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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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Linda Doerwald, Carla Onnekink, Siebe T. van Genesen, Wilfried W. de Jong, and Nicolette H. Lubsen‡
From the Department of Biochemistry, Faculty of Science, University of Nijmegen, 6500HB Nijmegen, The Netherlands

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, which are 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)

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‡ To whom correspondence should be addressed: Dept. of Biochemistry 161, Faculty of Science, P.O. Box 9101, 6500HB Nijmegen, The Netherlands. Tel: 31-24361-6850; Fax: 31-24354-0525; E-mail: nhl@sci.kun.nl.
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; MEM, Dulbecco’s modified Eagle’s medium; PKR, double-stranded RNA-activated kinase; IRES, internal ribosome entry site; FGF, fibroblast growth factor.

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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 Hsp70 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 of 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 Hsp.

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. Cells were harvested during recovery at 37 °C and assayed for β-galactosidase and luciferase activity.

Transfections—C2 cells were transfected with LipofectAMINE plus (Invitrogen). Approximately 6.5 × 10⁴ 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, Hsp-Cap-Luc or pHSF-IRE-Luc and expression vectors for αB-crystallin, Hsp27, Hsp70, βB2-crystallin, or the empty vectors as a 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.

"\textbf{RT-PCR.—} C2 cells were transfected 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. 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 H₂O 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) using 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, TGGATGGCTACTACCTGAGAC, and at position +1720, GCC-TTGGGCGCTTATGAGGATC) and, as a transfection control, β-galactosidase mRNA primers (at position +155, 3′-TTGACGCGCGCAGTCGTC-5′, and at position +630, TCGACGGCGACACTG). 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 μl of the diluted RNA to test for cDNA contamination. The PCR was denatured at 94 °C for 60 s, primers were annealed at 66 °C for 30 s, and then elongation was performed at 72 °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-
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 Hsp27 was not (Fig. 3 and data not shown). Rapamycin has been reported to inhibit cap-dependent translation initiation but not cap-independent mechanism. In support of this proposed difference in the mechanism of translation initiation, we found that 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. 3B 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. 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 352-bp long 5′ non-coding region of the GFG-2 mRNA. This region contains an IRES known to be used during stress (46). This construct thus reflects IRES-dependent translation after stress.

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.
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. 6B 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 βB-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, S90D, S19D/S45D/S90D, and Hsp27 S15E/S90E) αB-crystallin or Hsp27 were co-transfected with pHsp-Cap-Luc. As shown in Fig. 7A, mimicking constitutive phosphorylation of αB-crystallin abolishes the protection of translation, irrespective of the site at which phosphorylation is mimicked. The non-phosphorylatable...
mutants of βB-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. 7B). These data show that, for full activity in translation thermotolerance, Hsp27 needs to be phosphorylated, whereas βB-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 βB-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 rate of increase in β-galactosidase activity during recovery at 37 °C was then measured. As shown in Fig. 8A, in the presence of co-transfected βB-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 βB-crystallin and Hsp27 persists for at least 20 h (Fig. 8A). In C2 cells, using the pHsp-Cap-Luc reporter described above, luciferase values in control cells were co-transfected with pHsp-Cap-Luc with (CAP + Hsp27) or without (CAP − Hsp27) the Hsp27 expression construct. Cells were 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 βB-crystallin or Hsp27, respectively. Error bars, standard deviation.

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...
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. 9A). 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. 9A). In these cells, the level of Hsp70 was still increasing, and this increase was inhibited by 2-aminopurine (Fig. 9B).

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. 10A). In these cells, 2-aminopurine blocked the further accumulation of both Hsp70 and Hsp27, confirming that 2-aminopurine blocks the heat shock response (Fig. 10B). 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. 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.

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. 8B, −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
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. 10B), 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. 9A). 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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