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Translational Thermotolerance Provided by Small Heat Shock Proteins Is Limited to Cap-dependent Initiation and Inhibited by 2-Aminopurine*

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Heat shock results in inhibition of general protein synthesis. In thermotolerant cells, protein synthesis is still rapidly inhibited by heat stress, but protein synthesis recovers faster than in naive heat-shocked cells, a phenomenon known as translational thermotolerance. Here we investigate the effect of overexpressing a single heat shock protein on cap-dependent and cap-independent initiation of translation during recovery from a heat shock. When overexpressing αB-crystallin or Hsp27, cap-dependent initiation of translation was protected but no effect was seen on cap-independent initiation of translation. When Hsp70 was overexpressed however, both cap-dependent and -independent translation were protected. This finding indicates a difference in the mechanism of protection mediated by small or large heat shock proteins. Phosphorylation of αB-crystallin and Hsp27 is known to significantly decrease their chaperone activity; therefore, we tested phosphorylation mutants of these proteins in this system. αB-crystallin needs to be in its non-phosphorylated state to give protection, whereas phosphorylated Hsp27 is more potent in protection than the unphosphorylatable form. This indicates that chaperone activity is not a prerequisite for protection of translation by small heat shock proteins after heat shock. Furthermore, we show that in the presence of 2-aminopurine, an inhibitor of kinases, among which is double-stranded RNA-activated kinase, the protective effect of overexpressing αB-crystallin is abolished. The synthesis of the endogenous Hsps induced by the heat shock to test for thermotolerance is also blocked by 2-aminopurine. Most likely the protective effect of αB-crystallin requires synthesis of the endogenous heat shock proteins. Translational thermotolerance would then be a co-operative effect of different heat shock proteins.

Cells facing stress divert their resources to combating and surviving that stress. For example, during a heat shock, general macromolecular synthesis and processing is inhibited, and the set of transcription units that encode the heat shock proteins (Hsps)[1] is activated (1, 2). Synthesis of the Hsps is required for optimal survival of heat (or other) stress. The Hsps are a complex group of proteins, ranging in size between 90 and 20 kDa. The small heat shock proteins (sHsps) belong to a family of proteins distinguished by sharing a common protein domain, the so-called α-crystallin domain (for review, see Ref. 3). In man, there are 10 different sHsps (4); of these, Hsp27 and αB-crystallin are traditional heat shock proteins in the sense that their synthesis is induced by a heat shock (5). All sHsps, including the stress-inducible sHsps, are constitutively expressed in different tissues; αB-crystallin, for example, is abundant in lens, heart, skeletal muscle, and brain (5–7). The best known property of Hsp27 and of αB-crystallin is their *in vitro* chaperone activity: they keep their substrates in solution but cannot refold them (for review, see Refs. 6 and 8). *In vivo*, they might act as a reservoir of unfolded proteins for the large Hsps, which are ATP-dependent refoldases. The sHsps are also thought to stabilize the cytoskeleton during stress (9); in addition, they interact with some components of the apoptotic pathway, thereby protecting the cell from apoptosis (10).

sHsps can be regulated in their activity by phosphorylation. αB-crystallin has three phosphorylation sites. Serine 59 is phosphorylated when cells are stressed, and serines 19 and 45 are found phosphorylated when cells are going into mitosis (11, 12). The overall level of phosphorylation of αB-crystallin remains low (13). Hsp27 has two (rodents) or three (man) phosphorylation sites. At normal temperature, Hsp27 is mainly unphosphorylated: after different kinds of stress, including heat shock, Hsp27 is extensively phosphorylated (5, 14). For Hsp27 and αB-crystallin, phosphorylation has been shown to prevent complex formation *in vitro* (15–17), resulting in significant decrease in chaperone activity (16–18). Phosphorylation-mimicked Hsp27 protects cells from heat stress but not from oxidative stress (16, 19, 20).

Cells having a full complement of Hsps because of an earlier stress are more resistant to subsequent heat stress, a phenomenon known as thermotolerance. Thermotolerance can be induced by expression of a single Hsp, such as Hsp70, Hsp27, or αB-crystallin (21–25). In thermotolerant cells, protein synthesis is still rapidly inhibited by heat stress, but protein synthesis recovers faster than in naive heat-shocked cells, a phenomenon known as translational thermotolerance (26–29). The mechanism of inhibition of translation by a heat shock (as well as by other types of stress; Ref. 30) and thus also the mechanism of translational thermotolerance is still a matter of debate. The main block seems to be at the level of translation initiation.

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[1] The abbreviations used are: Hsp, heat shock protein; sHsp, small heat shock proteins; eIF2α, eukaryotic initiation factor 2α; SG, stress granules; RT-PCR, reverse transcriptase-PCR; DMEM, Dulbecco's modified Eagle's medium; PKR, double-stranded RNA-activated kinase; IRES, internal ribosome entry site; FGF, fibroblast growth factor.

Phosphorylation and thus inhibition of eIF2α is commonly found after stress (31, 32), but other factors must be affected as well because the inactivation of eIF2B in rat hepatoma cells did not correlate directly with the level of phosphorylation of eIF2α (33). The cap-binding complex must also be affected because cap-independent initiation of translation is more stress-resistant than is cap-dependent initiation of translation (34, 35). Deficiency in eIF4E strongly inhibits general translation in HeLa cells, but translation of Hsp mRNAs and cap-independent mRNAs still takes place (36), a situation that resembles the pattern of translation in cells recovering from heat stress and which suggests that eIF4E is down-regulated during a heat shock. Stalled translation initiation complexes containing almost all components of the 48S preinitiation complex but not the 60S ribosomal subunit accumulate in the cytoplasm as stress granules (SG). Hsp27 has been detected in SGs as well (37), possibly in complex with eIF4G (38). Assembly in SGs is a highly dynamic process, and untranslated mRNAs are thought to be sorted and processed there for either reinitiation, degradation, or packaging into nonpolysomal messenger ribonucleoprotein complexes (39). This indicates that during and after stress, SGs are important checkpoints for initiation of translation.

Thus far, translational tolerance has only been assayed at the level of the overall rate of protein synthesis, and no distinction has been made between cap-dependent and cap-independent translation initiation. We show here that overexpression of either αB-crystallin or Hsp27 protects cap-dependent but not cap-independent translation initiation, whereas overexpression of Hsp70 affects both. Further, we show that the phosphorylation state of αB-crystallin or Hsp27 affects its ability to confer translational tolerance. Finally, we show that 2-aminopurine, a kinase inhibitor that inhibits eIF2α kinases such as double-stranded RNA-activated kinase (PKR) (40, 41), blocks the establishment of translational tolerance by overexpression of an sHsp.

EXPERIMENTAL PROCEDURES

Cell Culture—C2 cells (mouse myoblast cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with penicillin and streptomycin (Roche Applied Science) and supplemented with 20% fetal calf serum (PAA Laboratories) to prevent differentiation of these cells. T-REx cells (HeLa cells stably transfected with tetracycline repressor protein; Invitrogen) were cultured in minimum Eagle's medium (BioWhittaker) with glutamax (Invitrogen), 10% fetal calf serum, penicillin, streptomycin, and blasticydin (Invitrogen).

SDS-PAGE and Western Blot Analysis of Heat-shocked Cells—For heat shock assays, 6-well plates were seeded with $\sim 2.5 \times 10^5$ cells/well for C2 cells and $\sim 6.5 \times 10^5$ cells/well for T-REx cells. The next day, cells were submitted to a heat shock by submerging plates in a 45 °C water bath. C2 cells were heat shocked for 30 min; T-REx cells were heat shocked for 60 min at 45 °C. Cells were harvested at various times during recovery at 37 °C by scraping in 100 μl of lysis buffer (20% glycerol, 6% SDS, and 120 mM Tris·HCl, pH 6.8). Protein concentrations were measured by using the BCA protein assay kit (Pierce) according to the manufacturer's protocol for 96-well plates. After addition of SDS sample buffer (20% glycerol, 4% SDS, 200 mM dithiothreitol, 200 mM Tris·HCl, pH 6.8, and bromphenol blue), 40 μg of the sample was loaded on gel, separated, and Western blotted. Blots were blocked for 1 h in blocking buffer (10 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20, and 5% dried low-fat milk) and incubated with primary antibodies in appropriate dilutions in blocking buffer (anti-αB-crystallin mouse monoclonal 1:500, anti-Hsp25 rabbit polyclonal 1:5000, anti-Hsp70 1:1000; Stressgen) for 1 h at room temperature. After three 10-min washes in blocking buffer, the blot was incubated with the secondary antibody with a conjugated alkaline phosphatase (Promega) for 1 h. The blots were washed again in blocking buffer, rinsed once with AP buffer (100 mM Tris·HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$) and stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate in AP buffer, dried, and analyzed with Molecular Analyst version 1.4.1 software (Bio-Rad) after scanning with a Bio-Rad GS700 imaging densitometer.

Pulse Labeling—C2 cells were cultured in DMEM with penicillin, streptomycin, and 20% fetal calf serum to 80% confluence in 25-cm^2 flasks. Cells were starved for 2 h in DMEM without methionine with 20% fetal calf serum, penicillin, and streptomycin. Cells were heat shocked for 30 min at 45 °C and assayed for the level of protein synthesis at different time points during recovery. At these time points, medium was replaced by 2 ml of DMEM without methionine with 20% fetal calf serum, penicillin, and streptomycin and 20 μCi of [^{35}S]methionine (Amersham Biosciences). After 1-h incubation at 37 °C, cells were harvested by scraping in 1 ml of phosphate-buffered saline and centrifuging for 2 min at 5000 rpm. The cell pellet was resuspended in 50 μl of SDS sample buffer. After 10 min at 95 °C and 15 min in a sonicator bath, 25 μl of the sample were loaded on an SDS-PAGE gel. To enhance the ^{35}S signal, the gel was incubated in Amplify (Amersham Biosciences) for 30 min after fixing with 0.1% Coomassie Brilliant Blue in 40% methanol and 10% acetic acid and destaining in 7.5% methanol and 7.5% acetic acid. After drying, the gel was autoradiographed overnight, and results were analyzed with Molecular Analyst software (Bio-Rad) after scanning with a Bio-Rad GS700 imaging densitometer.

Transfections—C2 cells were transfected with LipofectAMINE plus (Invitrogen). Approximately 6.5×10^4 cells were plated in DMEM with penicillin, streptomycin, and 10% fetal calf serum in 6-well plates. After 24 h, cells were transfected with a total of 1 μg of DNA per well using 4 μl of LipofectAMINE and 6 μl of LipofectAMINE plus reagent. As a transfection control, 0.1 μg of CMV-β-galactosidase was co-transfected, and the other 0.9 μg of DNA was divided over the various constructs, pHsp-Cap-Luc or pHsp-IRES-Luc and expression vectors for αB-crystallin, Hsp27, Hsp70, βB2-crystallin, or the empty vectors as control in a 1:1 ratio (except where indicated otherwise). 48 h after transfection, cells were heat shocked for 30 min at 45 °C (unless mentioned otherwise) and assayed for reporter gene activity during recovery at 37 °C.

Approximately 2.5×10^5 T-REx cells were plated in minimum Eagle's medium with glutamax, 10% fetal calf serum, penicillin, and streptomycin in 6-well plates and transfected after 24 h with 1 μg of DNA using 3 μl of Fugene reagent (Roche Applied Science) per well. As a control for the transfection efficiency, 0.2 μg of the pGL3 control vector (Promega) was co-transfected. The pcDNA4TO LacZ (Invitrogen) and αB-crystallin, Hsp27, or empty expression vectors were used in a 1:1 ratio. 48 h after transfection, cells were heat shocked for 1 h at 45 °C. Where indicated, cells were pre-heat shocked for 30 min at 45 °C 6 h earlier. Cells were treated with 1 μg/ml doxycycline for 3 h, starting 3.5 h before the heat shock to induce the pcDNA4TO LacZ construct. After 3 h of induction, cells were washed with phosphate-buffered saline and fresh DMEM with penicillin, streptomycin, and 10% fetal calf serum. Cells were harvested during recovery at 37 °C and assayed for β-galactosidase and luciferase activity.

Transfections were done in triplicate and repeated at least twice with different batches of DNA. Unless otherwise indicated, results of representative experiments are shown.

RT-PCR—Transfected cells were scraped in 0.5 ml of TRIzol (Invitrogen) per well. After transfer into a tube, 100 μl of chloroform was added and the mixture was vortexed for 15 s. After 15 min on ice and centrifuging for 15 min at 13,000 rpm at 4 °C, 200 μl of the upper phase was precipitated with an equal amount of isopropanol. At this step, material from three wells was pooled. Samples were left at −20 °C for at least 3 h. After centrifuging for 30 min at 13,000 rpm at 4 °C, the pellet was washed twice with cold 70% ethanol and then air-dried for 5 min. The pellet was dissolved in 45 μl of H_2O and stored at −20 °C. The RNA was treated with 7.5 units of RNase-free DNase per μg of RNA for 15 min at 37 °C, and DNase was then inactivated for 10 min at 70 °C. The reverse transcription reaction was performed using the 1st-strand cDNA synthesis kit for RT-PCR (Roche Applied Science) according to the manufacturer's instructions with 1 μg of RNA and random primers in a total volume of 20 μl.

Primers used for the PCR were luciferase mRNA primers (at position +1330, TGGATGGCTACATTCTGGAGAC, and at position +1720, CCTTCTTGGCCTTTATGAGGATC) and, as a transfection control, β-galactosidase mRNA primers (at position +155, TGGCGTTACCCAACTTAATC, and at position +630, TCAGACGGCAAACGACTGT). The PCR was performed in a total of 25 μl, using 5 μl of cDNA, a mixture of luciferase and β-galactosidase mRNA primers, cDNA PCR buffer, dNTPs, and Advantage HF polymerase (Advantage-HF PCR kit, BD Biosciences). A parallel reaction was performed on 0.25 μg of the DNase-treated RNA to test for DNA contamination. The DNA was denatured at 94 °C for 60 s, primers were annealed at 66 °C for 30 s, and then elongation was performed at 68 °C for 90 s. PCR samples were taken after 20, 25, and 30 cycles and separated on 5% acrylamide gels in 1× Tris-borate-EDTA buffer. Results were quantitated using Molec-

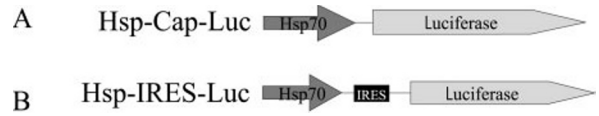

FIG. 1. **Reporter constructs to test translation efficiency after heat shock.** *A*, to test for cap-dependent translation after heat shock, the pHsp-Cap-Luc construct was made. This construct contains the luciferase gene of the pGL3 basic vector with the *D. melanogaster* Hsp70 promoter driving expression of the reporter gene. *B*, in the pHsp-IRES-Luc, the 5′ non-coding region of the pHsp-Cap-Luc is replaced by the 532-bp long 5′ non-coding region of the FGF-2 mRNA. This region contains an IRES known to be used during stress (46). This construct thus reflects IRES-dependent translation after stress.

ular Analyst software (Bio-Rad) after scanning on a Bio-Rad Geldoc 1000 system. Samples shown did not contain DNA contamination.

Treatment with 2-Aminopurine—For the pre-heat shock, cells were transfected with pcDNA4TO LacZ and pGL3 control vector in a 2:1 ratio with a total of 1 μg of DNA per well. 48 h after transfection, cells were pre-heat shocked for 30 min at 45 °C. The LacZ construct was induced as described in the transfection section of "Experimental Procedures." 2-Aminopurine (Sigma) was added to a final concentration of 10 mM after washing away the doxycycline. 2-Aminopurine was dissolved in phosphate-buffered saline/acetic acid (17.5 M; 200:1) to a 100 mM stock and adjusted to pH 7.5 with NaOH. As a negative control, phosphate-buffered saline/acetic acid (200:1 adjusted to pH 7.5 with NaOH) was added to non-treated cells. Cells were harvested after 6-h incubation at 37 °C. For the αB-crystallin-transfected cells, 2-aminopurine was added 30 min before heat shock. Cells were harvested after 6 h of recovery with 2-aminopurine at 37 °C. Cells were assayed for luciferase and β-galactosidase activity and Western blotted for Hsp expression.

Reporter Assays—After transfection in 6-well plates, heat shock and recovery, cells were harvested by scraping or vigorously shaking in 200 μl of reporter lysis mix (25 mM Bicine, pH 7.5, 0.05% Tween-20, and 0.05% Tween-80) per well.

For the β-galactosidase assay, 1:100 galacton (Tropix) was added to a 100 mM phosphate buffer, pH 8.1, with 5 mM $MgCl_2$; 200 μl of this mixture was added to 20 μl of the cell lysate. After 30-min incubation at room temperature, 300 μl of light emission accelerator (Tropix) was added. For the luciferase assay, 100 μl of luciferase reagent (Promega) was added to 20 μl of the cell lysate immediately before measurement. Measurements were performed on a Lumat LB 9507 luminometer for 10 s. In C2 cells, luciferase values were corrected for transfection efficiencies using the β-galactosidase values. In T-REx cells, β-galactosidase values were corrected for transfection efficiencies using the luciferase values of non-heat-shocked cells transfected in parallel.

Constructs—The two reporter constructs (Fig. 1) were made in the pGL3 basic vector (Promega). For the pHsp-Cap-Luc, the *Drosophila melanogaster* Hsp70 promoter was excised from the pBN247 construct (42) using HindIII blunt/SalI and cloned in front of the luciferase gene using SmaI and XhoI sites from the vector. For the pHsp-IRES-Luc construct, the rat FGF-2 IRES (532 bp) was excised from pRObFGF503 (43) using HindIII/NcoI and inserted between the Hsp70 promoter and the luciferase reporter gene. Rat αB-crystallin cDNA was cloned NcoI-XhoI/SalI into β-actin vector (44). The human αB-crystallin coding sequence (kindly provided by P. Muchowski, University of Washington, Seattle) and phosphorylation mutants, made by site-directed mutagenesis (Stratagene), were cloned into the pIRESneo vector (Clontech). The constructs pSV Ha Hsp27 wt and phosphorylation mutants S15A, S90A, S15A/S90A, and S15E/S90E were a kind gift from J. Landry (15). The Hsp70 clone was constructed by PCR on mRNA isolated from heat-shocked HeLa cells. The primers used were at position +1 (CAAGCTAACGGCTAGCCTGAGGAGC) and at position +2390 (AAGGCCCCTAATCTACCTCCTCAATGGTGGG) of the Hsp70 mRNA. PCR fragments were cloned into pGEM-T-Easy vector (Promega) and, after sequencing, the Hsp70 coding sequence was cloned into the β-actin vector using SphI blunt/SalI for the insert and NcoI blunt/SalI for the vector. The rat βB2-crystallin expression construct was described previously (45).

RESULTS

αB-crystallin Protects Cap-dependent but Not Cap-independent Translation—Overall translation in C2 cells (mouse myoblasts) was severely inhibited after a 30-min heat shock at 45 °C and did not recover within 7 h at 37 °C (Fig. 2). Accumulation of the endogenous small heat shock proteins Hsp27 and

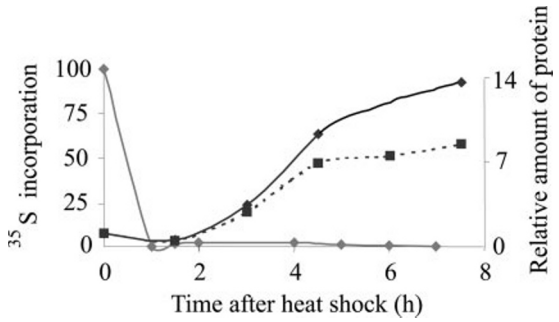

FIG. 2. **Effects of heat shock on translation in C2 cells.** Protein synthesis was measured after a 30-min 45 °C heat shock as described under "Experimental Procedures." The level of protein synthesis is expressed relative to synthesis before heat shock (*solid line* with *gray diamonds*). In a separate experiment, the endogenous levels of αB-crystallin (*solid line* with *black diamonds*) and Hsp27 (*dotted line* with *black squares*) were determined by analyzing heat-shocked cells during recovery at 37 °C, as described under "Experimental Procedures." Levels of αB-crystallin and Hsp27 are expressed relative to their level before heat shock.

αB-crystallin was first detectable after 3 h; the level of Hsp27 reached a steady state after about 4.5 h, while the level of αB-crystallin continued to increase up to 7.5 h (Fig. 2). Hence, even though general protein synthesis was inhibited, special mRNAs, such as those encoding heat shock proteins, were still translated under these conditions.

To determine whether prior expression of a small heat shock protein provides a measure of translational thermotolerance under these conditions, C2 cells were transfected with an expression construct for αB-crystallin together with constructs designed to report translation efficiency after heat shock (Fig. 1). Two different constructs were used. In one, pHsp-Cap-Luc, the luciferase coding region was placed under control of the Hsp70 promoter without altering the 5′ non-coding region of the pGL3 basic vector; in the second, pHsp-IRES-Luc, the 5′ non-coding region of the FGF-2 mRNA was inserted between the Hsp70 promoter and the luciferase coding region. Both reporter gene constructs are driven by the Hsp70 promoter, and the reporter mRNA should thus accumulate only after a heat shock; to decrease background resulting from possible promoter leakage prior to the heat shock, luciferase was used as the reporter. This enzyme is heat labile and inactivated by heat shock. The only difference between the mRNAs encoded by these two reporter constructs is in the 5′ non-coding region. The pHsp-Cap-Luc mRNA has a short, non-structured 5′ non-coding region and is predicted to be initiated via the default mechanism for translation initiation, namely, cap-dependent translation initiation. The pHsp-IRES-Luc mRNA contains the FGF-2 internal ribosome entry site (IRES) known to be used during stress (46) and should be initiated by means of a cap-independent mechanism. In support of this proposed difference in the mechanism of translation initiation, we found that the luciferase expression from the pHsp-Cap-Luc construct was inhibited by rapamycin, whereas that from the pHsp-IRES-Luc was not (Fig. 3 and data not shown). Rapamycin has been reported to inhibit cap-dependent translation initiation but not cap-independent translation initiation (47).

During recovery from a heat shock, the luciferase yield from the IRES-Luc mRNA was higher than that from the Cap-Luc mRNA. Overexpression of αB-crystallin significantly stimulated the luciferase expression from the Cap-Luc mRNA, but it did not affect that from the IRES-Luc mRNA (Fig. 3). As the luciferase mRNA levels did not differ between control cells and cells overexpressing αB-crystallin (Fig. 3*B* and data not shown), the increase in luciferase yield from the Cap-Luc mRNA must be because of an increase in the rate of translation.

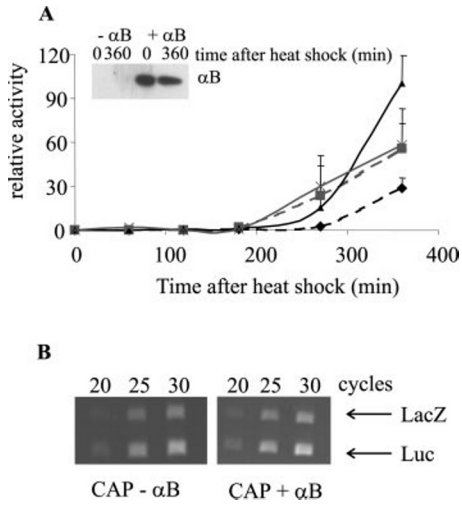

FIG. 3. **Expression of the cap- and IRES-dependent reporter constructs after heat shock and protection by αB-crystallin during recovery.** *A*, C2 cells were transfected with pHsp-Cap-Luc (*dotted line* with *black diamonds*), or with pHsp-IRES-Luc (*dotted line* with *black squares*) and empty expression vector, or co-transfected with the αB-crystallin expression construct and the pHsp-Cap-Luc (*solid line* with *black triangles*) or pHsp-IRES-Luc (*dotted line* with *Xs*) constructs. Reporter gene activity was measured at several time points during recovery at 37 °C. Values shown are relative to those obtained in cells transfected with pHsp-Cap-Luc and the αB-crystallin expression construct after 360 min of recovery. *Error bars*, standard deviation. *Insert*, Western blot for αB-crystallin after co-transfection with pHsp-Cap-Luc and empty vector (−αB) or the αB-crystallin expression vector (+αB). *B*, RT-PCR (after 20, 25, and 30 cycles) of luciferase mRNA (*Luc*) and β-galactosidase mRNA (*LacZ*) after transfection of pHsp-Cap-Luc with (*CAP + αB*) or without (*CAP − αB*) the αB-crystallin expression construct. Cells were harvested 6 h after heat shock. Note that the β-galactosidase mRNA level represents the control for transfection efficiency as well as for the efficiency of the RT-PCR.

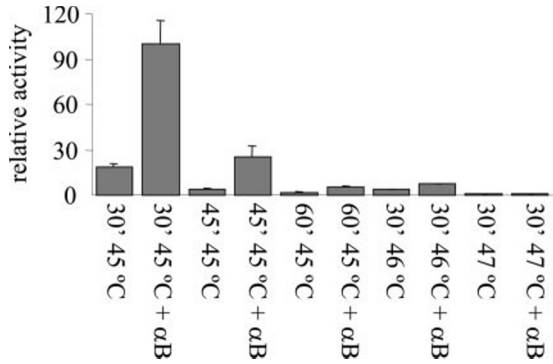

FIG. 4. **Translational thermotolerance provided by αB-crystallin depends on the severity of the heat shock.** C2 cells were transfected with the pHsp-Cap-Luc and the αB-crystallin expression construct or the empty vector. 48 h after transfection, cells were subjected to different heat shocks: 30, 45, and 60 min at 45 °C or 30 min at 46 °C or 47 °C. 6 h after recovery at 37 °C, cells were harvested and assayed for reporter gene activity. Values shown are relative to those obtained in cells transfected with pHsp-Cap-Luc and the αB-crystallin expression construct after a 30-min heat shock at 45 °C and 6 h of recovery at 37 °C. *Error bars*, standard deviation.

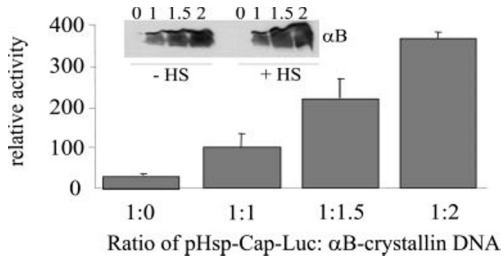

FIG. 5. **Dependence of protection on the level of αB-crystallin.** Different ratios of the pHsp-Cap-Luc and the αB-crystallin expression constructs were transfected. The total amount of DNA transfected was kept constant by adjusting for the amount of αB-crystallin expression construct with the empty vector. Cells were harvested after 6 h of recovery and assayed for reporter gene activity. Activities are expressed relative to the activity obtained after transfecting a 1:1 ratio of pHsp-Cap-Luc and αB-crystallin expression construct. *Error bars*, standard deviation. *Insert*, Western blot for αB-crystallin in non-heat shocked (−HS) and in heat-shocked cells (+HS) transfected with corresponding ratios of the pHsp-Cap-Luc and the αB-expression constructs.

The protective effect of αB-crystallin on the rate of translation of the Cap-Luc mRNA depended upon the severity of the heat shock: the level of protection was similar during recovery from a 30- or 45-min heat shock at 45 °C but decreased during recovery from a longer or more severe heat shock (Fig. 4). In subsequent experiments with C2 cells, a 30-min heat shock at 45 °C was used (note that cell lines differ in their resistance to heat stress; to obtain comparable data with HeLa cells, the HeLa cells need to be exposed to 45 °C for 1 h (data not shown)).

The level of protection by αB-crystallin depended upon the amount of αB-crystallin. When more of the αB-crystallin expression construct was co-transfected, the amount of activity obtained from the pHsp-Cap-Luc reporter increased (Fig. 5). Because of the fact that there is a maximum to the amount of DNA that can be transfected, it was not possible to increase the amount of the co-transfected αB-crystallin expression construct above that shown in Fig. 5, so it is not known what the maximal level of protection provided by αB-crystallin under these conditions is.

Translation Thermotolerance Provided by Hsp70 and Hsp27—Hsp70 and Hsp27 have also been reported to provide cells with translational thermotolerance (21, 27, 48). Therefore, we tested whether expression of these two heat shock proteins also increased the rate of translation of the Cap-Luc mRNA during recovery from a heat shock. As shown in Fig. 6, the results obtained after overexpression of Hsp27 are very similar to those obtained after overexpression of αB-crystallin as shown above: Hsp27 did not affect the level of mRNA from either the pHsp-Cap-Luc or pHsp-IRES-Luc construct (Fig. 6*B* and data not shown), but in the presence of Hsp27, the yield from the Cap-Luc mRNA increased, while that from the IRES-Luc mRNA stayed the same. In contrast, in the presence of Hsp70, the yield from both the Cap-Luc and the IRES-Luc mRNAs increased. As control, an expression construct for βB2-crystallin, a lens structural protein, was co-transfected. In the presence of βB2-crystallin, neither Cap-Luc nor IRES-Luc mRNA yielded more luciferase. Protection is thus specific for Hsps. Although the two sHsps as well as the large Hsp provide translational thermotolerance, the protection provided by the sHsps is more limited and apparently directed at a step required for cap-dependent initiation only.

The Role of Phosphorylation—Hsp27 and αB-crystallin are phosphorylated during stress. For Hsp27, the extent of phosphorylation is high (5, 14), and phosphorylation is required for thermotolerance (19, 49, 50). For αB-crystallin, the extent of phosphorylation is low (6, 13), and the role of phosphorylation in thermotolerance is not known. To test whether the phosphorylation state of αB-crystallin or Hsp27 influences their ability to protect the translation of the Cap-Luc mRNA, constructs expressing mutants mimicking non-phosphorylated (αB S45A, S19A/S45A/S59A, and Hsp27 S15A, S90A and S15A/S90A) or constitutive phosphorylated (αB S19D, S45D, S59D, S19D/S45D/S59D, and Hsp27 S15E/S90E) αB-crystallin or Hsp27 were co-transfected with pHsp-Cap-Luc. As shown in Fig. 7*A*, mimicking constitutive phosphorylation of αB-crystallin abolishes the protection of translation, irrespective of the site at which phosphorylation is mimicked. The non-phosphorylatable

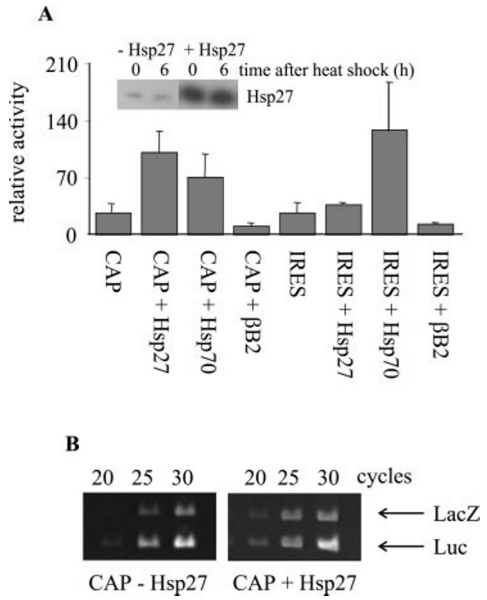

FIG. 6. **The effect of overexpression of Hsp27, Hsp70, and βB2-crystallin on protection of translation after heat shock.** *A*, C2 cells were transfected with the pHsp-Cap-Luc or pHsp-IRES-Luc together with Hsp27, Hsp70, or βB2 expression constructs or their empty vector. 48 h after transfection, cells were heat shocked and harvested after 6 h of recovery at 37 °C. *Bars*, values relative to those obtained in cells transfected with pHsp-Cap-Luc and the Hsp27 expression construct; *error bars*, standard deviation. *Inset*, Western blot for Hsp27 in cells co-transfected with pHsp-Cap-Luc and empty vector (−Hsp27) or the Hsp27 expression construct (+Hsp27). Data shown are from the same Western blot; intermediate lanes were removed for sake of clarity. *B*, RT-PCR (after 20, 25, and 30 cycles) of luciferase mRNA (*Luc*) and β-galactosidase mRNA (*LacZ*) after transfection of pHsp-Cap-Luc with (*CAP + Hsp27*) or without (*CAP − Hsp27*) the Hsp27 expression construct. Cells were harvested 6 h after heat shock. Note that the β-galactosidase mRNA level represents the control for transfection efficiency as well as for the efficiency of the RT-PCR.

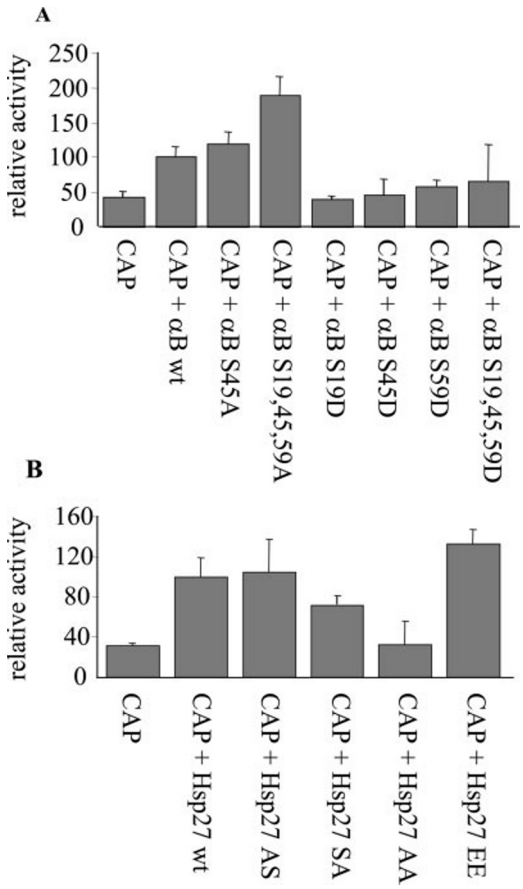

FIG. 7. **Effect of phosphorylation of αB-crystallin or Hsp27 on protection of Cap-dependent translation after heat shock.** *A*, mutants mimicking phosphorylation of αB-crystallin are indicated as αB S19D, S45D, S59D, and S19,45,59D (the S19D/S45D/S59D mutant); non-phosphorylatable αB-crystallin mutants are indicated as S45A and S19A,45,59A, (the S19A/S45A/S59A mutant) where the number indicates the amino acid residue mutated. Expression constructs of the mutants were co-transfected with pHsp-Cap-Luc. *B*, three mutants mimicking (partially) unphosphorylated Hsp27 (Hsp27 AS = Hsp27 S15A; Hsp27 SA = Hsp27 S90A, and Hsp27 AA = Hsp27 S15A/S90A) or one mimicking phosphorylation (Hsp27 EE = Hsp27 S15E/S90E) were co-transfected with the pHsp-Cap-Luc construct. Cells were heat shocked and harvested after 6 h of recovery. *Bars*, activities in the presence of the mutant proteins relative to activity of pHsp-Cap-Luc in the presence of overexpressed wild-type αB-crystallin or Hsp27, respectively. *Error bars*, standard deviation.

mutants of αB-crystallin, however, are more active in our translation protection assay. In contrast, the non-phosphorylatable mutant of Hsp27 (Hsp27 S15A/S90A) was inactive, whereas the mutant mimicking full phosphorylation (Hsp27 S15E/S90E) was as active as wild type. The mutant in which the most 5′ phosphorylation site was mutated (Hsp27 S15A) was as active as wild type, whereas the mutant in which the second phosphorylation site was mutated (Hsp27 S90A) was slightly less active than wild type (Fig. 7*B*). These data show that, for full activity in translation thermotolerance, Hsp27 needs to be phosphorylated, whereas αB-crystallin must be dephosphorylated.

Thermotolerance of Translation of mRNA Synthesized from a Non-heat-shock Promoter—In the experiments described above, we cannot give a quantitative measure of the extent of translational thermotolerance provided by αB-crystallin or Hsp27 as we have no way of determining what the yield of luciferase would be if translation were not inhibited (because we used a heat shock promoter). Nor can we determine whether the extent of translational thermotolerance obtained when αB-crystallin or Hsp27 is overexpressed is similar to that seen in thermotolerant cells, as the use of the Hsp promoter, and thus the expression of pHsp-Cap-Luc or pHsp-IRES-Luc, may differ between naive and thermotolerant cells. To have at least some measure of the relative translational thermotolerance, we turned to T-REx HeLa cells (HeLa cells stably expressing the tetracycline repressor). In these cells, we induced expression of a LacZ construct placed under control of the tetracycline repressor for 3 h to deliver a burst of β-galactosidase mRNA; we then washed away the inducer and, 30 min later, challenged the cells with a heat shock of 1 h at 45 °C. The rate of increase in β-galactosidase activity during recovery at 37 °C was then measured. As shown in Fig. 8*A*, in the presence of co-transfected αB-crystallin or Hsp27, the rate of accumulation of β-galactosidase was significantly higher than in cells co-transfected with an empty vector. As mRNA levels did not differ significantly (data not shown), this effect must be caused by an increase in translation, *i.e.* translational thermotolerance provided by the small heat shock proteins. (Note that, in this system, the protective effect of αB-crystallin and Hsp27 persists for at least 20 h (Fig. 8*A*). In C2 cells, using the pHsp-Cap-Luc reporter described above, luciferase values in control cells caught up with those in αB-crystallin-expressing cells after 20 h of recovery. This difference is most likely caused by differences in the stability of the reporter enzymes luciferase *versus* β-galactosidase and of their cognate mRNAs.)

The extent of translation of the β-galactosidase mRNA during recovery from a heat shock was also measured in thermotolerant cells, *i.e.* cells recovering from a mild prior heat shock. The rate of increase in β-galactosidase activity in these cells (pre-HS + HS) was not significantly different from that in cells

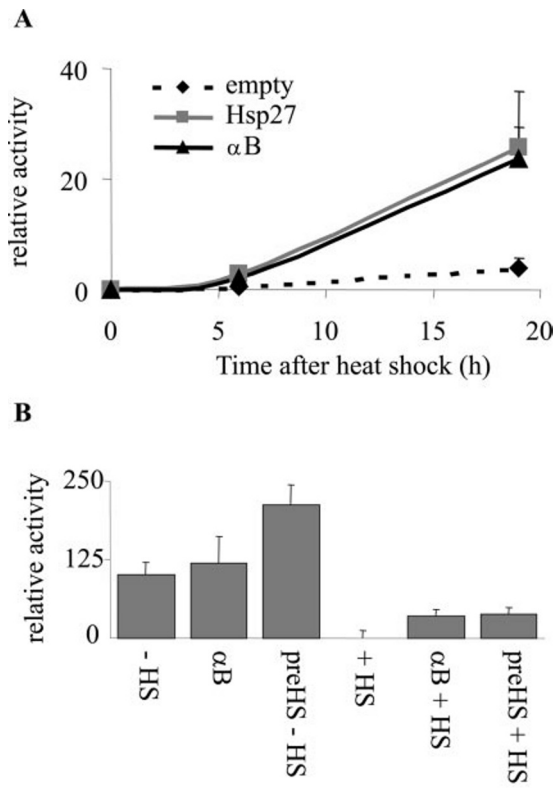

FIG. 8. **Thermotolerance of the translation of mRNA synthesized from a non-heat shock promoter.** *A*, T-REx cells were co-transfected with pcDNA4TO LacZ and αB-crystallin or Hsp27 expression constructs or their empty vectors. Cells were heat shocked for 1 h at 45 °C and harvested during recovery at 37 °C. Values shown are relative to those obtained in cells co-transfected with pcDNA4TO LacZ and the empty expression vector after 6 h of recovery. *B*, T-REx cells were co-transfected with the pcDNA4TO LacZ construct and the αB-crystallin expression construct or the empty vector. Where indicated (preHS), cells were subjected to a pre-heat shock (30 min, 45 °C) 6 h before heat shock. After heat shock (1 h, 45 °C), cells were harvested after 6 h of recovery at 37 °C. Activities shown here are relative to activities found in non-heat-shocked cells transfected in parallel and are the average of two experiments. *Error bars*, standard deviation.

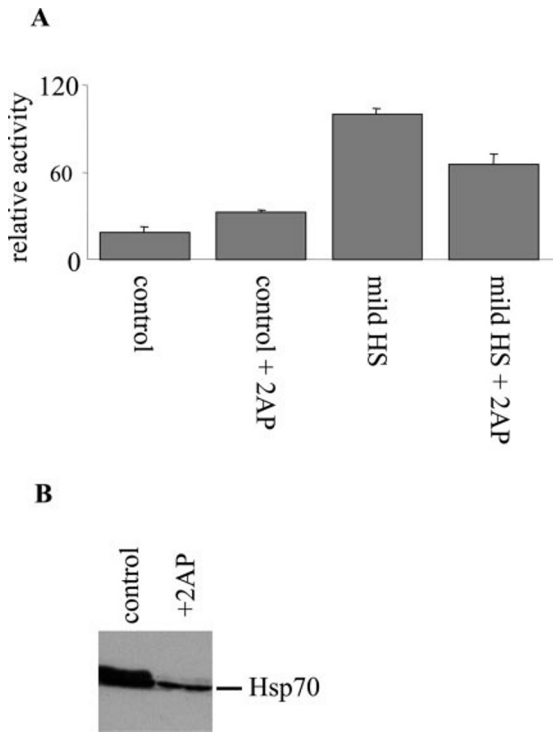

FIG. 9. **Effect of 2-aminopurine on translation.** *A*, T-REx cells were transfected with pcDNA4TO LacZ and, where indicated (*mild HS*), were subjected to a heat shock for 30 min at 45 °C. 3 h after the heat shock, LacZ was induced for 3 h; 2-aminopurine (*2-AP*) was added 6 h after the heat shock, and cells were harvested 6 h later. *Bars*, values relative to those obtained in cells subjected to a mild heat shock; *error bars*, standard deviation. *B*, equal amounts of extracts from control cells and from cells treated with 2-aminopurine (*2-AP*) were assayed on a Western blot for Hsp70 expression.

overexpressing αB-crystallin alone (αB + HS). In the presence of αB-crystallin or after a pre-heat shock, the translational activity was about 30% of that found in control non-heat-shocked cells (Fig. 8*B*, *−HS*).

The Kinase Inhibitor 2-Aminopurine Blocks Translational Thermotolerance—The data presented above suggest that both αB-crystallin and Hsp27 are capable of conferring translational thermotolerance equivalent to that found in thermotolerant cells. However, it must be remembered that during the heat shock applied to measure thermotolerance, the synthesis of endogenous heat shock proteins is also activated. Therefore, it is possible that the translational thermotolerance provided by one of these two small heat shock proteins requires the cooperation of endogenously synthesized heat shock proteins. To test this possibility, we sought a means of inhibiting the synthesis of endogenous heat shock proteins. It has been shown previously (51) that 2-aminopurine inhibits the synthesis of Hsp70, a finding in agreement with the fact that one of the kinases inhibited by 2-aminopurine, PKR, is required for the heat shock response (52, 53). Therefore, we tested the effect of 2-aminopurine. As 2-aminopurine inhibits eIF2α kinases (40, 41), and as phosphorylation of eIF2α is thought to be at least partially responsible for inhibition of protein initiation during stress (31, 32), treatment with 2-aminopurine should result in an increase in the rate of β-galactosidase synthesis, if the synthesis of endogenous Hsps is not required for the sHsp translational tolerance. As expected, 2-aminopurine enhanced the rate of accumulation of β-galactosidase in control cells, presumably because of dephosphorylation of eIF2α (Fig. 9*A*). Cells recovering from a heat shock are known to have a higher rate of translation than control cells, an effect that has been attributed to inhibition of PKR (54). However, when 2-aminopurine was added to cells which had been allowed to recover for 6 h from a mild heat shock, an inhibition of translation was found (Fig. 9*A*). In these cells, the level of Hsp70 was still increasing, and this increase was inhibited by 2-aminopurine (Fig. 9*B*).

When 2-aminopurine was added just before a heat shock and remained present during recovery, β-galactosidase synthesis was blocked completely, irrespective of the presence of αB-crystallin (Fig. 10*A*). In these cells, 2-aminopurine blocked the further accumulation of both Hsp70 and Hsp27, confirming that 2-aminopurine blocks the heat shock response (Fig. 10*B*). These results strongly suggest that the synthesis of the endogenous heat shock proteins is required for the translational thermotolerance seen in cells overexpressing αB-crystallin.

DISCUSSION

We have shown here that, during recovery from a heat shock, overexpression of αB-crystallin or Hsp27 increases expression from a luciferase reporter gene producing a cap-dependent transcript but not from a luciferase reporter gene producing a cap-independent transcript, whereas overexpression of Hsp70 increases expression from both constructs. For sHsps, it has been shown that they cannot refold luciferase under these conditions (55), and their effect must thus be on *de novo* synthesis. The effect of Hsp70 could, in principle, be because of

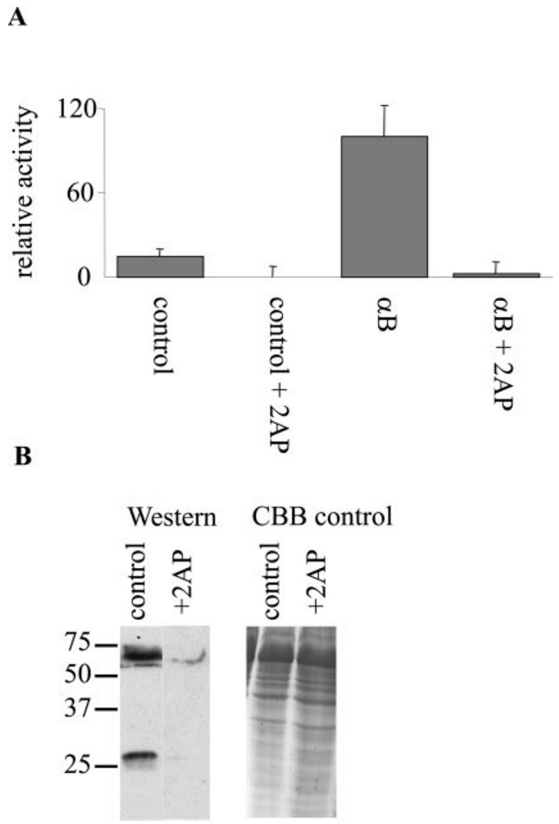

FIG. 10. **Effect of 2-aminopurine on the protection of translation by αB-crystallin.** *A*, T-REx cells were transfected with pcDNA4TO LacZ and αB-crystallin expression construct or the empty vector. 2-Aminopurine was added directly before the heat shock, and cells were harvested after 6 h of recovery at 37 °C. *Bars* show the activities relative to those found in αB-crystallin overexpressing cells in the absence of 2-aminopurine; *error bars*, standard deviations. *B*, control heat-shocked cells (*control*) and heat-shocked cells treated with 2-aminopurine (*2-AP*) were harvested after 6 h of recovery at 37 °C, and extracts were assayed on Western blot for Hsp70 and Hsp27 expression levels. A Coomassie Blue-stained gel of the same samples is shown as the loading control.

refolding of previously synthesized luciferase. However, the leakage of the Hsp70 promoter used in these reporter constructs is very low, as the level of luciferase in non-heat-shocked cells is only about 10% of that found in cells after recovery from a heat shock in the absence of an overexpressed Hsp (data not shown). The increase in luciferase activity in the presence of Hsp70 must therefore also be due to *de novo* synthesis. The Hsp70 isoform Hsp72 has been shown to be associated with polysomes after a heat shock (27), and the effect of Hsp70 could well be on elongation as well as on initiation. In the case of sHsps, only cap-dependent synthesis increases, making it very unlikely that these proteins affect elongation. The most likely interpretation of our data is that sHsps protect one of the steps in cap-dependent initiation. This suggestion seems at first glance to be in contrast to the data of Cuesta *et al.* (38), who showed that Hsp27 forms an insoluble complex with eIF4G. However, the experiments reported by Cuesta *et al.* were performed at the heat shock temperature, whereas we examined the effect of Hsp27 in cells recovering from a heat shock. Our data, combined with the data of Cuesta *et al.*, would suggest a model in which Hsp27 (or αB-crystallin) interacts with and stabilizes eIF4G during the heat shock, resulting in increased availability of eIF4G during recovery from a heat shock. Such a model is attractive, as it would also be in accordance with the presumed role of the sHsps as chaperones of non-native proteins. For αB-crystallin, there is indeed a correlation between protective activity and chaperone activity, as for this protein phosphorylation abolishes its ability to confer translational thermotolerance, and phosphorylation also decreases its chaperone activity (17, 18). However, we find that mimicking phosphorylation of Hsp27 does not affect its ability to provide translational thermotolerance, yet it has been clearly shown that such phosphorylation mutants lack *in vitro* chaperone activity (16). Conversely, mutants of Hsp27 that cannot be phosphorylated retain full chaperone activity but are inactive in our translation tolerance assay. Thus, for Hsp27, there is no correlation between chaperone activity and translational thermotolerance. Either αB-crystallin and Hsp27 differ in the mechanism by which they confer translational thermotolerance (they could, for example, target different components of the cap-binding complex) or, if their mechanism of action is the same, the establishment of translational tolerance by sHsps does not require chaperone activity.

The heat shock response is elicited by proteotoxic stress. The nature of the cellular damage sensors is still unclear, but one recently elucidated mechanism involves the activation of the double-stranded RNA-activated kinase through activation of RAX/PACT (56, 57). In the absence of double-stranded RNA-activated kinase, Hsp70 is not induced (53). Hsp70 induction is also blocked by 2-aminopurine (Ref. 51 and Fig. 10*B*), an effect possibly mediated by inhibition of double-stranded RNA-activated kinase. To our surprise, we found that treatment with 2-aminopurine abolished the protective effect of overexpressing αB-crystallin. The most likely explanation is that the protective effect of αB-crystallin requires the synthesis of endogenous heat shock proteins. Translational thermotolerance would then be a cooperative effect of different heat shock proteins. Present studies are directed at determining which heat shock proteins are required in addition to αB-crystallin or Hsp27.

The translational response to a heat shock is complex. Initially, translation is strongly inhibited, whereas translation is enhanced upon recovery from a heat shock (for example see Fig. 9*A*). It has been suggested that PKR is the controlling factor in both effects: its activation upon stress would cause phosphorylation of eIF2α and thus inhibition of translation initiation; its inhibition during recovery would lead to dephosphorylation of eIF2α and thus to enhanced translation initiation. It has been suggested that the presumed inhibition of PKR during heat shock recovery is due to enhanced transcription of Alu repeats and inhibition of PKR by those transcripts (54, 58). Indeed, this mechanism has been suggested to be an evolutionary advantage of maintaining short interspersed elements (59). Our data and other data strongly call this interpretation into question. Activation of PKR is required for synthesis of heat shock proteins and thus for the recovery of the cells; inhibition of PKR during heat shock and during recovery from the heat shock blocks rather than stimulates protein synthesis. The mechanism of the stimulation of protein synthesis in recovering cells thus remains obscure. If dephosphorylation of eIF2α is involved, then, in recovering cells, eIF2α is not a PKR substrate. Alternatively, GADD34 up-regulation in stressed cells might result in a higher rate of dephosphorylation of eIF2α than phosphorylation by PKR (60).

Hsp27 and αB-crystallin are constitutively expressed in a number of tissues. Our finding that these proteins provide these tissues with translational thermotolerance, albeit in cooperation with other heat shock proteins, is thus of physiological importance. We have further shown that the translational thermotolerance provided by the sHsps is specific for cap-dependent mRNAs. The sHsps thus can shift the translation pattern by maintaining translation of cap-dependent mRNAs during stress. It is becoming more and more apparent that

shifts in the activity of general initiation factors have a major effect on the translational efficiency of specific genes (32). It would be of considerable interest to determine whether there is also a subtle interaction between sHsps and the translation initiation in the absence of stress. The level of sHsps could then be one of the determinants of the translational pattern of a cell.

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REFERENCES

1. Laszlo, A. (1988) *Exp. Cell Res.* **178,** 401–414
2. Brostrom, C. O., and Brostrom, M. A. (1998) *Prog. Nucleic Acid Res. Mol. Biol.* **58,** 79–125
3. de Jong, W. W., Leunissen, J. A., and Voorter, C. E. (1993) *Mol. Biol. Evol.* **10,** 103–126
4. Kappé, G., Franck, E., Verschuure, P., Boelens, W. C., Leunissen, J. A. M., and de Jong, W. W. (2003) *Cell Stress Chaperones* **8,** 53–61
5. Kato, K., Ito, H., and Inaguma, Y. (2002) *Prog. Mol. Subcell. Biol.* **28,** 129–150
6. Van Montfort, R., Slingsby, C., and Vierling, E. (2001) *Adv. Protein Chem.* **59,** 105–156
7. Davidson, S. M., Loones, M. T., Duverger, O., and Morange, M. (2002) *Prog. Mol. Subcell. Biol.* **28,** 103–128
8. Narberhaus, F. (2002) *Microbiol. Mol. Biol. Rev.* **66,** 64–93
9. Quinlan, R. (2002) *Prog. Mol. Subcell. Biol.* **28,** 219–233
10. Arrigo, A. P., Paul, C., Ducasse, C., Manero, F., Kretz-Remy, C., Virot, S., Javouhey, E., Mounier, N., and Diaz-Latoud, C. (2002) *Prog. Mol. Subcell. Biol.* **28,** 185–204
11. Hoover, H. E., Thuerauf, D. J., Martindale, J. J., and Glembotski, C. C. (2000) *J. Biol. Chem.* **275,** 23825–23833
12. Kato, K., Ito, H., Kamei, K., Inaguma, Y., Iwamoto, I., and Saga, S. (1998) *J. Biol. Chem.* **273,** 28346–28354
13. Ito, H., Okamoto, K., Nakayama, H., Isobe, T., and Kato, K. (1997) *J. Biol. Chem.* **272,** 29934–29941
14. Gaestel, M. (2002) *Prog. Mol. Subcell. Biol.* **28,** 151–169
15. Lambert, H., Charette, S. J., Bernier, A. F., Guimond, A., and Landry, J. (1999) *J. Biol. Chem.* **274,** 9378–9385
16. Rogalla, T., Ehrnsperger, M., Preville, X., Kotlyarov, A., Lutsch, G., Ducasse, C., Paul, C., Wieske, M., Arrigo, A. P., Buchner, J., and Gaestel, M. (1999) *J. Biol. Chem.* **274,** 18947–18956
17. Ito, H., Kamei, K., Iwamoto, I., Inaguma, Y., Nohara, D., and Kato, K. (2001) *J. Biol. Chem.* **276,** 5346–5352
18. Kamei, A., Hamaguchi, T., Matsuura, N., and Masuda, K. (2001) *Biol. Pharm. Bull.* **24,** 96–99
19. Geum, D., Son, G. H., and Kim, K. (2002) *J. Biol. Chem.* **277,** 19913–19921
20. Arrigo, A. P., Paul, C., Ducasse, C., Sauvageot, O., and Kretz-Remy, C. (2002) *Prog. Mol. Subcell. Biol.* **28,** 171–184
21. Carper, S. W., Rocheleau, T. A., Cimino, D., and Storm, F. K. (1997) *J. Cell. Biochem.* **66,** 153–164
22. Lavoie, J. N., Gingras-Breton, G., Tanguay, R. M., and Landry, J. (1993) *J. Biol. Chem.* **268,** 3420–3429
23. Nollen, E. A., Brunsting, J. F., Roelofsen, H., Weber, L. A., and Kampinga, H. H. (1999) *Mol. Cell. Biol.* **19,** 2069–2079
24. Gabai, V. L., Meriin, A. B., Mosser, D. D., Caron, A. W., Rits, S., Shifrin, V. I., and Sherman, M. Y. (1997) *J. Biol. Chem.* **272,** 18033–18037
25. Haslbeck, M., and Buchner, J. (2002) *Prog. Mol. Subcell. Biol.* **28,** 37–59
26. Hallberg, E. M., and Hallberg, R. L. (1996) *Cell Stress Chaperones* **1,** 70–77
27. Beck, S. C., and De Maio, A. (1994) *J. Biol. Chem.* **269,** 21803–21811
28. De Maio, A., Beck, S. C., and Buchman, T. G. (1993) *Eur. J. Biochem.* **218,** 413–420
29. De Maio, A., Beck, S. C., and Buchman, T. G. (1993) *Circ. Shock* **40,** 177–186
30. Sheikh, M. S., and Fornace, A. J., Jr. (1999) *Oncogene* **18,** 6121–6128
31. Patel, J., McLeod, L. E., Vries, R. G., Flynn, A., Wang, X., and Proud, C. G. (2002) *Eur. J. Biochem.* **269,** 3076–3085
32. Dever, T. E. (2002) *Cell* **108,** 545–556
33. Scheper, G. C., Mulder, J., Kleijn, M., Voorma, H. O., Thomas, A. A., and van Wijk, R. (1997) *J. Biol. Chem.* **272,** 26850–26856
34. Fernandez, J., Yaman, I., Sarnow, P., Snider, M. D., and Hatzoglou, M. (2002) *J. Biol. Chem.* **277,** 19198–19205
35. Kim, Y. K., and Jang, S. K. (2002) *Biochem. Biophys. Res. Commun.* **297,** 224–231
36. Joshi-Barve, S., De Benedetti, A., and Rhoads, R. E. (1992) *J. Biol. Chem.* **267,** 21038–21043
37. Kedersha, N. L., Gupta, M., Li, W., Miller, I., and Anderson, P. (1999) *J. Cell Biol.* **147,** 1431–1442
38. Cuesta, R., Laroia, G., and Schneider, R. J. (2000) *Genes Dev.* **14,** 1460–1470
39. Kedersha, N., Cho, M. R., Li, W., Yacono, P. W., Chen, S., Gilks, N., Golan, D. E., and Anderson, P. (2000) *J. Cell Biol.* **151,** 1257–1268
40. Jarrous, N., Osman, F., and Kaempfer, R. (1996) *Mol. Cell. Biol.* **16,** 2814–2822
41. Ben-Asouli, Y., Banai, Y., Pel-Or, Y., Shir, A., and Kaempfer, R. (2002) *Cell* **108,** 221–232
42. Torok, I., and Karch, F. (1980) *Nucleic Acids Res.* **8,** 3105–3123
43. Shimasaki, S., Emoto, N., Koba, A., Mercado, M., Shibata, F., Cooksey, K., Baird, A., and Ling, N. (1988) *Biochem. Biophys. Res. Commun.* **157,** 256–263
44. Gunning, P., Leavitt, J., Muscat, G., Ng, S. Y., and Kedes, L. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 4831–4835
45. van den IJssel, P. R., Overkamp, P., Knauf, U., Gaestel, M., and de Jong, W. W. (1994) *FEBS Lett.* **355,** 54–56
46. Vagner, S., Touriol, C., Galy, B., Audigier, S., Gensac, M. C., Amalric, F., Bayard, F., Prats, H., and Prats, A. C. (1996) *J. Cell Biol.* **135,** 1391–1402
47. Beretta, L., Gingras, A. C., Svitkin, Y. V., Hall, M. N., and Sonenberg, N. (1996) *EMBO J.* **15,** 658–664
48. Chang, G. C., Liu, R., Panniers, R., and Li, G. C. (1994) *Int. J. Hyperthermia* **10,** 325–337
49. Landry, J., Chretien, P., Laszlo, A., and Lambert, H. (1991) *J. Cell. Physiol.* **147,** 93–101
50. Gabai, V. L., and Sherman, M. Y. (2002) *J. Appl. Physiol.* **92,** 1743–1748
51. Yamamoto, N., Smith, M. W., Maki, A., Berezesky, I. K., and Trump, B. F. (1994) *Kidney Int.* **45,** 1093–1104
52. Williams, B. R. (1999) *Oncogene* **18,** 6112–6120
53. Zhao, M., Tang, D., Lechpammer, S., Hoffman, A., Asea, A., Stevenson, M. A., and Calderwood, S. K. (2002) *J. Biol. Chem.* **277,** 44539–44547
54. Chu, W. M., Ballard, R., Carpick, B. W., Williams, B. R., and Schmid, C. W. (1998) *Mol. Cell. Biol.* **18,** 58–68
55. Jakob, U., Gaestel, M., Engel, K., and Buchner, J. (1993) *J. Biol. Chem.* **268,** 1517–1520
56. Patel, C. V., Handy, I., Goldsmith, T., and Patel, R. C. (2000) *J. Biol. Chem.* **275,** 37993–37998
57. Ito, T., Yang, M., and May, W. S. (1999) *J. Biol. Chem.* **274,** 15427–15432
58. Rubin, C. M., Kimura, R. H., and Schmid, C. W. (2002) *Nucleic Acids Res.* **30,** 3253–3261
59. Schmid, C. W. (1998) *Nucleic Acids Res.* **26,** 4541–4550
60. Novoa, I., Zhang, Y., Zeng, H., Jungreis, R., Harding, H. P., and Ron, D. (2003) *EMBO J.* **22,** 1180–1187