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Stress inhibits nucleocytoplasmic shuttling of heat shock protein hsc70

Mohamed Kodiha, Angel Chu, Omar Lazrak, and Ursula Stochaj
Department of Physiology, McGill University, Montreal, Quebec, Canada

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Kodiha, Mohamed, Angel Chu, Omar Lazrak, and Ursula Stochaj. Stress inhibits nucleocytoplasmic shuttling of heat shock protein hsc70. *Am J Physiol Cell Physiol* 289: C1034–C1041, 2005. First published June 1, 2005; doi:10.1152/ajpcell.00590.2004.—Heat shock proteins of the hsp/hsc70 family are essential chaperones, implicated in the stress response, aging, and a growing number of human diseases. At the molecular level, hsc70s are required for the proper folding and intracellular targeting of polypeptides as well as the regulation of apoptosis. Cytoplasmic members of the hsp/hsc70 family are believed to shuttle between nuclei and cytoplasm; they are found in both compartments of unstressed cells. Our experiments demonstrate that actin filament-destabilizing drugs trigger the nuclear accumulation of hsc70s in unstressed and heat-shocked cells recovering from stress. Using human-mouse heterokaryons, we show that stress inhibits shuttling and sequesters the chaperone in nuclei. The inhibition of hsc70 shuttling upon heat shock is only transient, and transport is reestablished when cells recover from stress. Hsc70 shuttling is controlled by hsc70 retention in the nucleus, a process that is mediated by two distinct mechanisms, ATP-sensitive binding of hsc70s to chaperone substrates and, furthermore, the association with nucleoli. The nucleolar protein fibrillarin and ribosomal protein rpS6 were identified as components that show an increased association with hsc70s in the nucleus upon stress exposure. Together, our data suggest that stress abolishes the exit of hsc70s from the nucleus to the cytoplasm, thereby limiting their function to the nuclear compartment. We propose that during recovery from stress hsc70s are released from nuclear and nucleolar anchors, which is a prerequisite to restore shuttling.

nuclear transport; chaperone; nuclear retention; nucleoli

HEAT SHOCK PROTEINS ARE INVOLVED in numerous cellular functions, including folding of newly synthesized polypeptides and targeting of proteins to their proper cellular location. In particular, chaperones of the hsp/hsc70 family are essential to these processes (6, 10, 11). The hsp/hsc70s play a crucial role in the appropriate response to stress and the survival of stress-induced damage, processes that are relevant to a large number of human diseases and pathophysiological conditions (2). Moreover, hsp/hsc70s are implicated in the regulation of apoptosis, tumorigenesis, and aging (1, 7, 9). In eukaryotic cells under normal growth conditions, cytoplasmic hsp/hsc70s are believed to move in and out of the nucleus, and it was demonstrated in *Xenopus* oocytes that they shuttle between nucleus and cytoplasm (13). Unlike other members of the hsp70/hsc70 family, hsc70 (also referred to as hsp73 or hsp70-8) is essential for the survival of normal and tumor cells (17). Hsc70s concentrate in nuclei when cells are exposed to stress, and heat shock is the most efficient treatment to induce their accumulation in nuclei (4). Although heat increases the steady-state concentration of hsc70s in nuclei, it is not known whether stress also controls their movement between nucleus and cytoplasm. However, this knowledge is required to understand the dynamics of hsc70 localization under different physiological conditions. To address this question, we have monitored the distribution of endogenous hsc70s and the reporter protein EGFP-hsc70 (enhanced green fluorescent protein fused to hsc70) in human cultured cells. Our results demonstrate that exit of hsc70s from the nucleus upon recovery from stress is an active process and that heat shock restricts the nucleocytoplasmic trafficking of hsc70s. After heat exposure, hsc70 shuttling is prevented but is restored when cells recover from this environmental insult. We have identified stress-induced nuclear retention of hsc70 as a mechanism that controls shuttling of the chaperone.

MATERIALS AND METHODS

Nuclear reporter proteins. The fluorescent protein NLS-NES-GFP2 carries SV40 nuclear localization sequence (NLS) and the nuclear export sequence (NES) of inhibitor of cAMP-dependent protein kinase (PKI) fused to two copies of GFP (18). The NLS-NES-GFP2 coding sequence was transferred to a vector that mediates inducible expression in mammalian cells (3). A plasmid encoding hsc70 (kindly provided by Drs. S. Wax and N. Kedersha, Harvard Medical School, Boston, MA) was used to generate a fusion between EGFP and hsc70. To this end, the hsc70 coding sequence was cloned into the *Bam*HI site of vector pEGFP-C1 (Clontech, Palo Alto, CA). The correctness of the construct was verified by DNA sequencing; the fusion protein is referred to as EGFP-hsc70. EGFP-hsc70 shows the same distribution as authentic hsc70 when analyzed in control and stressed cells (data not shown; see Fig. 4). A schematic representation of the constructs used for transfection is shown in Fig. 1.

Transfection of HeLa cells. At a confluency of ~70%, HeLa cells were transfected in six-well plates with Effectene (Qiagen, Mississauga, ON, Canada), following the manufacturer's recommendations. Transient gene expression in HeLa cells with a dexamethasone-inducible system was described in detail previously (3, 4). Transfected cells were grown for 24 h on polylysine-coated coverslips in six-well plates or on multiwell slides before exposure to stress.

Treatment with leptomycin B, latrunculin B, and cytochalasin B. To analyze the potential role of the importin-β family member Crm1, we have tested the effect of leptomycin B (LMB), a compound that selectively inhibits this exporter (12). To this end, cells were incubated with 10 ng/ml LMB (gift of M. Yoshida, University of Tokyo, Tokyo, Japan) dissolved in ethanol or with the solvent ethanol for 15 h at 37°C after heat stress. Unstressed cells were incubated with LMB

Address for reprint requests and other correspondence: U. Stochaj, Dept. of Physiology, McGill Univ., 3655 Promenade Sir William Osler, Montreal, QC, H3G 1Y6, Canada (e-mail: ursula.stochaj@mcgill.ca).

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Fig. 1. Nuclear reporter proteins used in this study. NLS-NES-GFP2 is a fluorescent protein that contains two copies of green fluorescent protein (GFP) fused to nuclear localization sequence (NLS) of SV40 T-antigen and nuclear export sequence (NES) of the inhibitor of cAMP-dependent protein kinase. The reporter protein shuttles and at steady state is concentrated in the cytoplasm. The reporter protein EGFP-hsc70 was generated by in-frame fusion of enhanced GFP (EGFP) to the 5′-end of the hsc70 coding sequence. The synthesis of NLS-NES-GFP2 is controlled by a regulatable promoter that contains 5 copies of glucocorticoid response elements (GRE). Addition of dexamethasone to the growth medium will induce gene expression. Expression of the EGFP-hsc70 gene is driven by a cytomegalovirus (CMV) immediate early promoter (P_{CMV}). Both plasmids carry an SV40 polyadenylation signal (SV40 polyA).

for up to 24 h at 37°C. Latrunculin B and cytochalasin B (Calbiochem, San Diego, CA) were dissolved in DMSO. Cells recovering from stress were incubated for 15 h at 37°C with 1 mM latrunculin B, 10 μM cytochalasin B, or the solvent DMSO. Unstressed cells were treated with latrunculin B, cytochalasin B, or DMSO for 3 h at 37°C, as indicated in Figs. 3 and 4. In control experiments, the effect of latrunculin B or cytochalasin B on actin polymerization was tested with FITC-labeled phalloidin, following the supplier's protocol (Sigma, Oakville, ON, Canada).

Immunofluorescence. All steps were carried out at room temperature essentially as described previously (4). In brief, cells were washed in PBS and fixed for 25 min at room temperature in 3.7% formaldehyde-PBS. Fixed cells were rinsed in PBS and permeabilized with 0.1% Triton X-100 in PBS-2 mg/ml BSA (5 min, room temperature). All subsequent steps were carried out in PBS-2 mg/ml BSA-0.05% Tween 20. Samples were incubated overnight with primary antibodies against hsc70s or fibrillarin [SPA-815, StressGen (Victoria, BC, Canada); diluted 1:1,000; Santa Cruz Biotechnology, sc-11335, diluted 1:1,000]; primary antibodies were detected with 1.5 μg/ml Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). NLS-NES-GFP2 was visualized with polyclonal rabbit antibodies to GFP (Clontech, diluted 1:200) and 5 μg/ml secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR). To detect rpS6 (Santa Cruz Biotechnology, sc-13007, diluted 1:500), cells were fixed in methanol-acetone (1:1, vol/vol) for 30 min at −20°C. All subsequent incubations and washes were carried out in PBS-BSA. DNA was located with 4′,6-diamidino-2-phenylindole (DAPI), and samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Cells were analyzed with a Nikon Optiphot at ×400 magnification and photographed with Kodak T-MAX 400 film. Negatives were scanned and processed with PhotoShop 5.5 and 8.0.

Human-mouse heterokaryons. Heterokaryons between HeLa and mouse NIH3T3 cells were generated by a modification of published procedures (5). In brief, HeLa cells transiently synthesizing EGFP-hsc70 were trypsinized and seeded on coverslips 24 h after transfection to reach ~60% confluency on the next day. HeLa cells were then exposed to 1 h of heat stress at 45.5°C, and 3×10^5 NIH3T3 cells were added to each well of a six-well plate. After 1.5 h, mouse cells adhered to the coverslips, and cycloheximide was added to 75 μg/ml for 30 min. Cells were fused subsequently for 2 min with 50% polyethylene glycol (PEG) 3350. After removal of PEG, samples were washed three times with PBS and incubated at 37°C in growth medium containing 100 μg/ml cycloheximide. Cells were fixed 3, 5, and 15 h after heat stress, i.e., 1, 3, and 13 h after fusion. Nuclei were stained with DAPI, and heterokaryons or homokaryons were monitored for the distribution of EGFP-hsc70.

Analysis of nuclear retention. Nontransfected HeLa cells were exposed for 1 h to 45.5°C and subsequently treated for 5 min with 40 μg/ml digitonin in *buffer B* on ice (19). Digitonin-extracted cells were incubated with *buffer B* [containing 5 mg/ml BSA and 0.05% Nonidet P-40 (NP-40)] for 15 min at room temperature. The buffer was supplemented with 2.5 mM ATP, 2.5 mM ADP, or 1 mM nonhydrolyzable ATP analog adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP), as indicated in Figs. 5 and 6. Samples were washed extensively in *buffer B*-BSA-NP-40, in *buffer B*, and twice in PBS and fixed and processed for indirect immunofluorescence with anti-hsc70 antibodies as described above. To monitor the intactness of nuclear envelopes, cells were extracted with digitonin, treated with *buffer B*-BSA-NP-40, and washed as described above. Washed samples were fixed, blocked with PBS-2 mg/ml BSA, and incubated with anti-lamin B antibodies (0.5 μg/ml; Santa Cruz Biotechnology, sc-6217). Control cells were treated with digitonin only before blocking and incubation with antibodies.

Protein cross-linking and immunoprecipitation. Control, stressed, and recovering cells were grown on 100-mm tissue culture dishes, washed with cold PBS, and extracted with 40 μg/ml digitonin in PBS for 5 min on ice. Samples were washed with PBS and incubated with 0.2 mM 3,3′-dithiobis(sulfosuccinimidylpropionate) in PBS for 1 h on ice. Dishes were washed with cold PBS and stored at −70°C until use. For immunoprecipitation, plates were incubated for 10 min on ice with IP buffer [in mM: 20 Tris·HCl, pH 8.0, 5 EDTA, and 150 NaCl with 1% NP-40, 10% glycerol, and protease inhibitors (aprotinin, antipain, chymostatin, leupeptin, pepstatin, each at 1 μg/ml; 1 mM PMSF)]. Samples were vortexed with glass beads, cleared by centrifugation (5 min, 13,000 rpm, 4°C), and incubated with 50 μl of protein G-Sepharose (Amersham Biosciences; 30 min, 4°C with gentle agitation). Resin was removed by centrifugation, and supernatants were incubated with 5 μg of anti-hsc70 for 2 h at 4°C, followed by addition of 50 μl of protein G-Sepharose and overnight incubation at 4°C. Beads were collected by centrifugation, washed three times in IP buffer, and incubated with gel sample buffer containing 1.4 M β-mercaptoethanol (15 min, 95°C). Material released from the resin was analyzed by Western blotting.

Western blot analysis. Western blotting and enhanced chemiluminescence detection was carried out essentially as described previously (4), using a Lumigen PS-3 detection kit (Amersham Biosciences).

RESULTS AND DISCUSSION

During recovery from heat stress, nuclear hsc70s relocate to the cytoplasm in temperature-dependent fashion that does not require de novo protein synthesis. Heat shock induces the rapid nuclear accumulation of hsc70s in HeLa cells, and a 1-h exposure to 45.5°C (severe heat shock) is sufficient to concentrate hsc70s in nuclei (Fig. 2). After heat stress, hsc70s relocate from nuclei to the cytoplasm when cells recover at 37°C, as monitored for different time points in Fig. 2. Several hours after heat treatment, hsc70s began to exit the nucleus; after 15 h of recovery, they distributed throughout the cell. This relocation of hsc70s was temperature dependent and was abolished when cells were incubated at 4°C, consistent with an active transport process (Fig. 2 and data not shown). Interestingly, when cells were kept at 4°C, hsc70s not only failed to relocate to the cytoplasm but also concentrated in nucleoli. During recovery at 37°C, nuclear export of hsc70s did not depend on de novo protein synthesis, because it was not abolished by cycloheximide (Fig. 2).

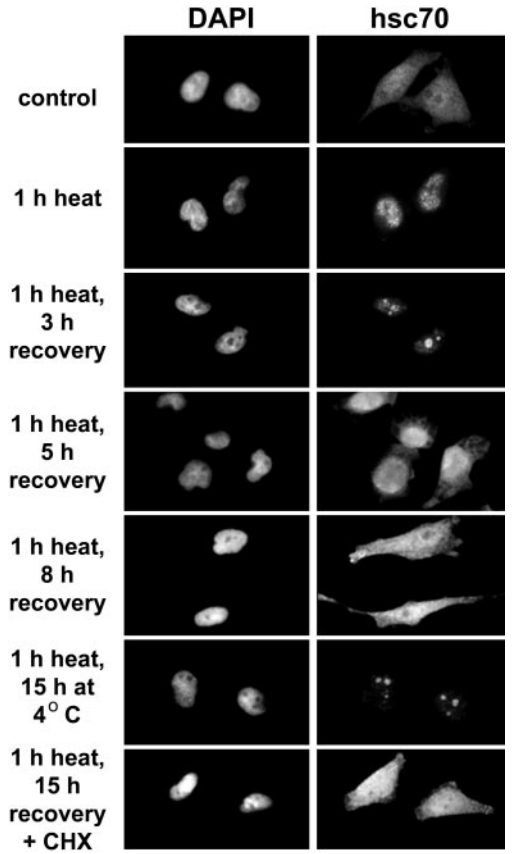

Fig. 2. Nuclear hsc70 relocation to the cytoplasm during recovery from heat shock is temperature dependent but does not require de novo protein synthesis. Hsc70s were localized by indirect immunofluorescence in unstressed cells (control) or upon exposure to severe heat shock (1 h at 45.5°C). After heat stress, cells recovered at 37°C for the times indicated. In addition, heat-shocked cells were kept for 15 h at 4°C or incubated at 37°C in the presence of 100 μg/ml cycloheximide (CHX). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

Hsc70 nuclear export does not require the transporter Crm1/exportin-1 in unstressed cells or during recovery from heat shock. Shuttling depends on nuclear import and export of proteins, and neither of these processes has been defined on a molecular level for members of the hsp/hsc70 family. The nuclear exporter Crm1/exportin-1 is involved in transport of many cargos, most of which contain a leucine-rich NES. This export pathway can be inhibited efficiently with LMB, a drug that covalently modifies the transporter Crm1/exportin-1 (12). Members of the hsc70/hsp70 families contain a conserved sequence element (i.e., positions 164–173 of mouse hsc70) that fits the consensus sequence for a hydrophobic NES recognized by Crm1. However, LMB did not prevent hsc70 export in cells recovering from heat stress (Fig. 3*A*). The same result was obtained when both LMB and cycloheximide were added during the recovery period. Likewise, LMB did not change the distribution of hsc70s in unstressed cells, even if the drug was present for up to 24 h (Fig. 3*C*).

In control experiments, LMB efficiently inhibited shuttling of NLS-NES-GFP2. This reporter protein carries a signal for nuclear localization (SV40-NLS) as well as nuclear export (PKI-NES); PKI-NES is recognized by Crm1. NLS-NES-

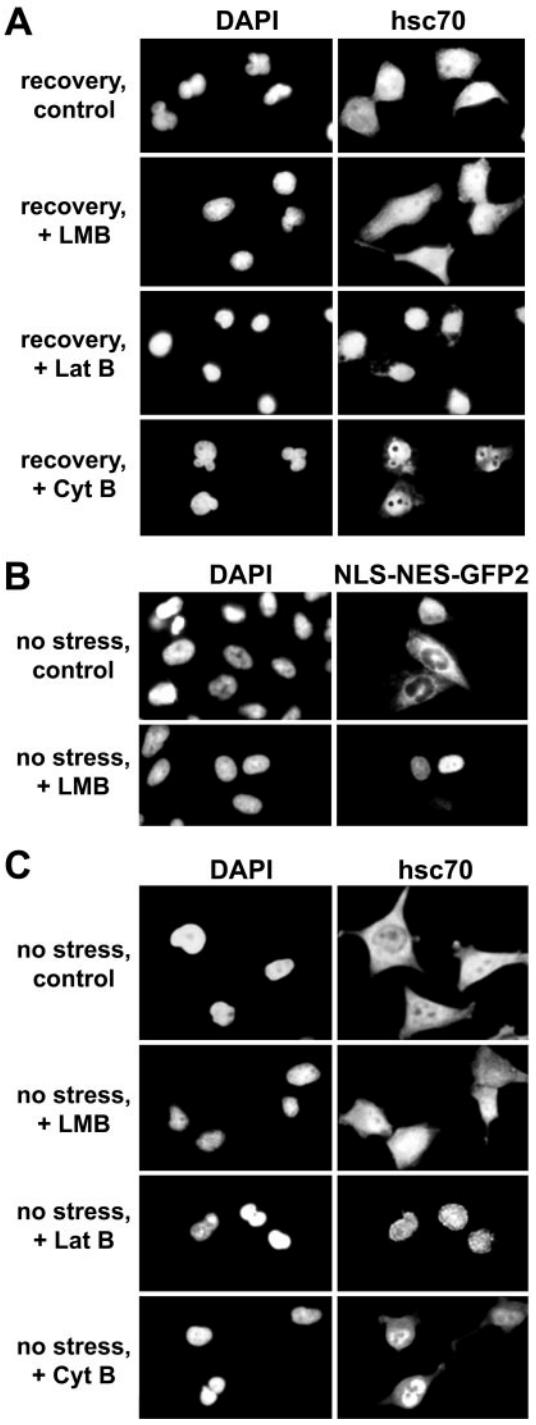

Fig. 3. Effect of leptomycin B (LMB), latrunculin B (Lat B), and cytochalasin B (Cyt B) on nuclear export of hsc70s and NLS-NES-GFP2. *A*: hsc70s were located in HeLa cells exposed to severe heat shock followed by 15-h recovery at 37°C. LMB, Lat B, and Cyt B were present throughout the recovery period as described in MATERIALS AND METHODS. *B*: HeLa cells transiently synthesizing NLS-NES-GFP2 were incubated for 15 h without or with 10 ng/ml LMB. *C*: unstressed cells were kept at 37°C and treated for 24 h with 10 ng/ml LMB. Alternatively, cells were incubated for 3 h at 37°C with 1 mM Lat B or 0.1 mM Cyt B. Hsc70s and NLS-NES-GFP2 were located by indirect immunofluorescence. Nuclei were visualized with DAPI.

GFP2 was both cytoplasmic and nuclear in the absence of LMB but accumulated in nuclei when LMB was added to the growth medium. Together, these results show that hsc70 nuclear export upon recovery from heat or under nonstress conditions does not rely on Crm1.

Actin filament-destabilizing drugs latrunculin B and cytochalasin B inhibit nuclear export of hsc70s in stressed and control cells. To further define hsc70 nuclear transport, we tested the effect of latrunculin B and cytochalasin B. These compounds are believed to affect actin located at the nuclear pore complex (NPC), thereby preventing nuclear export of various components (8). Incubation with latrunculin B or cytochalasin B drastically reduced the amount of actin filaments, which became obvious by the loss of phalloidin binding (not shown). Importantly, in cells recovering from heat shock, either drug prevented hsc70 export from the nucleus (Fig. 3*A*). Similarly, when unstressed cells were treated with latrunculin B or cytochalasin B, hsc70s concentrated in nuclei and nuclear accumulation were apparent after a 3-h treatment. These results support the idea that under normal physiological conditions, i.e., in the absence of stress, hsc70s shuttle between nucleus and cytoplasm in human culture cells. Furthermore, hsc70 export is abolished by the destabilization of filamentous actin, suggesting a role for actin in the translocation of nuclear hsc70s to the cytoplasm. In particular, actin located at the NPC could play a crucial role because it seems to be involved in nuclear export of multiple cargos (8).

Hsc70 shuttling is inhibited by heat shock and restored when cells recover from stress. Heterokaryons have been used to analyze the shuttling of proteins that are concentrated in nuclei at steady state under normal growth conditions. However, this approach has not been applied previously to monitor shuttling in heat-stressed cells. To achieve this, we used the fluorescent reporter protein EGFP-hsc70, which shares the biological properties of endogenous hsc70s when tested under a variety of stress conditions (Fig. 4*A*; M. Kodiha and U. Stochaj, unpublished observations). In unstressed cells, EGFP-hsc70 was distributed throughout nuclei and cytoplasm (Fig. 4*A*). Like endogenous hsc70s, EGFP-hsc70 accumulated in nuclei when cytochalasin B or latrunculin B was added to the growth medium (Fig. 4*A*).

Human-mouse heterokaryons were used to evaluate EGFP-hsc70 shuttling after stress exposure; these heterokaryons contain nuclei from both species, which share the same cytoplasm (5). EGFP-hsc70 was first concentrated in nuclei of HeLa cells

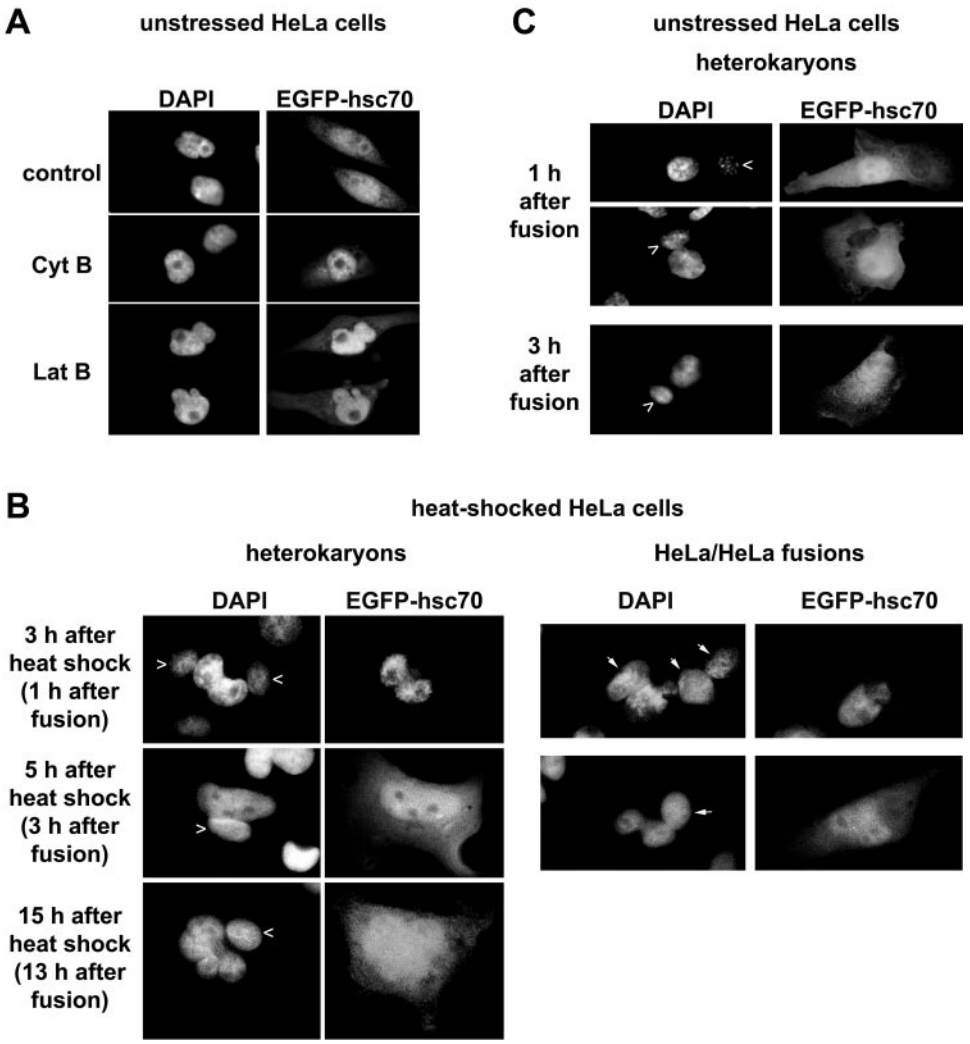

Fig. 4. Hsc70 shuttles in unstressed, but not heat-shocked, cells. *A*: HeLa cells synthesizing EGFP-hsc70 were incubated with the solvent DMSO, Cyt B, or Lat B for 3 h at 37°C. In fixed cells, EGFP-hsc70 and nuclei (DAPI) were located by fluorescence microscopy. *B*: HeLa cells synthesizing EGFP-hsc70 were heat shocked and fused to mouse NIH3T3 cells. EGFP-hsc70 was localized in fixed heterokaryons 3, 5, and 15 h after heat treatment, equivalent to 1, 3, and 13 h after fusion. Homokaryons generated by fusion of several HeLa cells were analyzed in parallel. Arrowheads mark the position of mouse nuclei in heterokaryons, and arrows point to the nuclei of nontransfected HeLa cells in homokaryons. *C*: heterokaryons obtained after fusion with unstressed HeLa cells were fixed at 1 and 3 h after fusion, comparable to samples shown for 3 and 5 h after heat shock in *B*. Mouse nuclei are labeled with arrowheads.

by heat shock for 1 h at 45.5°C. HeLa cells were returned subsequently to the normal growth temperature and fused to mouse cells. In these heterokaryons, we localized EGFP-hsc70 at different time points during their recovery from heat exposure. (It should be noted that EGFP-hsc70 synthesized in HeLa cells is the only source of fluorescence seen in Fig. 4*B*, because cycloheximide prevents de novo synthesis of EGFP-hsc70 in heterokaryons.) Three hours after heat shock, EGFP-hsc70 remained restricted to human nuclei in human-mouse heterokaryons (Fig. 4*B*). By contrast, EGFP-hsc70 was absent from mouse nuclei and the common cytoplasm, demonstrating that the translocation from human nuclei to the cytoplasm was prevented at this point. To determine whether this export inhibition, and thereby the block in shuttling, was reversible, heterokaryons were allowed to recover for a longer period of time. At 5 h after heat shock, EGFP-hsc70 began to migrate out of the human nucleus and appeared in the common cytoplasm. After a 15-h recovery period, human and mouse nuclei displayed comparable signals for EGFP-hsc70, showing that shuttling of the chaperone had resumed.

The absence of EGFP-hsc70 from mouse nuclei at early time points after cell fusion is not simply a failure of the nonstressed mouse nuclei to import the chaperone. While generating heterokaryons, we also obtained fusions originating from a mixture of transfected and nontransfected HeLa cells, the latter were not synthesizing EGFP-hsc70. In these multinucleated cells, or homokaryons, 3 h after heat shock we detected nuclei that did not contain EGFP-hsc70 (Fig. 4*B*). As observed for heterokaryons, EGFP-hsc70 appeared in the common cytoplasm of homokaryons at 5 h upon heat exposure and began to migrate into all of the nuclei present. At 15 h after heat shock, EGFP-hsc70 was present in all of the nuclei of multinucleated cells, and nuclei from transfected and nontransfected cells could no longer be distinguished.

For comparison, heterokaryons were generated with the protocol described above, but with unstressed instead of heat-treated HeLa cells. When inspected 1 and 3 h after fusion, the times equivalent to 3 and 5 h after heat shock, EGFP-hsc70 was detected at the earlier time point in all mouse nuclei present in heterokaryons (Fig. 4*C*). At 1 h after fusion, the amount of EGFP-hsc70 in mouse nuclei was somewhat variable between different heterokaryons. Three hours after fusion, the time equivalent to 5 h after heat exposure for the stressed cells shown in Fig. 4*B*, the signal for EGFP-hsc70 was comparable to that for mouse and HeLa nuclei of heterokaryons (Fig. 4*C*). Together, the data obtained for heterokaryons support the idea that EGFP-hsc70 appears faster in mouse nuclei when unstressed HeLa cells are the source of the fusion protein.

Hsc70s are retained in nuclei of heat-shocked cells. Shuttling between nucleus and cytoplasm can be regulated on different levels; this includes import, export, and retention of the shuttling protein. As such, the movement of nuclear hsc70s to the cytoplasm could be controlled by retention in the nucleus. The liberation from nuclear anchors would be a rate-limiting step for shuttling because this release is a prerequisite for subsequent export to the cytoplasm.

Heat shock is likely to trigger hsc70 binding to a large number of nuclear proteins that require chaperone activity, a process that may contribute to nuclear retention of hsc70s. To test this hypothesis, we have developed an assay for hsc70 release from nuclear anchors, which is not complicated by transport across the nuclear envelope (see MATERIALS AND METHODS for details). To this end, control and heat-shocked HeLa cells were first extracted with digitonin, which permeabilizes the plasma membrane and removes most of the cytoplasmic proteins but leaves the nuclear membranes intact. After digitonin extraction, the nonionic detergent NP-40 was used to solubilize the nuclear envelope, which would no longer restrict the movement of proteins. The proper permeabilization of membranes in our assays was verified in control experiments (Fig. 5*A*). As expected, anti-laminin B antibodies do not have

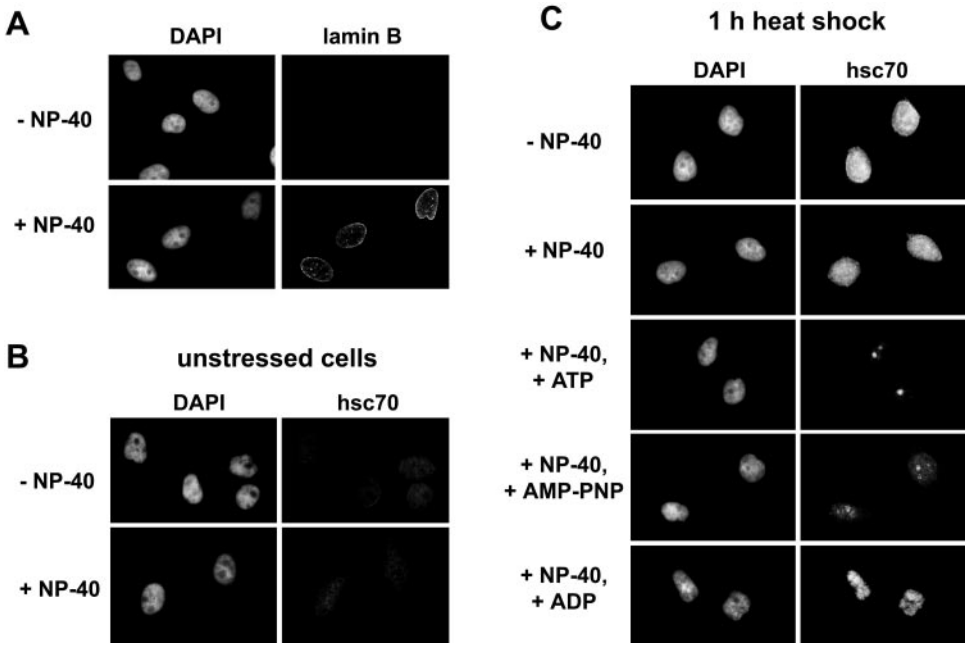

Fig. 5. Hsc70s are retained in nuclei of heat-stressed cells. *A*: the nuclear envelope of digitonin-treated cells was permeabilized with Nonidet P-40 (NP-40). Heat-shocked HeLa cells extracted with digitonin were incubated in the absence or presence of NP-40. Cells were fixed and binding of anti-laminin B antibodies was tested by indirect immunofluorescence. Unstressed controls (*B*) and heat-shocked cells (*C*) were treated with digitonin. Samples were incubated subsequently with buffer containing NP-40 and ATP, adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP), or ADP as indicated. Specimens were fixed, and hsc70s were detected by indirect immunofluorescence.

Table 1. *Hsc70s are retained in nucleoplasm of heat-shocked cells*

	Control	1-h Heat			
		No recovery	3-h Recovery	5-h Recovery	15-h Recovery
No nucleotide added	(+)	+++*	++ Nucleoli	+ Nucleoli for some cells	(+)
ATP	(+)	(+) Nucleoli	(+) Nucleoli	(+) Nucleoli for some cells	(+)
AMP-PNP	(+)	(+) Nucleoli	(+) Nucleoli	(+) Nucleoli for some cells	(+)
ADP	(+)	+++*	++ Nucleoli	+ Nucleoli for some cells	(+)

AMP-PNP, adenosine 5′-(β,γ-imido)triphosphate. Results for the distribution of hsc70s in nuclei of digitonin-treated cells for the experiments shown in Figs. 5 and 6 are summarized. Control, heat-stressed, and recovering cells were extracted with digitonin and incubated in buffer containing the nonionic detergent Nonidet P-40 and the different nucleotides shown. The presence of hsc70s was monitored by indirect immunofluorescence. Similar results were obtained for at least 3 independent experiments. Signals for hsc70s in nucleoplasm: +++, strong; ++, intermediate; (+), weak. For some of the conditions, hsc70s could be detected in nucleoli. *The strong fluorescence in the nucleoplasm of stressed cells could mask the presence of hsc70s in nucleoli.

access to the nuclear lamina in digitonin-treated cells, but subsequent incubation with NP-40 led to antibody binding.

We next used this assay to determine whether hsc70s are retained in nuclei of control and heat-shocked cells (Fig. 5, *B* and *C*). Samples were treated with digitonin followed by incubation in the absence or presence of NP-40. Heat-shocked samples retained most of the hsc70s even in the presence of NP-40, suggesting that binding to nuclear anchors contributes to hsc70 accumulation in nuclei. By contrast, little hsc70 was found in nuclei of unstressed cells under any of the conditions tested (Fig. 5*B* and Table 1).

ATP and its nonhydrolyzable analog AMP-PNP release hsc70s from nuclear anchors. One way to retain hsc70s in nuclei is their binding to substrates that need to be refolded. This chaperone-substrate interaction is known to be stabilized by ADP, whereas ATP induces the rapid dissociation and binding of substrates (reviewed in Refs. 6 and 15). Digitonin-extracted cells were incubated for 15 min in NP-40-containing buffer supplemented with ATP, AMP-PNP, or ADP followed by localization of hsc70s (Fig. 5*C*). Unlike ADP, both ATP and AMP-PNP efficiently released the chaperone from nuclei, suggesting that ATP binding, but not cleavage, is required to liberate hsc70s from nuclear anchors. Interestingly, ATP failed to release hsc70 completely from nucleoli, suggesting that binding to nucleolar components is more complex than a chaperone-unfolded protein interaction.

Nuclear retention of hsc70s changes during recovery from heat shock. We next monitored hsc70 nuclear retention in cells recovering from stress. To this end, HeLa cells exposed to heat were analyzed after 3-, 5-, and 15-h incubation at 37°C. The amount of hsc70s present in the nucleoplasm decreased during recovery, and nucleoplasmic chaperone could be liberated with ATP or AMP-PNP (Fig. 6). In addition, hsc70s transiently concentrated in nucleoli, albeit with kinetics different from their accumulation in the nucleoplasm. The hsc70 levels increased in nucleoli of most cells after a 3-h recovery period, but only in few nucleoli after 5 h (Fig. 6, Table 1). As observed after heat exposure, hsc70 associated with nucleoli was not fully liberated by incubation with ATP or AMP-PNP. At 15 h after heat shock, hsc70 distribution was similar to that in unstressed controls and no accumulation was seen in nuclei or nucleoli. Results of these in vitro experiments (summarized in Table 1) suggest that hsc70 binding to chaperone substrates

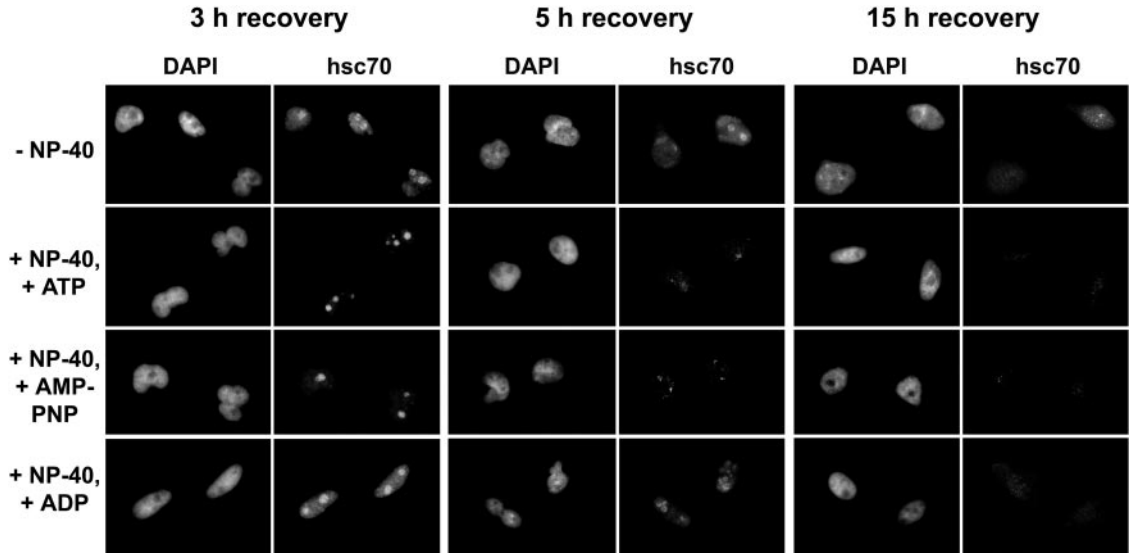

Fig. 6. Nuclear retention of hsc70s changes in cells recovering from heat stress. Heat-shocked cells were allowed to recover for 3, 5, and 15 h before digitonin extraction and incubation with NP-40-containing buffer in the presence of ATP, AMP-PNP, or ADP. Hsc70s were located by indirect immunofluorescence as described for Fig. 5.

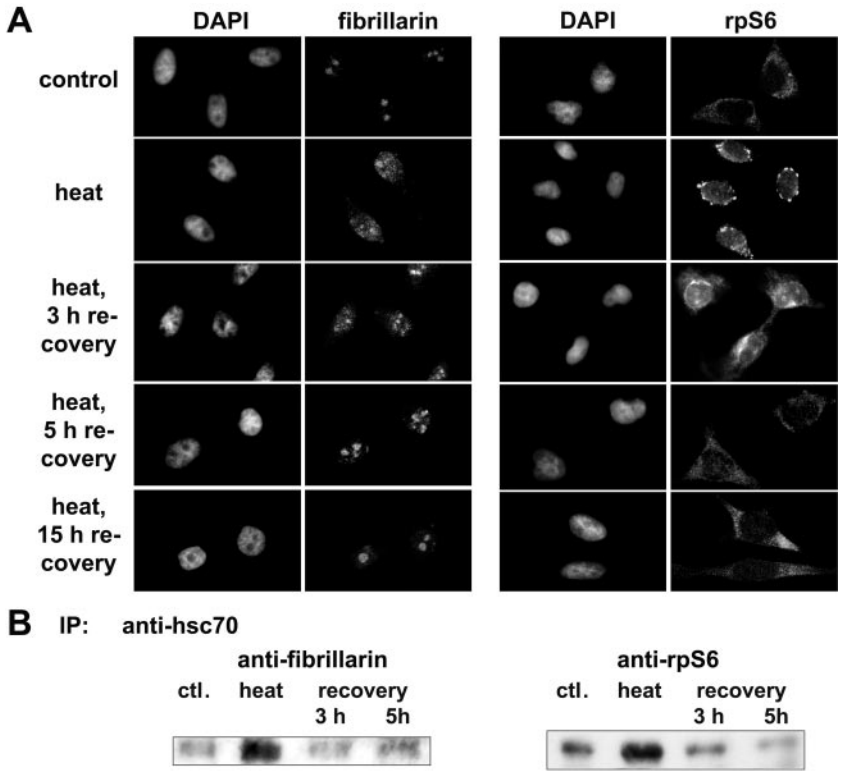

Fig. 7. Nucleolar protein fibrillarin and ribosomal protein rpS6 redistribute in stressed cells and associate with hsc70 upon heat shock. *A*: fibrillarin and rpS6 were localized by indirect immunofluorescence in unstressed, heat-shocked, and recovering HeLa cells. *B*: nuclear proteins were immunoprecipitated (IP) with antibodies against hsc70. Samples containing comparable amounts of hsc70 were tested for the presence of fibrillarin and rpS6 by Western blot analysis.

contributes to its nuclear retention immediately after heat treatment and at early stages of recovery.

Binding of hsc70s to nuclei of stressed cells. Members of the hsp/hsc70 family are involved in multiple interactions in the nucleus, and in response to heat stress hsc70s can be expected to interact with a large variety of nuclear components. For instance, the importance of hsc70s for the organization of nucleoli is well established, and chaperones are implicated in restoring nucleolar function upon stress (13, 16). On the basis of these earlier observations, nucleolar proteins were candidates for the interaction with hsc70s in heat-treated cells. To test this idea, we examined fibrillarin, a bona fide component of nucleoli, and the ribosomal protein rpS6, which is assembled into the small ribosomal subunits in nucleoli. When analyzed by indirect immunofluorescence (Fig. 7*A*), fibrillarin was concentrated in nucleoli of control cells but redistributed throughout nucleus and cytoplasm in response to heat exposure. During recovery, fibrillarin relocated to nucleoli, and after 15 h at 37°C, its distribution was similar to that in unstressed controls. In parallel, nuclear proteins were immunoprecipitated with antibodies against hsc70s, and immunoprecipitates that contained comparable amounts of hsc70 were probed with antibodies against fibrillarin (Fig. 7*B*). Although the nucleolar protein copurified with hsc70s for control, stressed, and recovering cells, clearly the highest amount of fibrillarin associated with hsc70s in heat-shocked cells.

Like fibrillarin, rpS6 redistributed after heat stress. As part of the 40S ribosomal subunit, rpS6 is mostly cytoplasmic under control conditions; however, heat treatment resulted in the formation of large structures containing rpS6 at the cytoplasmic side of the nuclear periphery. Increased amounts of rpS6 were detected in the nucleus as well. During stress recovery rpS6 relocated, and after 15 h its distribution was similar to that in unstressed cells (Fig. 7*A*). Similarly to fibrillarin, the association of rpS6 with hsc70 in nuclei was enhanced transiently after heat stress, as demonstrated by the coimmunoprecipitation of both proteins (Fig. 7*B*). Together, the data obtained for the interaction of nuclear hsc70s with fibrillarin and rpS6 are consistent with a role of the chaperone in restoring nucleolar function after heat exposure.

In conclusion, our study demonstrates that the nucleocytoplasmic shuttling of chaperones of the hsp/hsc70 family is inhibited by heat shock but restored when cells recover from

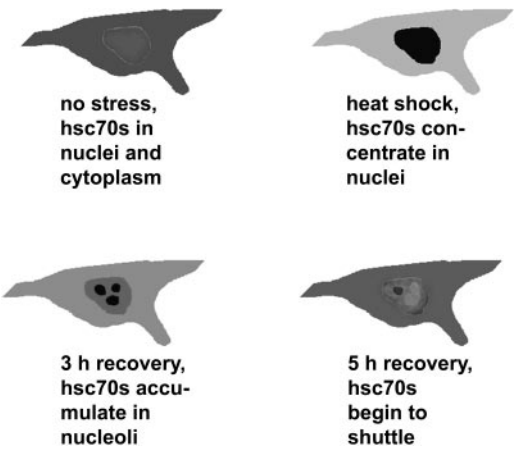

Fig. 8. Simplified model for the changes in nucleocytoplasmic shuttling of hsc70s upon heat shock and during recovery from stress. See text for details.

stress-induced damage. Importantly, stress alters not only the steady-state distribution but also the movement of hsc70s between nucleus and cytoplasm. Herein we have shown that hsc70 retention in the nucleus is drastically increased in response to heat exposure, a process that prevents export of the chaperone to the cytoplasm and thereby shuttling. We have identified two different forms of hsc70 interaction with nuclear anchors, both of which can be expected to contribute to the sequestration of chaperone in nuclei. First, hsc70s bind to nuclear proteins in an ATP-sensitive fashion, which most likely represents binding of the chaperone to folding substrates. Second, hsc70s associate with nucleoli, and at least a portion of the nucleolar chaperone cannot be liberated by the addition of ATP. This could indicate an association of hsc70s with nucleolar components in a fashion that is distinct from a chaperone-folding protein interaction. Independent of the type of association that underlies hsc70s retention in nuclei, we have shown that this retention is low in control cells, high after heat shock, and gradually reduced during recovery from stress. These changes in nuclear retention of hsc70s upon stress and during recovery can be expected to affect a variety of biological processes that require chaperone activity. For instance, immediately after stress, the proper folding of chaperone substrates in the cytoplasm may be impaired until de novo synthesis or shuttling of hsp/hsc70s resumes. Moreover, stress may interfere with the chaperone-dependent targeting of cytoplasmic proteins to various organelles, including mitochondria and peroxisomes, both of which require cytoplasmic hsp/hsc70s for protein import.

On the basis of the results described herein, we have developed a simplified model for hsc70 shuttling (Fig. 8). Hsc70s accumulate in nuclei of heat-stressed cells, where they are initially retained in the nucleoplasm by binding to chaperone substrates in an ATP-sensitive fashion. During recovery from heat, hsc70s relocate within the nucleus and transiently concentrate in nucleoli; this interaction cannot be prevented by the addition of ATP. As recovery progresses, hsc70s are liberated from nuclear and nucleolar anchors, which precedes their relocation to the cytoplasm. We propose that the release from nuclear anchors is a limiting factor that regulates hsc70 nuclear export and thereby shuttling of the chaperone in cells exposed to heat.

Taken together, the stress-induced sequestration of hsc70s in nuclei possibly affects repair processes in the cytoplasm as well as the proper assembly and maintenance of several organelles. These consequences of stress exposure are likely to impinge on different aspects of physiology and ultimately survival of each cell.

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