

Association of α B-Crystallin, a Small Heat Shock Protein, with Actin: Role in Modulating Actin Filament Dynamics *in Vivo*

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Disruption of cytoskeletal assembly is one of the early effects of any stress that can ultimately lead to cell death. Stabilization of cytoskeletal assembly, therefore, is a critical event that regulates cell survival under stress. α B-crystallin, a small heat shock protein, has been shown to associate with cytoskeletal proteins under normal and stress conditions. Earlier reports suggest that α B-crystallin could prevent stress-induced aggregation of actin *in vitro*. However, the molecular mechanisms by which α B-crystallin stabilizes actin filaments *in vivo* are not known. Using the H9C2 rat cardiomyoblast cell line as a model system, we show that upon heat stress, α B-crystallin preferentially partitions from the soluble cytosolic fraction to the insoluble cytoskeletal protein-rich fraction. Confocal microscopic analysis shows that α B-crystallin associates with actin filaments during heat stress and the extent of association increases with time. Further, immunoprecipitation experiments show that α B-crystallin interacts directly with actin. Treatment of heat-stressed H9C2 cells with the actin depolymerizing agent, cytochalasin B, failed to disorganize actin. We show that this association of α B-crystallin with actin is dependent on its phosphorylation status, as treatment of cells with MAPK inhibitors SB202190 or PD98059 results in abrogation of this association. Our results indicate that α B-crystallin regulates actin filament dynamics *in vivo* and protects cells from stress-induced death. Further, our studies suggest that the association of α B-crystallin with actin helps maintenance of pinocytosis, a physiological function essential for survival of cells.

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Introduction

Cells respond to environmental stress by preferential synthesis and accumulation of a family of proteins called heat shock proteins (HSPs), which assist them to survive under these unfavorable conditions.^{1–3} Small HSPs (sHSPs), a subset of the HSP family, have been shown to be involved in many cellular processes that help in the survival of cells under conditions of stress.^{4,5} In spite of the increasing number of reports, the exact mechanism

by which sHSPs confer stress protection to cells is not clearly understood. Stresses like ischemia and hyperthermia lead to enhanced expression of α B-crystallin, a member of the sHSP family, implying its importance in cell survival.^{6–8} Functionally, α B-crystallin acts as a molecular chaperone and prevents the stress-induced aggregation of target proteins *in vitro*.^{9,10} Earlier studies from our laboratory have shown that the chaperone-like activity of α -crystallin in preventing aggregation of target proteins increases several fold at heat shock temperatures.^{11,12} In addition to its chaperone functions *in vitro*, the role of α B-crystallin is well documented in many cellular events such as cell division, differentiation and apoptosis.^{13–15}

Both ischemia and heat-stress cause extensive damage to the cytoskeleton that includes collapse of the intermediate filament network, disruption of the

Abbreviations used: HSP, heat shock protein; sHSP, small HSP; FITC, fluorescein isothiocyanate; MAP, microtubule-associated protein; MAPK, MAP kinase.

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microtubules, and rearrangement and/or disorganization of actin microfilaments.^{16–18} It has been observed that a brief period of hyperthermia is associated with enhanced post-ischemic ventricular recovery in rats.¹⁹ Cardiac ischemia results in rapid translocation of α B-crystallin from the cytosolic pool to intercalated discs and Z-lines of the myofibrils,²⁰ implicating its critical role in the protection of myocardium. A point mutation (R120G) in α B-crystallin leads to its abnormal interaction with desmin and causes desmin-related myopathies in the heart tissue.²¹ Studies from our laboratory as well as those of others have shown that reduced chaperone activity of R120G α B-crystallin might be the molecular basis for the observed pathology.^{22,23} Reducing α B-crystallin gene expression using anti-sense strategy leads to disruption of the actin microfilament network,²⁴ further supporting the important role played by this protein in maintenance of microfilament integrity and cellular survival. In addition to its regulation of microfilament stability, α B-crystallin has been shown to interact with microtubules and proteins of the intermediate filament family and confer stability during conditions of stress.^{25,26}

Disruption of the cytoskeleton and disaggregation of actin fibers are among the most immediate effects of stress, such as heat shock.^{16,17} Stabilization of actin fibers under stress conditions, therefore, is one of the critical events required for cellular survival. Moreover, several cellular processes, like motility and pinocytosis, involve extensive actin polymerization-depolymerization.^{27,28} α B-crystallin has been reported to interact with actin *in vitro*, and this interaction increases with increasing temperature.²⁹ α B-Crystallin has been shown to regulate actin polymerization-depolymerization dynamics and stabilize them in a phosphorylation-dependent manner *in vitro*.^{30,31} However, the molecular mechanism of interaction of α B-crystallin with actin filaments *in vivo* is not clearly understood.

In the present study, we have investigated the effect of heat stress on the localization of α B-crystallin in the H9C2 rat cardiomyoblast cell line. Our study shows that upon heat stress, α B-crystallin co-localizes with actin stress fibers. Immunoprecipitation experiments show that α B-crystallin interacts with actin filaments upon heat stress. This association of α B-crystallin with actin depends on its phosphorylation. Our results suggest that α B-crystallin plays an important role in processes such as pinocytosis. Thus, α B-crystallin has a major role in protecting cells from stress-induced damage and in the preservation of cellular architecture.

Results

Differential partitioning of α B-crystallin upon heat stress

In order to investigate the partitioning of α B-crystallin between the soluble, cytosolic and insol-

uble cytoskeletal fractions upon heat shock, we have incubated H9C2 cells at 43 °C for 15 min, 30 min, 45 min, 60 min or 75 min. The cells were lysed, the soluble and insoluble fractions separated and the levels of α B-crystallin was determined by Western blotting. We observed an increase in the level of α B-crystallin in the total cell lysate as the duration of heat shock increased (Figure 1(a)). When we analyzed the soluble and insoluble fractions of the cell lysates, α B-crystallin was found to be present in both fractions. While we observed a decrease in the level of α B-crystallin in the soluble fraction as the

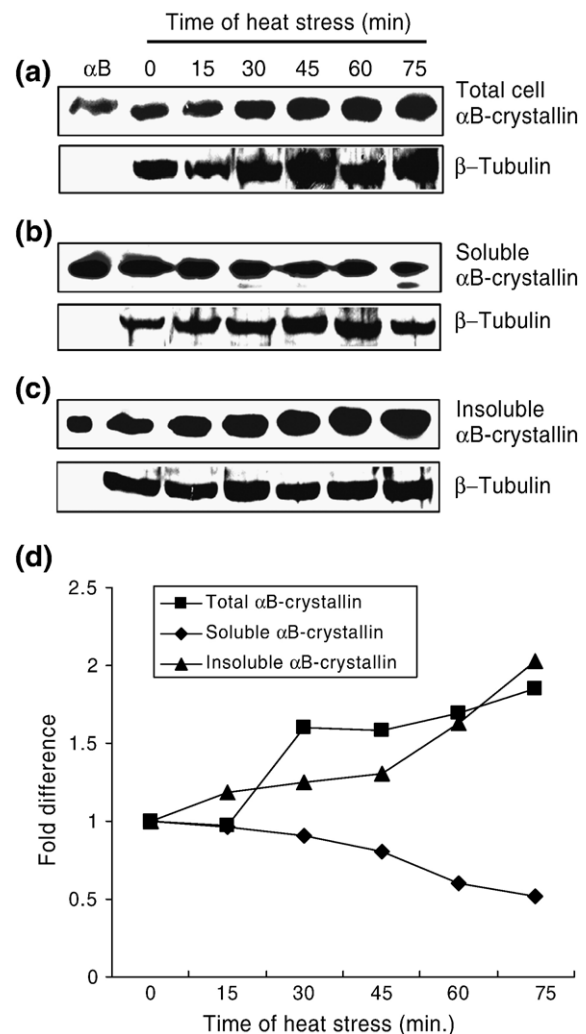


Figure 1. Western blot analysis to demonstrate the differential partitioning of α B-crystallin in H9C2 cells during heat stress. H9C2 cells, unstressed (0 min) or heat-stressed at 43 °C for 15 min, 30 min, 45 min, 60 min or 75 min were lysed and fractionated into soluble and insoluble fractions as described in Materials and Methods. (a) Total cell lysate, (b) the soluble fraction, and (c) the insoluble fraction were immunoblotted for α B-crystallin. Lane α B shows the band corresponding to recombinant α B-crystallin that served as a positive control. β -Tubulins as loading controls are also shown. (d) Graphic representation of band intensities of α B-crystallin from total cell lysates, soluble and insoluble fractions of unstressed, and heat-stressed H9C2 cells quantified by densitometry.

duration of heat shock increased (Figure 1(b)), we found a gradual increase in its level in the insoluble fraction (Figure 1(c)).

α B-Crystallin forms strands upon heat treatment

Since α B-crystallin increasingly partitioned to the insoluble fraction after heat stress, we analyzed its localization by confocal microscopy. Figure 2(a) shows that in unstressed H9C2 cells, α B-crystallin is found predominantly in the cytoplasm, with almost no staining in the nucleus. After heat stress for 1 h, α B-crystallin exhibits strand-like morphology in the cytoplasm and migrates into the nucleus (Figure 2(b)). The formation of strands in the cytoplasm as well as the translocation of α B-crystallin into the nucleus seems to be reversible upon recovery. After 3 h of recovery, α B-crystallin is found to be distributed uniformly in the cytosol as well as in the nucleus (Figure 2(c)). However, 6 h after recovery, most of the α B-crystallin translocates from the nucleus to the cytosol (Figure 2(d)). Similar experiments were performed with primary cultures of neonatal rat ventricular myocytes. Even in these differentiated cells, α B-crystallin forms strands in the cytosol upon heat stress (data not shown). Our results showing that α B-crystallin partitions into the insoluble fraction (Figure 1) and forms filaments upon heat stress (Figure 2(b)) suggest that α B-crystallin associates with cytoskeletal proteins.

α B-Crystallin associates with actin filaments *in vivo* upon heat stress

The actin microfilament network is an early target of cellular stress and forms transient stress fibers,

which disappear when the stress is released. It is likely that sHSPs such as α B-crystallin may contribute to the regulation of actin dynamics under normal and stressful conditions. We performed dual staining of H9C2 cells for α B-crystallin and actin after heat stress for various periods of time ranging from 15 min to 75 min, and analyzed their extent of co-localization by confocal microscopy. In unstressed cells, α B-crystallin is distributed uniformly in the cytosol and shows 23% co-localization with actin fibers (Figure 3(a)). As the duration of heat stress increases, we observe increased fiber-like morphology of α B-crystallin as well as its increased co-localization with actin stress fibers, with the extent being 86% after 75 min of heat stress (Figure 3(c)–(f)). At the earliest time-point of heat stress studied (15 min), α B-crystallin also shows translocation into the nucleus. Earlier studies from our laboratory have shown that α B-crystallin, which is present mostly in the cytoplasm, migrates to the nucleus upon stress, where it plays an important role in the stabilization of the nucleo-skeletal assembly.³² Figures 2 and 3 show fiber-like morphology of α B-crystallin and its colocalization with actin filaments in the cytoplasm upon stress. In the present study, we have investigated the importance of this association of α B-crystallin with actin filaments and its role in the organization of cytoskeletal assembly.

The increased partitioning of α B-crystallin upon heat stress to the insoluble fraction and its co-localization with actin filaments suggest that it interacts with actin under these conditions. We have subjected H9C2 cells to heat stress at 43 °C for 1 h, lysed and performed immunoprecipitation using antibodies to α B-crystallin both in the soluble

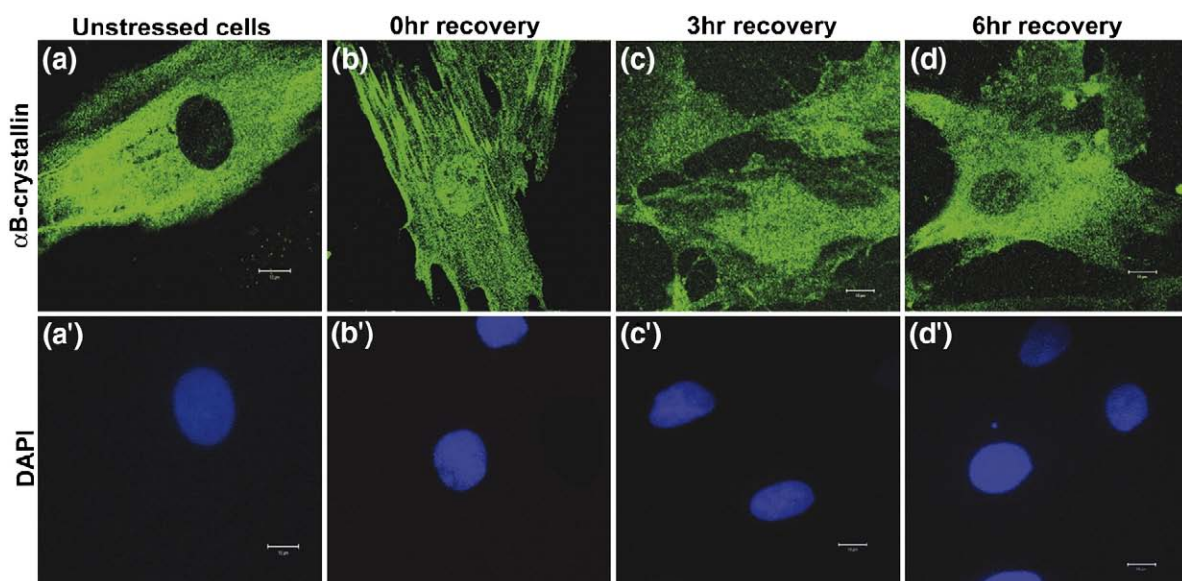


Figure 2. Immunolocalization of α B-crystallin upon heat stress. H9C2 cells, grown on coverslips were (a) unstressed or (b) subjected to heat stress at 43 °C for 60 min. The heat-stressed cells were allowed to recover at 37 °C for a period of (c) 3 h or (d) 6 h. (a)–(d) The cells were fixed in 3.7% (v/v) formaldehyde and immunostained using α B-crystallin-specific antibodies; (a')–(d') the nuclei were counterstained with DAPI. The scale bar represents 10 μ m.

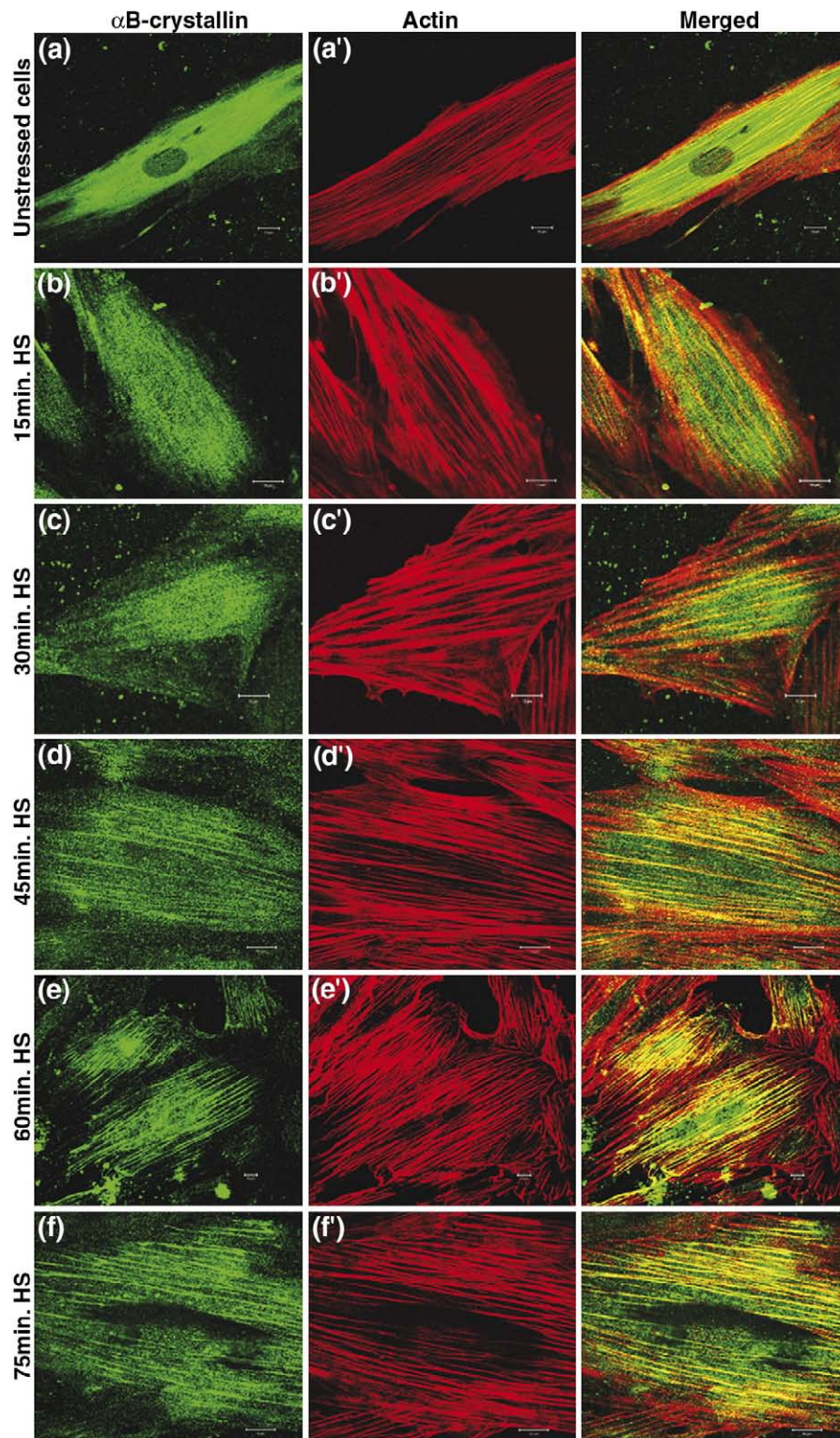


Figure 3. Co-localization of α B-crystallin with actin fibers. H9C2 cells, (a) unstressed or heat-stressed at 43 °C for (b) 15 min, (c) 30 min, (d) 45 min, (e) 60 min, or (f) 75 min were fixed with 3.7% (v/v) formaldehyde, permeabilized with chilled acetone and stained for α B-crystallin or for actin fibers. (a)–(f) α B-crystallin is shown in green and (a')–(f') actin fibers are shown in red. Merged panels show the overlays of confocal images of dual-stained cells in a single optical section of 0.3 μ m. The scale bar represents 10 μ m. The extent of co-localization was quantified using LSM-FCS software.

and insoluble fractions. Our results show actin band in the immunoprecipitated complex (Figure 4). Immunoblot analysis of this complex using antibody to actin (Figure 4, upper panel) shows detec-

table levels of actin in the soluble fraction of heat-stressed H9C2 cell lysates. Interestingly, the band intensity of actin in the immunoprecipitated complex from the insoluble fraction is significantly

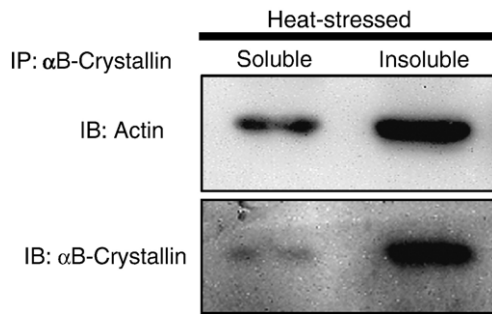


Figure 4. Immunoblot showing the interaction of α B-crystallin with actin. Lysates from heat-stressed H9C2 cells were fractionated, and soluble and insoluble fractions were separated by centrifugation (15,000g for 10 min at 4 °C). Proteins from the insoluble fraction solubilized in IP buffer (as described in Materials and Methods), and the soluble fraction were incubated with rabbit polyclonal α B-crystallin antibodies at 4 °C for 8 h. Subsequently, they were incubated with protein-G magnetic beads for 4 h. The proteins from the immunoprecipitated complexes were separated by SDS-PAGE (12% polyacrylamide gel) and transferred onto Nitrocellulose membrane. The membranes were immunoblotted with monoclonal actin antibodies (upper panel) or with monoclonal α B-crystallin antibodies (lower panel). Lane 1 shows bands from the soluble fraction and lane 2 shows the bands from the insoluble fractions.

higher. As expected, the band intensities of α B-crystallin in the immunoprecipitated complex show a similar pattern (Figure 4, lower panel).

Dynamic shift of phosphorylated α B-crystallin upon heat stress

α B-Crystallin is known to be phosphorylated at three serine residues (19, 45 and 59) *in vivo* and the extent of phosphorylation increases under conditions of stress.^{33–35} In order to understand the role of the phosphorylation status of α B-crystallin in its interaction with actin filaments, we have analyzed the soluble and insoluble fractions after heat stress for different lengths of time. Using antibodies specific for Ser59-phosphorylated α B-crystallin, we observed that the level of Ser59-phosphorylated α B-crystallin increases 3.4-fold in the soluble fraction up to 30 min of heat shock and gradually decreases thereafter (Figure 5(a) and (c)). On the other hand, the level of Ser59-phosphorylated α B-crystallin in the insoluble fraction remains more or

less constant during 30 min of heat stress and increases significantly from 45 min to 75 min (Figure 5(b) and (c)), the increase in the insoluble fraction being 2.8-fold at 75 min of heat shock. Western blot analysis of Ser45-phosphorylated α B-crystallin in the soluble fraction showed a gradual decrease and

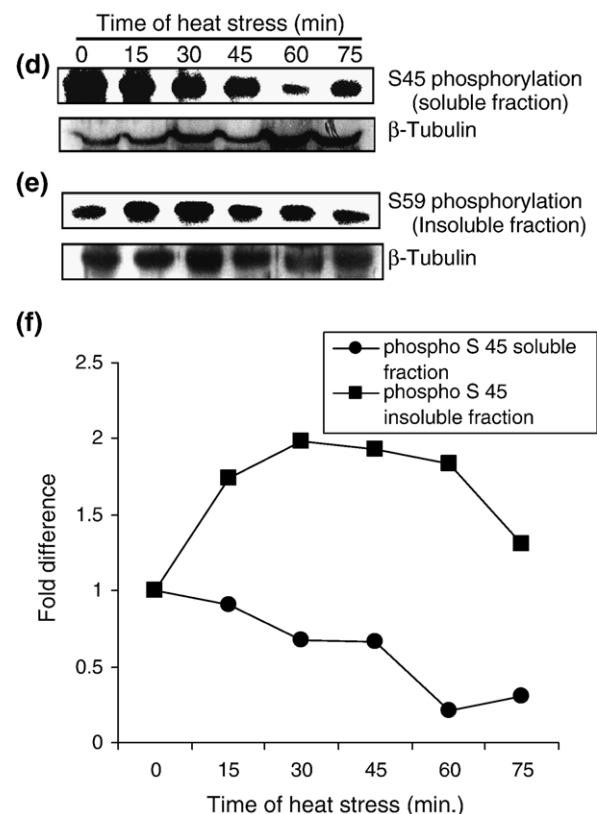
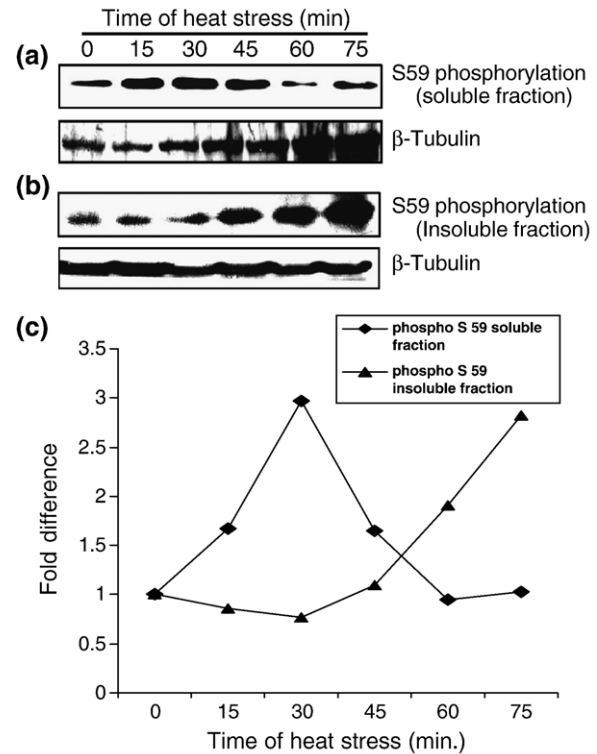


Figure 5. Western blots of phosphorylated α B-crystallin in the soluble and the insoluble fractions from lysates of unstressed and heat-stressed H9C2 cells. The soluble and insoluble fractions from lysates of unstressed H9C2 cells, or heat-stressed at 43 °C for 15 min, 30 min, 45 min, 60 min or 75 min were isolated as described earlier. Western blots show sSer59- and Ser45-phosphorylated α B-crystallin in (a) and (d) the soluble and (b) and (e) the insoluble fractions. The β -tubulin bands as loading control are also shown. (c) and (f) Graphic representation of band intensities as quantified by densitometry.

it reduced to 0.76-fold after 30 min of heat shock (Figure 5(d) and (f)), while its level in the insoluble fraction increased to 1.8-fold until about 60 min of heat stress, after which there was a decrease (Figure 5(e) and (f)). These results suggest that α B-crystallin is differentially phosphorylated at different serine residues and may influence its association with actin stress fibers.

Association of α B-crystallin with actin filaments during heat stress depends upon its phosphorylation

In order to study the role of phosphorylation of α B-crystallin in its association with actin stress fibers, we have transiently transfected H9C2 cells with an HA-tagged non-phosphorylatable mutant of α B-crystallin (the three serine residues are substituted by alanine). Our data show that the distribution of the 3A-mutant is similar to that seen for wild-type α B-crystallin in unstressed cells (Figure 6(a)). However, unlike wild-type α B-crystallin, the 3A-mutant fails to co-localize with actin stress fibers even after heat stress for 60 min (Figure 6(b)). Thus, phosphorylation of α B-crystallin appears to be important for its association with actin stress fibers.

We have used specific inhibitors of the p44 (PD98059) and p38 (SB202190) microtubule-associated protein (MAP) kinases (MAPK) that were shown earlier to be involved in the site-specific phosphorylation of Ser45 and Ser59 residues, respectively.³⁴ Our results show that inhibition of Ser59 (Figure 7(a)) as well as Ser45 phosphorylation of α B-crystallin almost completely inhibits (Figure 7(b)) its association with actin stress fibers. Thus, our study suggests that the association of α B-crystallin with actin filaments is phosphorylation-dependent, and that phosphorylation of both Ser45 and Ser59 is essential for this association.

Functional significance of α B-crystallin association with actin filaments

Disruption of actin filaments is among the most immediate early effects of various stresses such as hypoxia and hyperthermia, which ultimately lead to cell death.^{16–18,36} We, therefore, hypothesize that α B-crystallin may stabilize actin microfilaments by associating with them and hence prevent their disorganization. We treated normal, unstressed, or heat-stressed H9C2 cells with cytochalasin B (3 μ g/ml), an inhibitor of actin polymerization, and studied its effect on the association of α B-crystallin with actin stress fibers. In unstressed cells, α B-crystallin shows cytoplasmic localization (Figure 8(a)). Figure 8(b) shows that actin stress fibers are reduced significantly in H9C2 cells not subjected to heat stress but treated with cytochalasin B. Under these conditions, the degree of co-localization of α B-crystallin with actin is minimal. On the other hand, when the cells are subjected to heat stress and then treated with cytochalasin B, cells retain stress fiber morphology and show significant association with α B-crystallin (Figure 8(c)). Thus, our results suggest that α B-crystallin prevents heat-induced disorganization and helps in the maintenance of filamentous architecture of actin.

Pinocytosis analysis

The results obtained so far demonstrate that association of α B-crystallin with actin stress fibers protects the cytoskeletal network and prevents heat-induced damage to the cells. Cellular processes like pinocytosis and cell motility involve extensive polymerization and depolymerization of actin microfilaments.^{27,28} Therefore, we investigated the possible role of α B-crystallin in regulating physiological activities such as pinocytosis during heat stress as reflected by the uptake of fluorescein

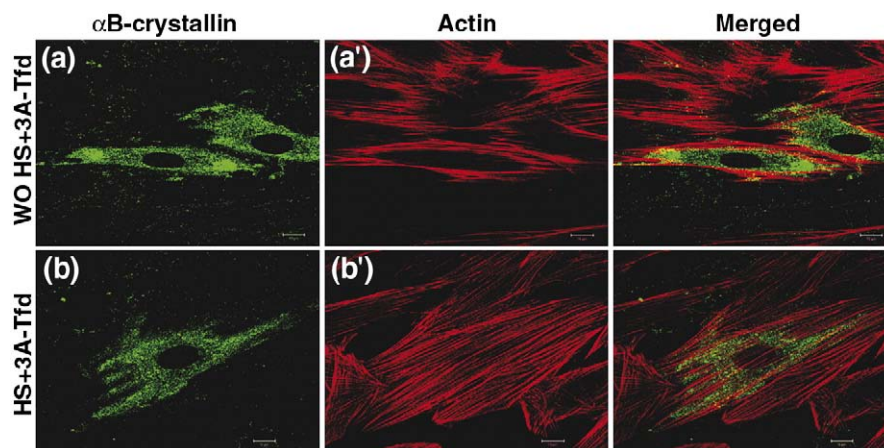


Figure 6. Phosphorylation status of α B-crystallin affects its association with actin fibers. H9C2 cells grown on glass coverslips were transfected with a non-phosphorylatable HA-3A-mutant of α B-crystallin. The transfected cells, either unstressed or heat-stressed at 43 °C for 60 min, were fixed and stained with anti-HA antibodies for the mutant α B-crystallin and with rhodamine-phalloidin for actin fibers. (a) and (a') Staining for the HA-epitope and actin fibers in unstressed cells. (b) and (b') The staining pattern in cells subjected to heat-stress. Merged panels show the overlays of confocal images of dual-stained cells in a single optical section of 0.3 μ m. The scale bar represents 10 μ m.

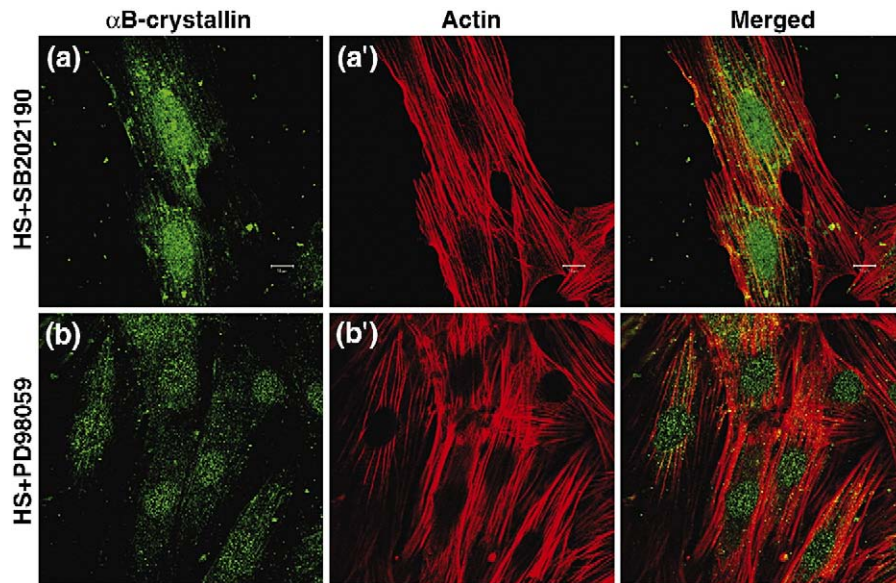


Figure 7. Effect of MAPK inhibitors on the association of α B-crystallin with actin fibers. H9C2 cells were treated with SB202190 (p38 MAPK inhibitor) or PD098059 (p44 MAPK inhibitor) for 18 h and later subjected to heat stress at 43 °C for 60 min and fixed with 3.7% (v/v) formaldehyde permeabilized and stained with rabbit polyclonal antibodies for α B-crystallin and with rhodamine-phalloidin for actin fibers. (a) and (b) The staining pattern for α B-crystallin or (a') and (b') for actin after treatment of cells with SB202190 and or PD098059, respectively. Merged panels show the overlays of confocal images of dual-stained cells in a single optical section of 0.3 μ m. The scale bar represents 10 μ m.

isothiocyanate (FITC)-dextran particles.³⁷ Cytochalasin B treatment is known to dissociate actin filaments and hence interfere with several physio-

logical activities including pinocytosis.³⁸ Our study shows that in unstressed cells treated with cytochalasin B, the uptake of FITC-lysine dextran is

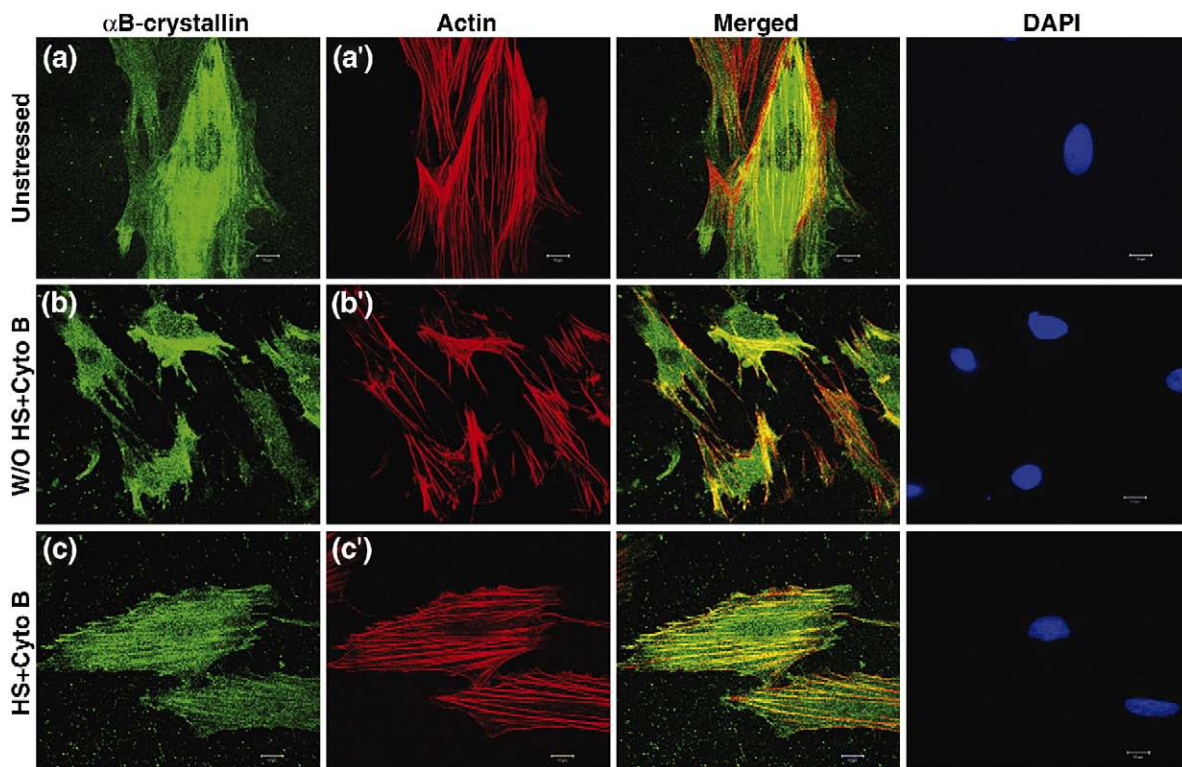


Figure 8. Effect of cytochalasin B, on the association of α B-crystallin with actin fibers. H9C2 cells, unstressed or heat-stressed at 43 °C for 60 min, were subsequently incubated with 3 μ g/ml of cytochalasin B for 15 min, fixed with 3.7% (v/v) formaldehyde, permeabilized and stained for (a)–(c) α B-crystallin (Panels a–c) or for (a')–(c') actin fibers. Merged panels show the overlays of confocal images of dual-stained cells in a single optical section of 0.3 μ m. The scale bar represents 10 μ m.

approximately 0.54-fold (Figure 9(c) and Table 1) compared to that by normal, untreated cells (Figure 9(a)). On the other hand, there is a 1.57-fold increase in the uptake of the FITC-dextran particles, when cells are subjected to heat stress (Figure 9(b)). Interestingly, the uptake of FITC-particles in cells subjected to heat stress and subsequently treated with cytochalasin B is 1.4-fold (Table 1), showing no significant decrease (Figure 9(d)). Thus, the enhanced association of α B-crystallin with actin fibers in heat-stressed H9C2 cells reduces the inhibitory effects of cytochalasin B on pinocytosis. Our results, therefore, suggest that, in addition to protecting the organization of the cytoskeletal network, the association of α B-crystallin with actin fibers also helps in maintaining the functional integrity of cells subjected to stress.

Discussion

α B-Crystallin, a member of the small heat shock protein family, has been implicated in prevention of aggregation of non-native proteins and in several diverse cellular functions such as cell division, differentiation and apoptosis.^{10,11,13–15} Studies have shown that α B-crystallin interacts with proteins of cytoskeleton and modulates their dynamics *in vitro* and *in vivo*.^{26,39} The association of α B-crystallin with cytoskeletal proteins alters under conditions of stress and helps in maintenance and regulation of their dynamics.⁴⁰ Actin, a cytoskeletal protein, is

Table 1. Uptake of FITC-dextran particle by H9C2 cells

Treatments	Total fluorescence Intensity	Fold difference from unstressed cells
Unstressed cells	392.75	1.00
Heat-stressed cells	618.7	1.57
Unstressed cytochalasin B-treated cells	214.8	0.54
Heat Stressed cytochalasin B-treated cells	551.25	1.4

highly susceptible to stressors such as heat stress and acidic environment.^{16,18} Bennardini *et al.* demonstrated that α B-crystallin could interact with actin and desmin filaments *in vitro* and their binding affinity increases after heat treatment.²⁹ However, such interaction of α B-crystallin with actin filaments *in vivo* and the consequence of this interaction in stress protection is not clearly understood. Here, we report the *in vivo* interaction of α B-crystallin with actin filaments, temporal changes during heat stress and its protective role. We find that this interaction of α B-crystallin prevents heat-induced disorganization of actin filaments. Thus, our results suggest an important role for α B-crystallin in the maintenance of cellular functions such as pinocytosis.

Our present data together lend further support to the view that α B-crystallin plays a significant role in protection of the cytoskeletal organization. Figure 1 shows the differential partitioning of α B-crystallin

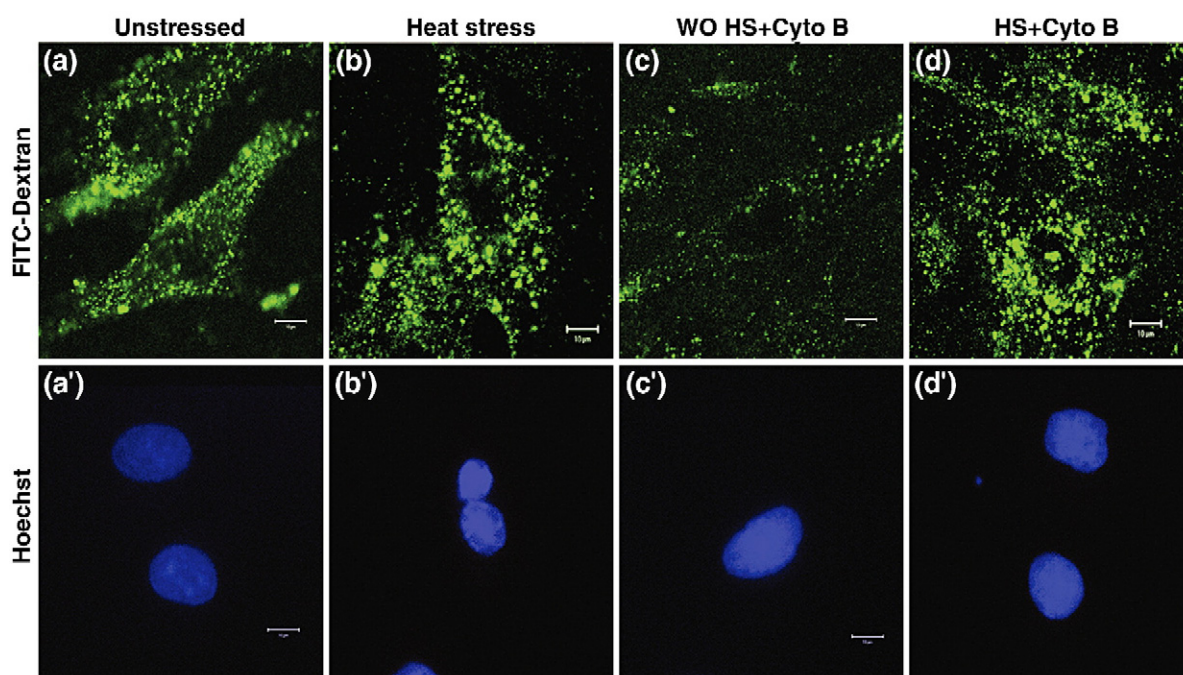


Figure 9. Role of α B-crystallin in pinocytosis. Pinocytosis in H9C2 cells was studied by the uptake of FITC-lysine dextran particles and visualized by confocal laser microscopy. H9C2 cells, (a) unstressed and (c) treated with 3 μ g/ml of cytochalasin B for 15 min or (b) heat-stressed at 43 °C for 60 min and (d) treated with cytochalasin B were subsequently incubated with 10 mg/ml of FITC-lysine dextran for 20 min. The cells were fixed with 3.7% (v/v) formaldehyde and analyzed by confocal microscopy. (a')–(d') The nuclei stained with Hoechst 33258. The scale bar represents 10 μ m.

into the soluble and insoluble fraction in a time-dependent manner; under conditions of stress, α B-crystallin partitions preferentially into the insoluble fraction. Dual-staining confocal image analysis shows that α B-crystallin appears as cytoplasmic strands and aligns with actin stress fibers (Figures 2 and 3). The formation of strands in the cytoplasm upon heat stress and the co-localization of α B-crystallin with actin filaments clearly demonstrate its association with cytoskeletal elements *in vivo*. Further, immunoprecipitation experiments have shown that α B-crystallin interacts with actin filaments (Figure 4). Earlier studies have shown that the interaction of α B-crystallin with actin filaments *in vitro* is phosphorylation-dependent.³¹

Phosphorylation of α B-crystallin seems to play a major role in its localization, interaction with target proteins and its cellular functions.^{41–43} Western blot analysis using Ser59 and Ser45 phospho-specific antibodies show that heat stress leads to phosphorylation of these residues. Interestingly, we observed stress-induced temporal changes in the partitioning of phosphorylated α B-crystallin from the soluble to the insoluble fraction, the partitioning being dependent on phosphorylation of Ser59 and Ser45 residues (Figure 5). These data suggest that upon stress, α B-crystallin is phosphorylated at Ser59 and Ser45 residues and subsequently it binds to actin filaments, thereby depleting its concentration in the soluble fraction. On the other hand, concentration of α B-crystallin phosphorylated at Ser45 decreases gradually in the soluble fraction, while in the insoluble fraction it increases up to 45 min and decreases thereafter. The reason for the observed decrease in Ser45-phosphorylated α B-crystallin from the insoluble pool is not clear. Phosphorylation of serine residues is necessary for α B-crystallin binding to actin. In contrast, phosphorylation of HSP27 leads to its dissociation from actin.⁴⁴ Further studies are necessary to investigate whether dephosphorylation of α B-crystallin might have a role in its dissociation from actin. Phosphorylation leads to extensive redistribution of α B-crystallin upon heat stress in a time-dependent manner. It appears that phosphorylation at Ser45 or Ser59 is essential for its interaction with actin fibers, since inhibition of phosphorylation at either of these sites using specific inhibitors abrogates this interaction. Inhibition of α B-crystallin phosphorylation by MAPK inhibitors (SB202190 and/or PD98059) may affect other proteins as well, and could result indirectly in the failure of α B-crystallin to localize with stress fibers. The fact that non-phosphorylatable 3A- α B-crystallin fails to associate with actin filaments even in the absence of MAPK inhibitors, suggests that the association of α B-crystallin with actin is phosphorylation-dependent (Figures 6 and 7). It is possible that phosphorylation makes α B-crystallin competent to associate with actin filaments. Interestingly, we observed that both phosphorylated α B-crystallin and the 3A-mutant exhibit similar oligomeric size (smaller than α B-crystallin) in a glycerol density-gradient centrifugation using H9C2 cell lysates (data

not shown). This result implies that change in size *per se* is not sufficient for the association of α B-crystallin with actin filaments. Rather, it appears that phosphorylation and hence its effects, if any, on the local conformation may be important for such interaction. Our studies show also that α B-crystallin protects the filamentous architecture of actin fibers from cytochalasin B-induced depolymerization (Figure 8). This protection seems to be phosphorylation-dependent as, upon treatment of cells with p38/p44 MAPK inhibitors, the cellular architecture was lost and very few actin filaments were seen (data not shown). Launay *et al.* have reported that treatment of cells with agents that disturb the cytoskeleton can lead to the activation of p38 MAPK and phosphorylation of Ser59 residue of α B-crystallin, which in turn protects the cytoskeleton.⁴⁵ Similar results have been shown for HSP27, where the regulation of the actin filament dynamics is p38 MAPK-dependent;⁴⁶ whether these two sHSPs function in a coordinated or independent manner is not clear. It has been shown that α B-crystallin interacts with MAPs and protects the microtubules from nocodazole-induced or calcium-induced disassembly.²⁵ We speculate that α B-crystallin might form a coat around actin filaments and prevent cytochalasin B-induced disorganization of the actin stress fiber architecture. Stress fiber formation and membrane ruffling are associated with increased pinocytosis, an essential cellular function.^{47,48} We have investigated the effect of heat stress and cytochalasin B in the pinocytotic process. We find that unstressed, cytochalasin B-treated cells show dramatically reduced pinocytosis compared to heat-preconditioned, cytochalasin B-treated cells as reflected by FITC-dextran particle uptake (Figure 9). Heat stress effects on pinocytosis have been shown for other cells, such as B-cells and dendritic cells. In these cells, increased pinocytosis leads to increased antigen presentation.⁴⁹ Pinocytosis plays a significant role in nutrient and drug uptake in several types of cells.^{50,51} Our studies suggest that α B-crystallin maintains the cellular architecture by stabilizing actin stress fibers and helps in the preservation of functional integrity of H9C2 cells subjected to heat stress. Altered physicochemical properties and dynamics of actin filaments can lead to acute myocardial infarction and heart failure.⁵² Hence, association of α B-crystallin with actin stress fibers may perform a crucial role in maintaining structural and functional integrity of cardiac tissue and help to prevent myocardial cell death.

Materials and Methods

Antibodies and reagents

Rabbit polyclonal antibodies for α B-crystallin, phosphoserine-59- α B-crystallin and phosphoserine-45- α B-crystallin were obtained from Stressgen Biotechnologies, Canada; monoclonal anti-HA antibodies and Protease

Inhibitor Cocktail were from Roche Applied Sciences, USA. FITC-dextran, rhodamine-phalloidin conjugates, and Alexa-488-conjugated secondary antibodies were from Molecular Probes, Oregon, USA. DMEM, fetal calf serum and cytochalasin B were from Sigma Chemical Company, USA. Lipofectamine-2000 was from Invitrogen, USA. HRPO-conjugated anti rabbit secondary antibodies and Enhanced Chemiluminescence (ECL) Western blot detection kit were from Amersham Biosciences, USA. Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories, USA. Actin monoclonal antibodies, inhibitors of p44 MAPK (PD098059) and p38 MAPK (SB202190) were from Calbiochem, EMB Biosciences Inc., Germany.

Cell culture and heat treatment

The H9C2 rat myoblast cell line was maintained at sub-confluent densities in DMEM supplemented with 10% (v/v) fetal calf serum, 100 units/ml of penicillin and 100 μ g/ml of streptomycin at 37 °C in a humidified chamber containing 5% (v/v) CO₂. Cells grown in T75 flasks were incubated at 43 °C in a waterbath for different lengths of time as indicated. Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), containing 50 mM NaCl, 5 mM EDTA, 1 mM PMSF, 50 mM NaF, 0.2 mM Na₃VO₄, 0.2% (v/v) NP-40 and Protease Inhibitor Cocktail). For immunofluorescence studies, H9C2 cells grown on coverslips were subjected to heat shock at 43 °C for different lengths of time, as indicated. For recovery, H9C2 cells subjected to heat stress for 60 min were incubated at 37 °C in a humidified CO₂ incubator. Cells were fixed with 3.7% (v/v) formaldehyde after each time-point and processed for confocal analysis.

Plasmid and transient transfection

The plasmid pCDNA 3.1HA-3A α B-crystallin was a kind gift from Dr C. Glembotski.⁴³ H9C2 cells were grown on coverslips and transfected with HA-3A α B-crystallin mutant using lipofectamine reagent according to the manufacturer's protocol. At 48 h post-transfection, the cells were given a heat shock (43 °C for 1 h) and fixed immediately with 3.7% formaldehyde. These cells were stained with rhodamine-phalloidin for actin and with anti-HA mAb for HA-epitope.

Immunofluorescence microscopy

H9C2 cells were grown on coverslips and given a heat-shock as described above. Cells were then washed twice with ice-cold cytoskeleton buffer (10 mM Pipes (pH 7.0), 150 mM NaCl, 5 mM glucose, 5 mM MgCl₂) and fixed with 3.7% formaldehyde. For recovery, heat-stressed cells were kept at 37 °C in a humidified CO₂ incubator. The fixed cells were permeabilized with 100% acetone at -20 °C for 8 min. After blocking with 2% (w/v) BSA, cells were incubated with rabbit α B-crystallin polyclonal antibodies followed by incubation with Alexa 488 secondary antibody. For dual staining, the cells were incubated with rhodamine-phalloidin conjugate for 1 h to visualize F-actin after immunostaining for α B-crystallin. The cells were mounted in Vectashield medium containing DAPI. Confocal laser scanning microscopy was performed with a Carl Zeiss inverted microscope. Image analysis, including crossover subtractions and estimation of the

extent of co-localization was done using LSM-FCS software (version 3.2).

Immunoprecipitation

The immunoprecipitation experiments were performed essentially as described.⁵³ H9C2 cells were heat-stressed at 43 °C for 1 h then lysed in 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM PMSF, 0.2 mM Na₃VO₄, 0.2% NP40, 10% (v/v) glycerol for 15 min at 4 °C and centrifuged at 14,000 rpm for 10 min. The supernatant containing soluble proteins was collected separately. The pellet was solubilized in IP buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM PMSF, 0.2 mM Na₃VO₄, 0.2% NP40, 1% (w/v) SDS, 0.5% (v/v) Triton X-100, 10% glycerol and Protease Inhibitor Cocktail). The supernatant and proteins solubilized from the pellet were incubated at 4 °C overnight with rabbit polyclonal α B-crystallin antibodies. Subsequently, protein-G magnetic beads were added to these samples and they were incubated further for 4 h. The magnetic bead complex was retrieved using a magnetic separator kit (Bangs Laboratories Inc. USA). The immunoprecipitated complex was washed three times with ice-cold IP buffer and suspended in Laemmli buffer. The samples were heated in a boiling water-bath for 5 min, subjected to SDS-PAGE and processed for Western blotting using appropriate antibodies.

F-actin stability test

H9C2 cells grown on glass coverslips were incubated either at 37 °C or subjected to heat stress at 43 °C for 1 h and further treated with cytochalasin B (3 μ g/ml) for 15 min. Immediately after treatment, the cells were fixed with 3.7% formaldehyde, stained for α B-crystallin and F-actin, and analyzed by confocal microscopy. H9C2 cells not subjected to any of the above treatments served as controls.

Treatment with kinase inhibitors

H9C2 cells grown on glass cover-slips, were treated either with 20 μ M SB202190 (a p38 MAPK inhibitor) or 50 μ M PD098059 (a p44 MAPK inhibitor) for 18 h before and during the heat treatment. Heat-stressed and control cells were fixed with formaldehyde for staining as described earlier. Concentrations of the vehicle, DMSO, used to dissolve the inhibitors, did not have any significant effect on the staining pattern.

SDS-PAGE and Western blot analysis

Heat-stressed and control cells were harvested and lysed in ice-cold lysis buffer, sonicated and centrifuged at 11,000g for 10 min at 4 °C. The supernatant represents the soluble fraction, and the pellet represents the insoluble protein fraction. Total cell lysates were prepared by solubilizing the cell pellet directly in Laemmli buffer. The total cell lysate, the soluble fraction and the insoluble fraction were subjected to SDS-PAGE (12 polyacrylamide gel) and transferred to nitrocellulose membrane using a semi-dry transfer apparatus (Amersham Pharmacia). The membrane was blocked with 10% (w/v) milk protein, incubated sequentially with appropriate primary antibodies and HRPO-conjugated secondary antibodies, and visualized by chemiluminescence (Amersham Biosciences,

USA) according to the manufacturer's instructions. The band intensities were quantified by densitometry. The ratio of band intensities of α B-crystallin and that of the corresponding loading control, expressed in arbitrary units was used for comparison. The values represent an average of a minimum of three independent experiment.

Pinocytosis analysis

H9C2 cells were grown on glass cover-slips. Unstressed or heat-stressed (43 °C for 1 h) H9C2 cells were treated with cytochalasin B (3 μ g/ml for 15 min) and further incubated with FITC-lysine dextran (10 mg/ml for 20 min). Unstressed or heat-stressed H9C2 cells incubated with FITC-lysine dextran served as controls. The cells were then fixed with 3.7% formaldehyde and incubated with Hoechst-33258 for staining nuclei. Uptake of FITC-lysine dextran was visualized by confocal microscopy.

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