applied Biosystems amino acid sequencer model 477A (Perkin Elmer Corp., Foster City, CA).

RESULTS AND DISCUSSION

Immunoblot analysis of the cell extract showed that mH3 reacted with a 27-kDa component in both interphasic and mitotic HeLa cell extracts (Fig. 1A). To identify the mH3 antigen, immunoscreening of a HeLa cell cDNA library with mH3 was performed. The nucleotide sequence of the cDNA insert of the positive clone was determined. The deduced amino acid sequence was identical to that of HSP27 from human fibroblast and more than 80% similarity to those of HSP27 from other organisms (Fig. 1B). To confirm that the mH3 antigen is HSP27, following three assays were performed. First, the mH3 antigen changed its localization from the cytoplasm to the nuclei after heat shock treatment (data not shown) in the same manner as HSP27 does [20, 21]. Second, mH3 reacted with the recombinant HSP27 by immunoblotting (Fig. 1C, a). Third, polyclonal anti-HSP27 antibody reacted with a 27-kDa component in the immunoprecipitates of mH3 from HeLa cell extract (Fig. 1C, b). These data confirm that the mH3 antigen is HSP27.

Next, the localization of HSP27 was observed in HeLa cells by immunofluorescence microscopy using mH3. Interestingly, in the mitotic cells, mH3 stained the structure similar to the mitotic spindle, suggesting that HSP27 localizes to the mitotic spindles. To confirm this, the localization of HSP27 was compared to that of microtubules by double staining using mH3 and polyclonal anti-tubulin antibody in HeLa cells (Fig. 2). In interphasic cells, HSP27 distributed throughout the cytoplasm but not in the nuclei. Colocalization of HSP27 with microtubules was not apparent, though the distribution of HSP27 was overlapped with microtubules especially in the peripheral of the nuclei (Fig. 2, upper three panels). In mitotic cells, the colocalization of HSP27 with mitotic spindles was clearly observed (Fig. 2, lower three panels). This is the first observation showing the spindle localization of small HSPs. Examples of other proteins that selectively associate with microtubules in the mitotic spindle include kinesin-related protein Eg5, protein phosphatase γ 1, and vault poly (ADP-ribose) polymerase [22–24]. Among these proteins, it is reported that the associa-

tion of Eg5 with mitotic spindle requires mitotic specific phosphorylation at Thr-927 by $p34^{cdc2}$. The $p34^{cdc2}$ is also known to phosphorylate microtubule-associated protein 4 and regulate its microtubules-binding and -stabilizing abilities during mitosis [25, 26]. Thus $p34^{cdc2}$ is a key mediator for regulation of microtubule function during mitosis. Although phosphorylation is the major post-translational modification in HSP27, it is not known that $p34^{cdc2}$ phosphorylates HSP27. As we have no evidence for such mitotic specific phosphorylation of this protein, the phosphopeptide-specific antibody to HSP27 will be required for further analysis.

Furthermore, we biochemically investigated the association of HSP27 with microtubules in HeLa cells. First, immunoprecipitation of the cell extract with mH3 was performed. The immunoprecipitates of mH3 contained five major proteins with molecular mass of 55, 50, 42, 27, and 25-kDa, respectively (Fig. 3A, lane 2). Among these components, the 42-kDa band was also detected even in the control experiment using only protein A-Sepharose beads (Fig. 3A, lanes 1 and 2). The 50- and 25-kDa bands were heavy and light chain of immunoglobulin, respectively. The 27-kDa band was HSP27 itself judging from its reactivity with polyclonal anti-HSP27 antibody (see above; Fig. 1C, b). To identify the 55-kDa protein (Fig. 3A, lane 2), the band was excised from SDS–PAGE gel and digested into four major fragments with V8 protease. These fragments were separated on SDS–PAGE, transferred to a PVDF membrane, and stained with CBB (Fig. 3B, a). The N-terminus amino acid sequences of these fragments were matched against those of human α- or β-tubulin (Fig. 3B, b). This strongly suggests that the 55-kDa protein is α- and β-tubulin. To confirm this, 55-kDa protein was probed with monoclonal anti- α- and β-tubulin antibodies and a polyclonal anti-tubulin antibody. These three anti-tubulin antibodies reacted with the 55-kDa band (Fig. 3A, lanes 4, 5, and 6). These data showed that HSP27 associated with α- and β-tubulin *in vivo*. At low temperature, it is well known that microtubules depolymerize and tubulin exists as soluble forms, containing $\alpha\beta$ heterodimer and some oligomers. As preparation of cell extracts and successive immunoprecipitation were conducted at 4°C, it is likely that the tubulin was co-precipitated with HSP27 as depolymerized forms. However, it remains unknown whether HSP27 also binds to microtubules. To examine this, microtubules were sedimented using taxol from HeLa cell extract and were probed with mH3 to detect HSP27. As shown in Fig. 4A, a strong positive signal was detected in lane 2 but not in lane 4, showing that HSP27 associates with microtubules. HSP27 was further co-sedimented with microtubules prepared from not only mitotic but also interphasic HeLa cells (Fig. 4B). From this result, we can not explain the difference in the colocalization of HSP27 with microtubules between interphasic and mitotic cells (see above; Fig. 2). This point must be tested by further investigation.

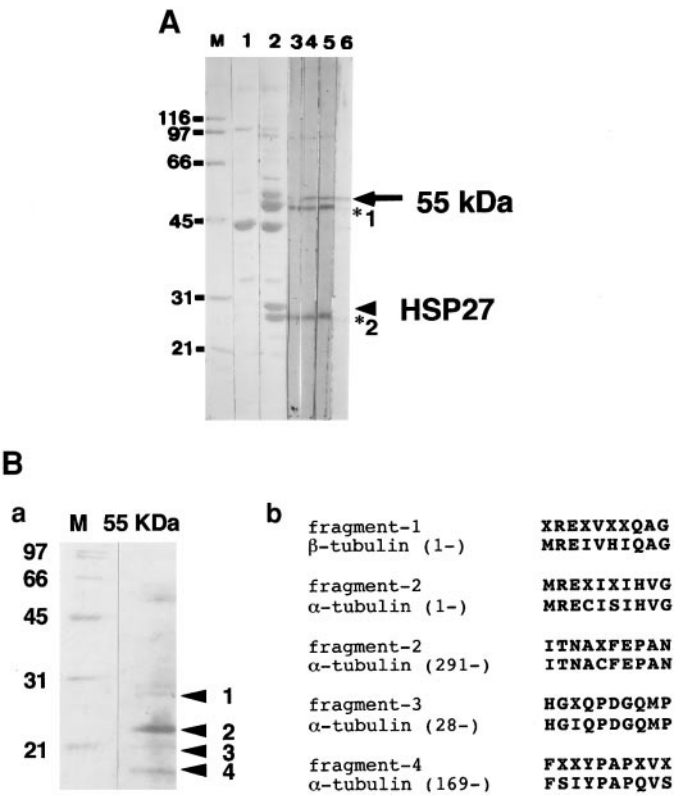

FIG. 3. Association of HSP27 with tubulin in HeLa cells. (A) Immunoprecipitation of HeLa cell extract with mH3. The aliquots of the cell extract were incubated with either only protein A-Sepharose beads (lane 1), or mH3 followed by protein A-Sepharose beads (lanes 2–6). The immunoprecipitated proteins were subjected to SDS–PAGE, transferred to a PVDF membrane and stained with CBB (lanes 1 and 2) or detected by immunoblotting (lanes 3–6). In lanes 3–6, one-tenth of the sample used in lane 2 were loaded in each lane. Immunoblot using anti-α-tubulin monoclonal antibody (lane 4), anti-β-tubulin monoclonal antibody (lane 5), anti-tubulin polyclonal antibody (lane 6) and immunoblot only with the second antibody (lane 3) as a control. The heavy (*1) and the light chain (*2) of IgG, respectively, are also shown. (B) Identification of the 55-kDa component coprecipitated with HSP27 as α-and β-tubulin. The 55-kDa component of the mH3 immunoprecipitate was digested into 4 major fragments by V8 protease. (a) Fragments were subjected to SDS–PAGE, transferred to a PVDF membrane, and stained with CBB. The N-terminus amino acid sequences of the fragment 1, 2, 3, and 4 were determined. (b) Comparison of partial amino acid sequences between the fragment 1–4 and human α-or β-tubulin. The amino acids are indicated by one letter code and X represents undetermined amino acid.

Arai and Atomi showed that αB-crystallin has chaperone activity to prevent aggregation of tubulin [13]. They concluded that αB-crystallin recognizes denatured tubulin in its very early step(s) of denaturation and suppresses further aggregation of tubulin. In this report, we present histochemical and biochemical evidences showing the association of HSP27 with tubulin and/or microtubules in HeLa cells. We consider that

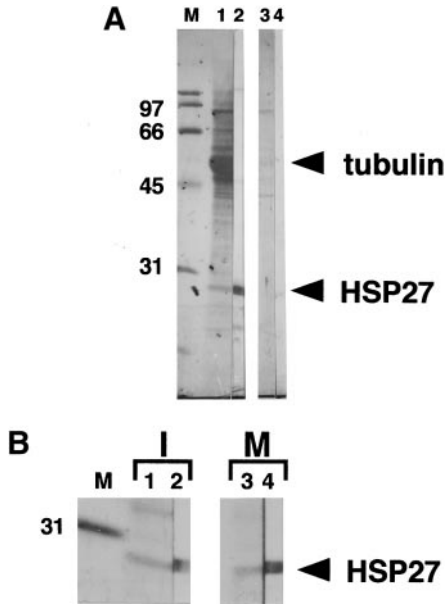

FIG. 4. Co-sedimentation of HSP27 with microtubules. Microtubules was sedimented from HeLa cells and was probed with mH3 to detect HSP27. (A) HeLa cell extract was mixed with (lanes 1 and 2) or without (lanes 3 and 4) taxol and the microtubules formed were sedimented through a 15% sucrose-cushion by ultra centrifugation. Lanes 1 and 3, CBB staining; lanes 2 and 4, immunoblot analysis using mH3. (B) Microtubules from interphasic (lanes 1 and 2) and mitotic (lanes 3 and 4) HeLa cell extract were also probed with mH3. Lanes 1 and 3, CBB staining; lanes 2 and 4, immunoblot analysis using mH3; lane M, carbonic anhydrase as a molecular weight marker (31-kDa).

HSP27 binds to native tubulin, because 1) immunofluorescence observation shows the colocalization of HSP27 with mitotic spindle at non-stressed condition, 2) immunoprecipitation conducted at 4°C shows the coprecipitation of tubulin with HSP27, while in the case of αB-crystallin, coprecipitation with tubulin is not observed in this condition, and 3) HSP27 binds to taxol-stabilized microtubules composed of native but not denatured tubulin.

Kato *et al.* reported that exposure of C6 glioma cells to microtubule depolymerization drugs increases the contents of αB-crystallin but not HSP27 [11]. It is also reported that over expression of αB-crystallin but not HSP27 protects microtubules integrity at ischemic stress in rat neonatal cardiac myocytes [12]. Taken together, it might be concluded that at stress condition αB-crystallin rather than HSP27 protects microtubules by binding to denatured tubulin. If so, what is the role of HSP27 in the cell? While HSP27 localizes in mitotic spindles, we suppose that HSP27 plays a role in the progression of mitosis. We are now conducting experiments according to this line.

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