Cell. Mol. Life Sci. 63 (2006) 735–743
1420-682X/06/060735-9
DOI 10.1007/s00018-005-5582-5

Cellular and Molecular Life Sciences

Research Article

The effect of αB-crystallin and Hsp27 on the availability of translation initiation factors in heat-shocked cells

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Received 9 December 2005; received after revision 16 January 2006; accepted 23 January 2006
Online First 23 February 2006

Abstract. The mechanism of the translational thermotolerance provided by the small heat shock proteins (sHsps) αB-crystallin or Hsp27 is unknown. We show here that Hsp27, but not αB-crystallin, increased the pool of mobile stress granule-associated enhanced green fluorescent protein (EGFP)-eukaryotic translation initiation factor (eIF)4E in heat-shocked cells, as determined by fluorescence recovery after photobleaching. Hsp27 also partially prevented the sharp decrease in the pool of mobile cytoplasmic EGFP-eIF4G. sHsps did not prevent the phosphorylation of eIF2α by a heat shock, but promoted dephosphorylation during recovery. Expression of the C-terminal fragment of GADD34, which causes constitutive dephosphorylation of eIF2α, fully compensated for the stimulatory effect of αB-crystallin on protein synthesis in heat-shocked cells, but only partially for that of Hsp27. Our data show that sHsps do not prevent the inhibition of protein synthesis upon heat shock, but restore translation more rapidly by promoting the dephosphorylation of eIF2α and, in the case of Hsp27, the availability of eIF4E and eIF4G.

Keywords. eIF2B, FRAP, eIF4E, eIF4G, stress granules, eIF2α.

Introduction

Cells in stress down-regulate protein synthesis using various strategies [1, 2]. A common factor in most types of stress is the activation of an eukaryotic translation initiation factor (eIF)2α kinase. Phosphorylation of eIF2α leads to inhibition of the activity of eIF2B, the guanine nucleotide exchange factor responsible for regenerating eIF2-GTP. This results in a limited availability of eIF2-GTP-tRNA-Met, and thus a reduced rate of translation initiation (for review see [1, 3]). Heat stress also directly inactivates eIF2B [4]. Stress is usually also accompanied by dephosphorylation of the eIF4E binding proteins. These then sequester eIF4E, thereby inhibiting the cap-binding complex [5, 6]. Cap-dependent translation under at least some conditions of stress is also inhibited by phosphorylation of eIF4G by protein kinase Pak2 [7]. The modulation of the activity of various initiation factors in stressed cells explains why translation initiation via an internal ribosome entry site (IRES) is more resistant to inhibition by heat stress than cap-dependent translation initiation [8–10].
Inhibition of translation initiation results in the accumulation in the cytoplasm of phase dense granules, the stress granules (SGs; for review see [11, 12]). In these SGs, stalled translation initiation complexes containing almost all components of the 48S pre-initiation complex, but not the 60S ribosomal subunit, are found [11–13]. Other proteins such as the RNA-binding proteins TIA-1 and TIAR

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as well as the endoribonuclease G3BP are also found in SGs, and appear to play a role in their formation [14–16]. The assembly of SGs is a highly dynamic process; untranslated mRNAs are thought to be sorted and processed in SGs for reinitiation, degradation or packaging into non-polysomal mRNP complexes [15]. It has been recently shown that SGs are linked with cytoplasmic sites of RNA degradation, the processing bodies [17]. Processing bodies are also induced by stress, although not by heat stress [17]. Accumulation of proteins and mRNA in SGs is irreversible when cells are lethally stressed, but SG formation is reversible in cells recovering from sublethal stresses [14, 15]. To disaggregate SGs, Hsp70 and ATP are necessary [11, 12]. Small heat shock proteins (sHsps) may also play a role, as at least Hsp27 has been detected in SGs [15, 18, 19]. Stress due to amino acid starvation also induces processing bodies, the autophagic lysosomal bodies. These are distinct from the stress-induced processing bodies and granules. Interestingly, the eIF2α kinase signaling pathway plays a regulatory role in the formation of all these structures [13, 14, 20].

Stress due to unfolding cytoplasmic proteins induces the synthesis of protective proteins, the Hsps (for review see [21, 22]). These proteins are required for recovery from stress, but also protect cells from subsequent stress, a phenomenon known as thermotolerance [23]. Thermotolerance manifests itself not only by increased cellular survival after stress, but also through a lesser inhibition of macromolecular synthesis by stress ([24, 25]; for review see [26]). We and others have previously shown that the small heat shock proteins (sHsps) Hsp27 and αB-crystallin protect translation initiation against a heat stress [9, 25]. The mechanism of this protection is unknown but must involve increasing, directly or indirectly, the availability of eIF4E and/or eIF2-GTP-tRNA-Met. Using fluorescence recovery after photobleaching (FRAP), we have looked at the dynamics of enhanced green fluorescent protein (EGFP)-eIF4E sequestered in SGs in cells recovering from heat stress, and show here that Hsp27, but not αB-crystallin, increases the pool of free EGFP-eIF4E and EGFP-eIF4G. We also assayed the effect of sHsps on the phosphorylation of eIF2α, and tested whether phosphorylation of eIF2α is rate limiting in the recovery of protein synthesis after a heat shock, both in the absence and in the presence of sHsps. We show that inducing dephosphorylation of eIF2α by expression of the constitutively active C-terminal fragment of GADD34, the regulatory subunit of the eIF2α phosphatase complex [27], can fully compensate for the effect of αB-crystallin but only partially for that of Hsp27.

Materials and methods

Cell culture. T-REx αB or Hsp27 cells [T-REx HeLa cells (Invitrogen) stably transfected with an expression construct for αB-crystallin (pcDNA4-TO-αB) or Hsp27 (pcDNA4-TO-Hsp27) under the control of the tetracycline repressor] were cultured in minimum essential medium Eagle (BioWhittaker) with glutamax (Gibco), 10% fetal calf serum, penicillin, streptomycin and blasticydin (Invitrogen).

FRAP experiments. Approximately 2.5×10^5 T-REx αB or Hsp27 cells were plated in minimum essential medium Eagle with glutamax, 10% fetal calf serum, penicillin and streptomycin in 3.5-cm glass dishes (HBSt3522; WillCo-dish) and transfected after 24 h with 0.2 µg pEGFP-eIF4E DNA or pEGFP-4G DNA and 0.8 µg pBluescript DNA as carrier using 6 µl dimethyldioctadecylammonium bromide (DDAB):dioleoyl-L-alpha-phosphatidylethanolamine (DOPE) liposomes [28] per dish. Where indicated, after 24 h, 1 µg/ml doxycycline was added to the medium for 24 h to induce αB-crystallin or Hsp27 expression before the heat shock (1 h at 45 °C). After the heat shock, the medium was replaced by medium lacking phenol red. FRAP experiments were performed on the cells during recovery at 37 °C using a Zeiss LSM510 meta microscope. Excitation wavelength was 488 nm and the band pass filter was 500–550 nm. The fluorescence of the region of interest was measured for 10 s using the laser beam reduced to 1.1% of the 25% setting, and then bleached for 4 s using the 488 laser line at maximal intensity. Recovery of fluorescence was measured for 200 s after bleaching with the laser beam again reduced to 1.1% of the 25% setting. Fluorescence recovery was measured of at least three SGs and two granule-free cytoplasmic regions in one cell. The data from at least three independent experiments were pooled.

SG formation. T-REx-αB or Hsp27 cells were transfected with EGFP-eIF4E or eIF4G fusion constructs as described above. Where indicated, after 24 h, 1 µg/ml doxycycline was added to the medium of the T-Rex αB cells or Hsp27 for 24 h to induce sHsp expression before the heat shock (1 h at 45 °C). Cells were either fixed with 3% paraformaldehyde in PBS at various times after heat shock and recovery, and photographed using a Zeiss Axiovert 135 TV microscope, or living cells were photographed directly after heat shock and during recovery using a Zeiss LSM510 meta microscope.

Reporter assays. At 24 h before transfection, 2.5×10^5 T-REx αB or Hsp27 cells were plated per well in a 6-well plate. Cells were transfected with 1 µg DNA using 6 µl DDAB:DOPE liposomes [28]. As a transfection control, 0.1 µg CMV-β-galactosidase was co-transfected; the other 0.9 µg DNA was used for the various constructs: pHsp-Cap-Luc (0.2 µg) and expression vectors for the factors indicated (0.7 µg), or the corresponding empty vectors as control. At 24 h after transfection, the expression of

αB-crystallin or Hsp27 was induced by adding 1 µg/ml doxycycline. At 48 h after transfection, cells were heat shocked for 1 h at 45 °C. Cell survival after heat shock was 80% as measured by the WST-1 assay (Roche), and independent of the induced expression of αB-crystallin or Hsp27 (data not shown). Cells were harvested after 6 h of recovery at 37 °C by vigorously shacking in 200 µl 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, galacton (Tropix) was diluted 1:100 in 100 mM phosphate buffer pH 8.1, 5 mM $MgCl_2$, and 200 µl was added to 20 µl cell lysate. After 30-min incubation at room temperature, 300 µl of light emission accelerator (Tropix) was added. In addition, 20 µl cell lysate was also used for the luciferase assay. Immediately before measurement, 100 µl luciferase reagent (Promega) was added to the lysate, and measurements were performed on a Lumat LB 9507 luminometer for 10 s.

Western blots. Cells were harvested directly in SDS sample buffer (20% glycerol, 4% SDS, 200 mM dithiothreitol, 200 mM Tris-HCl pH 6.8, and bromophenol blue). The protein content was measured using the BCA protein assay kit (Pierce). Samples were heated for 5 min at 95 °C. Equal amounts of protein (50 µg) were loaded on a 12% acrylamide:bisacrylamide (30:0.8) gel. After separation, the proteins were transferred to Protran membranes (Schleicher and Schuell). The Western blots were stained for eIF2α, eIF2α-P (antibody from Sigma) or S6-P (antibody from Cell Signaling) using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce).

Constructs. The pHsp-Cap-Luc has been described previously [9]. The pAD4G vector was a kind gift of Dr. R. E. Rhoads. The insert was recloned *Sal*I/*Not*I (bl) in the *Sal*I and *Sma*I sites of the pEGFP-C1vector (Stratagene) to generate pEGFP-eIF4G. The eIF4E coding region from the pTCEEC 4E construct (a kind gift of Dr. R. E. Rhoads) was amplified using PCR such that it was flanked by a *Bgl*II and a *Hind*III site. The PCR fragment was ligated into the pGem-T Easy vector (Promega). After verification of the sequence, the eIF4E coding region was cloned in frame 3′ of the EGFP coding region into the pEGFP-C1 vector (Clontech) using the *Bgl*II and *Sac*II sites. To obtain the constitutively active GADD34 construct, the insert of pBabe A1 [27] (construct generously provided by Dr. C. Koumenis) was amplified by PCR, creating *Eco*RI and *Sal*I flanking sites. After verification of the sequence, the insert was cloned *Eco*RI/*Sal*I in frame with, and downstream of, the VSV tag in pCIneo-VSV. The expression clones for eIF2B and eIF2B-S535A [29] were a kind gift of Dr. G. M. Cooper. The EGFP-Ro60 expression construct was provided by Dr. G. J. Pruijn.

Results

Effect of overexpression of αB-crystallin or Hsp27 on SG formation after heat shock. To assess the effect of exogenous expression of sHsps on the location or activity of eIFs during recovery from a heat shock, we have made use of T-REx HeLa cells stably transfected with expression constructs for (hamster) Hsp27 (T-REx Hsp27) or (human) αB-crystallin (T-REx αB) under the control of the tetracyclin repressor. Upon induction, these cells express abundant amounts of the corresponding sHsp, reaching fully induced levels within about 24 h. For Hsp27, the level is about ten times the endogenous level in non-stressed cells. The induced αB-crystallin level is the same as the induced Hsp27 level; HeLa cells have little or no endogenous αB-crystallin [30]. To follow the behavior of eIF4E in living cells as a function of sHsp levels, we fused it with EGFP (EGFP-eIF4E) and transiently expressed the fusion protein in T-REx αB or Hsp27 cells. In non-stressed cells, EGFP-eIF4E was found uniformly distributed through the cytoplasm. No difference was seen between non-induced T-REx αB or Hsp27 cells or cells induced for the expression of the corresponding Hsps (data not shown). After a heat shock, EGFP-eIF4E was located in irregularly shaped cytoplasmic granules, presumably SGs, irrespective of the presence of αB-crystallin or Hsp27 (Fig. 1; note that due to the heat shock the cells appear swollen and rounded). With time, the SGs disappeared: after 6 h of recovery from a heat shock about 50% of the cells still contained SGs. The rate of disappearance of SGs tended to be higher in the presence of sHsps, but the effect was not statistically significant due to the variability between experiments (data not shown).

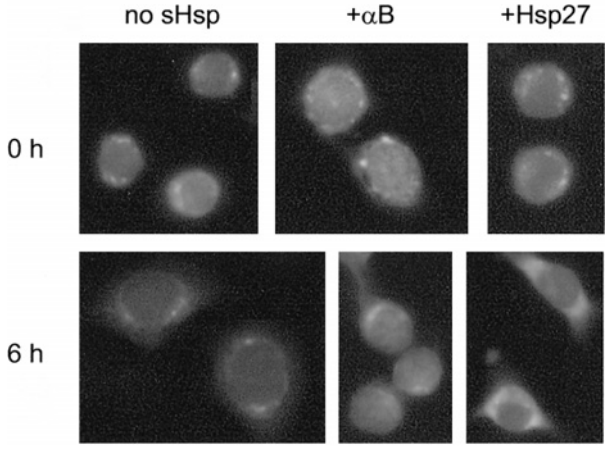

Figure 1. The effect of αB-crystallin or Hsp27 on stress granule (SG) formation in cells recovering from a heat shock. T-REx αB or Hsp27 cells (see Materials and methods) were transfected with the EGFP-eIF4E expression construct. Small heat shock protein (sHsp) expression was induced for 24 h prior to heat shock as indicated on the top. Cells were fixed either directly after heat shock (60 min 45 °C) or after 6 h of recovery as indicated on the right.

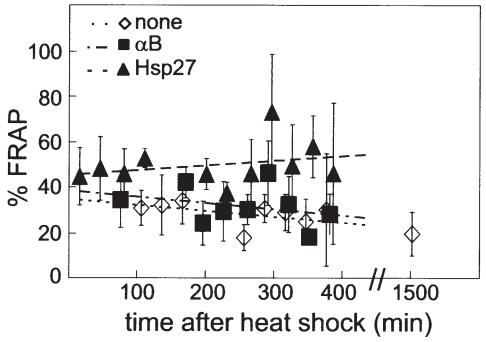

Figure 2. The effect of αB-crystallin or Hsp27 on the mobility of EGFP-eIF4E in stress granules in cells recovering from a heat shock. T-REx αB or Hsp27 cells (see Materials and methods) were transfected with the enhanced green fluorescent protein-eukaryotic translation initiation factor 4E (EGFP-eIF4E) expression construct. sHsp expression was induced for 24 h prior to heat shock as indicated. The fluorescence recovery after photobleaching (FRAP) was measured at various times during recovery at 37 °C. The results shown are the average and standard deviation of the FRAP. The data obtained at 30-min intervals (the data point is at the center of that interval) and obtained in three independent experiments were pooled. The lines are the best linear fit through the data points obtained for cells lacking sHsp (dotted line), expressing αB-crystallin (dot-dash line) or Hsp27 (dashed line).

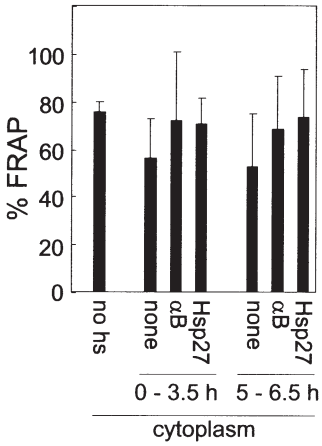

Figure 3. The effect of αB-crystallin or Hsp27 on the mobility of EGFP-eIF4E in the cytoplasm of cells recovering from a heat shock. T-REx αB or Hsp27 cells (see Materials and methods) were transfected with the EGFP-eIF4E expression construct and sHsp expression was induced for 24 h prior to heat shock as indicated. The FRAP was measured at various times during recovery at 37 °C. The results shown are the average and standard deviation of the FRAP during the first 3.5 h of recovery and between 5 and 6.5 hours of recovery. The data obtained in three independent experiments were pooled.

SGs are dynamic assemblies [15]. To determine whether sHsps have an effect on the amount of exchangeable EGFP-eIF4E in SGs, we measured the FRAP of EGFP-eIF4E in SGs. The extent of recovery of fluorescence of SGs was variable, even within a single cell. Nevertheless, a clear trend was seen. The FRAP of EGFP-eIF4E in SGs shortly after heat shock in control cells was, on average, about 34% (Fig. 2), and slowly decreased during the first 6 h of recovery. In the few SGs remaining after 24 h of recovery the FRAP was only about 22%. Apparently, in heat-shocked cells, only about a third of the EGFP-eIF4E in SGs is in rapid exchange with the cytoplasm. When cells expressing αB-crystallin were used, the extent of FRAP of EGFP-eIF4E in SGs after heat shock was, on average, not significantly higher than in control cells (37% *vs* 34%). Again a slow decrease in the extent of FRAP was seen during the first 6 h of recovery (Fig. 2). Thus, αB-crystallin did not affect the dynamics of EGFP-eIF4E in SG. However, when the same experiments were performed using cells expressing exogenous Hsp27, the extent of FRAP of EGFP-eIF4E in SGs after heat shock was significantly higher, 62% on average, than in control cells or αB-crystallin expressing cells. Furthermore, the extent of FRAP increased slowly during the first 6 h of recovery (Fig. 2). These data indicate that a larger fraction of the EGFP-eIF4E associated with SGs is mobile in the presence of Hsp27 than in the presence of αB-crystallin or in the absence of exogenous sHsps; in the presence of Hsp27, two-thirds of the bleached molecules are replaced, in control or αB-crystallin expressing cells only one-third.

One reason for the observed decrease in exchangeable EGFP-eIF4E associated with SGs could be that EGFP-eIF4E is trapped in the cytoplasm. We therefore also measured the extent of FRAP of cytoplasmic EGFP-eIF4E. In non-heat-shocked cells, the extent of FRAP was 75%. The FRAP of EGFP-eIF4E in cells recovering from heat shock was more difficult to measure, as most of the EGFP-eIF4E was located in SGs, and the fluorescence of the cytoplasm was weak. On average, a heat shock did lower the extent of FRAP (by about 25%) in control cells but not in cells expressing sHsps (Fig. 3). We conclude from these experiments that EGFP-eIF4E in the cytoplasm is mobile even in heat-shocked cells.

The data presented above show that the lower FRAP of EGFP-eIF4E in SGs in control or αB-crystallin-expressing cells than in Hsp27-expressing cells is not the result of a large difference in the pool of mobile EGFP-eIF4 in the cytoplasm. The exchange of EGFP-eIF4E between SGs and cytoplasm must thus be limited by another factor. As the cap-binding complex contains not only eIF4E but also the scaffold protein eIF4G, we measured whether EGFP-eIF4G is mobile. In non-stressed cells, EGFP-eIF4G was uniformly distributed throughout the cytoplasm and mobile, as the bleached area of the cytoplasm rapidly regained 80% of the fluorescence. In heat-shocked cells, EGFP-eIF4G remained uniformly distributed in the cytoplasm, both in control cells and in cells expressing either αB-crystallin or Hsp27 (Fig. 4a). Nevertheless, the pool of freely mobile EGFP-eIF4G, as determined from FRAP, was sharply reduced in heat-shocked cells (Fig. 4b). In control cells or in cells expressing αB-crys-

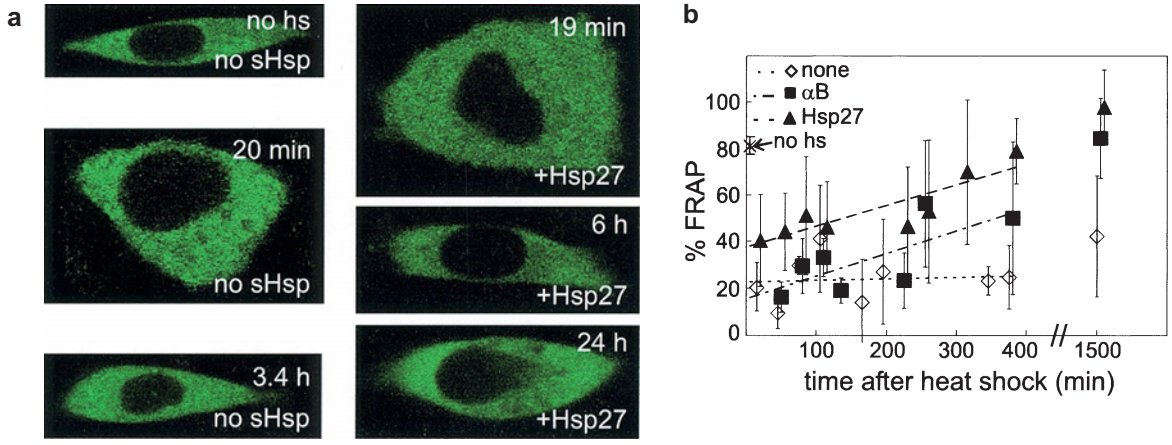

Figure 4. (*a*) The distribution of EGFP-eIF4G in cells recovering from a heat shock in the presence or absence of sHsps. T-REx αB or Hsp27 cells (see Materials and methods) were transfected with the EGFP-eIF4G expression construct, and sHsp expression was induced for 24 h prior to heat shock as indicated (bottom). Cells were photographed live before heat shock or during recovery at 37 °C after heat shock (60 min 45 °C) at the times indicated (top). (*b*) The effect of αB-crystallin or Hsp27 on the mobility of cytoplasmic EGFP-eIF4G in cells recovering from a heat shock. T-REx αB or Hsp27 cells (see Materials and methods) were transfected with the EGFP-eIF4G expression construct, and sHsp expression was induced for 24 h prior to heat shock as indicated. The FRAP was measured at various times during recovery at 37 °C. The results shown are the average and standard deviation of the FRAP. The data obtained at 30-min intervals (the data point is at the center of that interval) and obtained in three independent experiments were pooled. The lines are the best linear fit through the data points obtained for cells lacking sHsp (dotted line), expressing αB-crystallin (dot-dash line) or Hsp27 (dashed line).

tallin, the extent of FRAP was only 25%, indicating that little EGFP-eIF4G is freely diffusing. In cells expressing Hsp27, the pool of free EGFP-eIF4G was larger, as in this case the extent of FRAP was 50%. During the first 6 h of recovery, the extent of FRAP and thus pool of free EGFP-eIF4G increased in sHsps-expressing cells, but not in control cells. Even after 24 h of recovery, the extent of FRAP was still lower in control cells than in non-heat-shocked cells (Fig. 4b).

As a control, we also determined whether a heat shock decreased the freely diffusible pool of another protein, EGFP-Ro60, a protein that is part of the Ro RNP and is present both in the cytoplasm and in the nucleus. In this case no change in the extent of FRAP before or after a heat shock was seen in the cytoplasm, although it was sharply decreased in the nucleus after a heat shock (data not shown).

The effect of phosphorylation of eIF2α. The data presented above show that, in the presence of Hsp27, the freely mobile pool and therefore the availability of eIF4E and eIF4G in heat-shocked cells was higher than in control or αB-crystallin-expressing cells, which could explain the effect of Hsp27 on translational thermotolerance. However, αB-crystallin also promotes the recovery of translation after a heat shock [9]. An effect on the availability of eIF4E and eIF4G thus does not suffice to explain the effect of the two sHsps on the restoration of protein synthesis after heat shock. Our observation that in the presence of either of the two sHsps the SGs tended to disappear faster, suggested that these sHsps might also affect the phosphorylation of eIF2α, as phosphorylation of eIF2α suffices to induce SG formation [13, 14]. We therefore determined the changes in the level of phosphorylation of eIF2α in heat-shocked cells in the absence or presence of sHsps by staining Western blots of extracts of T-REx αB or Hsp27 cells recovering from a heat shock for eIF2α-P. As expected, the eIF2α-P level increased upon heat shock and then gradually decayed during recovery at 37 °C (Fig. 5). Hsp27 or αB-crystallin did not prevent phosphorylation of eIF2α during the heat stress, but the level of eIF2α-P decreased more rapidly than in control cells, an effect that is most clearly visible after 18 h of recovery (Fig. 5). For comparison, the change in the phosphorylation of ribosomal protein S6 after a heat shock is also shown. In heat-shocked cells, the level of S6-P dropped sharply, even though a heat shock has been reported to have little effect on p70 S6 kinase activity [5]. After 24 h of recovery the level was restored. sHsps had no effect on the level of S6-P.

As the data presented above indicated that sHsps did have some effect on the level of eIF2α phosphorylation, we determined whether eIF2α phosphorylation is limiting in the effect of sHsps on protein synthesis after heat shock. We manipulated the phosphorylation level of eIF2α by expressing the C-terminal fragment of GADD34, the regulatory subunit of the eIF2α phosphatase complex [27]. The efficacy of the C-terminal fragment of GADD34 in the dephosphorylation of eIF2α was demonstrated by testing for the presence of SGs. Directly after heat shock only 45% of the cells expressing C-terminal GADD34 contained SGs as compared to 90% of the control cells

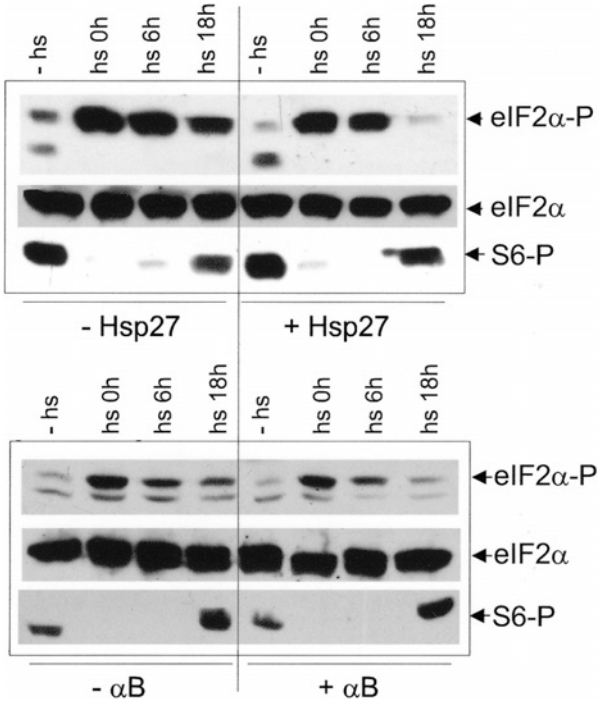

Figure 5. The effect of αB-crystallin or Hsp27 on the level of phosphorylation of eIF2α in cells recovering from a heat shock. sHsp expression was induced in T-REx αB or Hsp27 cells (see Materials and methods) for 24 h prior to heat shock as indicated. Cells were harvested directly prior to heat shock (lane: – hs), directly after heat shock (lane: hs 0h), or after 6 (lane: hs 6h) or 18 h (lane: hs 18h) of recovery. Western blots (see Materials and methods) were stained for eIF2α-P, eIF2α or S6-P as indicated.

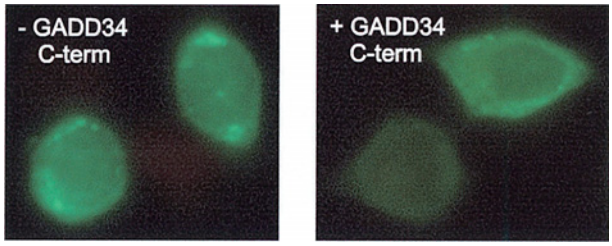

Figure 6. Expression of the C-terminal fragment of GADD34 blocks SG formation in heat-shocked cells. T-REx cells were co-transfected with expression constructs for EGFP-eIF4E and the C-terminal fragment of GADD34, and heat-shocked 48 h after transfection. Cells were fixed directly after heat shock and photographed.

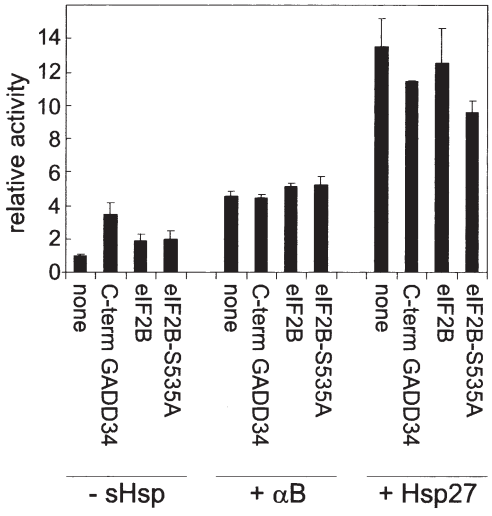

Figure 7. The effect of expression of the C-terminal fragment of GADD34 or eIF2B on protein synthesis during recovery from a heat shock. T-REx αB or Hsp27 cells (see Materials and methods) were co-transfected with pHsp-Cap-Luc and the expression constructs, or the corresponding empty vector, as indicated in the Figure. sHsp expression was induced for 24 h prior to heat shock as indicated. Cells were harvested after 6 h of recovery at 37 °C. The luciferase activity was corrected for transfection efficiency as determined from the β-galactosidase activity. The luciferase activity shown is relative to that found in non-induced T-REx cells transfected with empty vector, which was set arbitrarily at one. Data shown are the average and standard deviation of at least two independent experiments.

(Fig. 6 and data not shown). To measure the effect of manipulating the extent of eIF2α phosphorylation on the level protein synthesis after heat shock, we determined the yield of luciferase from the pHsp-Cap-Luc construct, in which expression of the luciferase reporter gene is driven by the Hsp70 promoter. We have previously shown that the yield of luciferase from this construct is a measure of the level of protein synthesis in cells recovering from a heat shock [9]. Co-transfection of pHsp-Cap-Luc with the expression construct for the C-terminal region of GADD34 resulted in a marked increase in luciferase activity (Fig. 7). This increase was only seen in the absence of sHsps; in the presence of sHsps the C-terminal region of GADD34 had no added effect. Hence, the effect of sHsps on the restoration of protein synthesis after heat shock is, at least in part, due to a faster dephosphorylation of eIF2α.

Heat shock also inhibits eIF2B directly [4], and we therefore also determined the effect of overexpressing the ε subunit of eIF2B or the non-phosphorylatable mutant thereof (eIF2B-S535A; [29]). A twofold increase in the luciferase activity was seen. Again, the effect of exogenous expression of eIF2B or the non-phosphorylatable mutant was only seen in the absence of sHsps. These data thus also show that sHsps act, at least in part, by restoring eIF2B activity.

Discussion

Previous studies of the effect of sHsps on translational thermotolerance have either assayed the effect of sHsps on total protein synthesis in heat-shocked cells [25], or followed the effect on the expression of reporter gene constructs [9]. In those studies, a distinction between a

lesser inhibition or a more rapid relief of the inhibition in the presence of sHsps could not be clearly made. Here we have looked at the effect of αB-crystallin and Hsp27 on the cytological manifestation of a stress-induced block in translation initiation, the SGs. We have used stable cell lines in which the ectopic expression of αB-crystallin or Hsp27 was placed under the control of the TetR. This approach has the advantage that sHsp expression is induced only 24 h prior to the experiments, thus avoiding a long-term adaptation of cells to an increased level in sHps. Our data clearly show that under these circumstances, and using the criteria of SG formation, neither Hsp27 nor αB-crystallin protects translation initiation from a heat stress: all cells still contain SGs irrespective of the presence of sHsps. Rather, sHsps promote a more rapid recovery of translation initiation.

It has been previously shown that the SGs are not static entities, but that GFP-tagged TIA-1 and PABP, both RNA binding proteins, rapidly shuttle in and out of these structures in arsenite-treated cells [15]. EGFP-eIF4E in heat-shocked cells behaves like GFP-PABP in arsenite-treated cells: part of the bound protein rapidly exchanges, another part is not mobile, indicating that SGs contain dynamic as well as static complexes. Surprisingly, the pool of mobile EGFP-eIF4E in SGs was increased only in the presence of Hsp27, not in that of αB-crystallin, while the pool of mobile EGFP-eIF4E in the cytoplasm was higher in the presence of Hsp27 and of αB-crystallin. From the cytoplasmic behavior of EGFP-eIF4E one would predict that the fluorescence recovery of EGFP-eIF4E in the SGs would also be more extensive in the presence of αB-crystallin. As this is not the case, the dynamics of association of EGFP-eIF4E with SGs is not merely dictated by its availability in the cytoplasm and must be determined by association with another SG component of which the behavior is not affected by αB-crystallin. Our results thus suggest that EGFP-eIF4E associates with SGs as a larger complex. The limiting component in the association of EGFP-eIF4E could be eIF4G as the pool of mobile EGFP-eIF4G was consistently larger in the presence of Hsp27 than in control or αB-crystallin-expressing heat-shocked cells. This finding is in agreement with previous reports showing association of Hsp27, but not αB-crystallin, with eIF4G upon heat shock [31, 32]. In contrast to the results reported by Cuesta et al. [31], overexpression of Hsp27 did not inhibit protein synthesis in the cells used in this study (as measured by co-transfection of luciferase reporter genes). Our data thus support the suggestion of Cowan and Morley [32] that the association of eIF4G with Hsp27 has a protective rather than an inhibitory function. We did not see an association of EGFP-eIF4G with SGs, either after heat shock (Fig. 5A) or after arsenite treatment (data not shown). Others have found that eIF4G is associated with SGs [33]. A possible explanation for this discrepancy is that in living cells as used here, the cytoplasmic signal dominates the signal from SGs, while in fixed cells some of the cytoplasmic signal is lost, thereby unmasking the SG (see for example [13]).

A common cause of inhibition of protein synthesis upon stress is phosphorylation of eIF2α by an eIF2α kinase (for review see [2]). The importance of this mechanism is emphasized by the fact that there are four mammalian eIF2α kinases, each of which responds to a different spectrum of stressful stimuli [34–38]. For the unfolded protein response (UPR) and arsenite stress, it has been shown that eIF2α phosphorylation is required for recovery from stress. In the case of the UPR and during amino acid starvation, eIF2α phosphorylation allows reinitation of translation and, thereby, the selective translation of mRNA regions coding for proteins required for recovery [2, 35, 39]. Whether a similar regulatory mechanism is operative in the recovery from arsenite stress is unknown. A heat shock activates two eIF2α kinases: HRI and PKR [34, 36, 37]. The activation of PKR is required for the synthesis of Hsp70 and thus for the recovery from a heat shock [40]. However, the mechanism involved is mRNA stabilization rather than translation reinitiation.

Various studies have pointed to a number of different factors as the cause of inhibition of protein synthesis upon a heat shock: sequestration of eIF4E by eIF4E binding proteins, inhibition of eIF2B by phosphorylation of the ε subunit, phosphorylation of eIF2α, *etc*. As expression of the constitutively active form of GADD34 relieves the inhibition at least partially, phosphorylation of eIF2α must play a significant role. In the presence of the C-terminal fragment of GADD34, αB-crystallin had no additional effect, indicating that phosphorylation of eIF2α is the limiting factor in the presence of αB-crystallin. Overexpression of Hsp27 did result in a larger stimulation than overexpression of the C-terminal domain of GADD34, but no additive effect between Hsp27 and the C-terminal domain of GADD34 was seen. Hence, phosphorylation of eIF2α accounts for part, but not all, of the inhibition relieved by Hsp27. Our results thus show that both αB-crystallin and Hsp27 act to restore protein synthesis after a heat shock by increasing the activity of eIF2B, most likely by promoting the dephosphorylation of eIF2α. The additional effect of Hsp27 as compared to αB-crystallin is probably due to an increase in the availability of eIF4E and eIF4G by prevention of the immobilization of these factors.

Acknowledgements. We thank Dr. R. E. Rhoads for the pAD4G and the TCEEC 4E constructs, Dr. C. Koumenis for the GADD34 clone, Dr. G. M. Cooper for the clones for eIF2B and eIF2B-S535A, and Dr. G. J. Pruijn for the EGFP-Ro60 expression construct. We thank the MIC facility at the Department of Cell Biology of the UMC St. Radboud, Nijmegen for use of the Zeiss LSM510 meta microscope, and the GI facility at the faculty of Science, Radboud University Nijmegen for use of the Zeiss Axiovert 135 TV mi-

croscope and for technical assistance. These experiments were financially supported by the BioMed program of the European Community (BMH4-CT98-3895).

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