Inactivation of eukaryotic initiation factor 2B *in vitro* by heat shock

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Protein synthesis in rat H35 Reuber hepatoma cells is rapidly inhibited on heat shock. At mild heat-shock temperatures the main cause for inhibition is the inactivation of the guanine nucleotide exchange factor eukaryotic initiation factor 2B (eIF2B); under more severe heat-shock conditions the activity of several initiation factors is compromised. eIF2B is required for GDP/GTP exchange on eIF2, which delivers methionyl-tRNA to the 40 S ribosomal subunit. We have tried to elucidate the mechanism underlying the inactivation of eIF2B by assays *in vitro*. Incubation of cell extracts at 41 °C or higher led to the inactivation of eIF2B. In agreement with observations in cells exposed to mild heat shocks, the thermal inactivation of eIF2B could be ascribed to neither eIF2α phosphorylation nor the induction of another inhibitor. With the use of glycerol gradients we show that eIF2B forms aggregates in heat-treated extracts. Furthermore eIF2B activity is protected against heat shock in thermotolerant cells. Taken together, these results suggest a role for chaperones in the control of eIF2B activity.

INTRODUCTION

Exposure of cells to stressful conditions leads in most cases to the so-called heat-shock response. This response is characterized by the onset of synthesis of a family of proteins, the heat-shock proteins (HSPs). Some HSPs function as chaperones, with an important role in the proper folding of proteins (reviewed in [1]). Induction of the synthesis of the HSPs is regulated mainly at the transcriptional level [2]. However, heat shock or other stressful conditions also affect protein synthesis (reviewed in [3]). Translation of most proteins is decreased, whereas synthesis of the HSPs seems to be less affected [4]. Especially in *Drosophila* cells, preferential translation initiation occurs on the HSP mRNA species after heat shock [5].

The inhibition of global protein synthesis seems to be achieved through the modification of several eukaryotic initiation factors (eIFs). The phosphorylation state of eIF4E, eIF2α and eIF4B changes [6], negatively influencing the activity of these proteins. We have recently investigated the heat-shock-induced inhibition of protein synthesis in rat hepatoma cells and found that these changes in phosphorylation of eIF4E and eIF2α did not occur at mild heat-shock temperatures [7], as also described earlier for HeLa cells [6]. Under the mild stress conditions applied, protein synthesis was regulated mainly by the activity of the guanine nucleotide exchange factor eIF2B [7].

During the initiation phase of protein synthesis eIF2, in complex with GTP, delivers the initiator methionyl-tRNA to the 40 S ribosomal subunit. The GTP on eIF2 is hydrolysed to GDP on joining of the 60 S ribosomal subunit [8]. This GDP must be exchanged for GTP by eIF2B before eIF2 can participate in a next round of initiation [9]. One of the major regulatory pathways by which eIF2B activity is controlled is by the phosphorylation of the α-subunit of eIF2 [9,10]. Phosphorylation of this subunit leads to the sequestering of eIF2B in an inactive state. The activity of eIF2B can also be regulated directly by the phosphorylation of its ϵ-subunit [10,11]. Neither process seemed to be involved in the inactivation of eIF2B under mild heat-shock conditions ([7], and G. C. Scheper, unpublished work).

Besides the phosphorylation of proteins, many means are employed of activating or inactivating proteins under stressful conditions, such as glycosylation, methylation, acetylation and ubiquitination (reviewed in [12]). Inactivation of proteins, even at mild heat-shock temperatures, might also occur by direct heat-denaturation. In particular the thermolabile luciferase from the firefly has been used in thermal denaturation studies [13,14]. Proteins that are easily denatured by heat often show a decreased sensitivity for heat shock in thermotolerant cells, in which increased amounts of the HSPs are present. This state of thermotolerance can be achieved either by exposing cells to a heat shock and allowing these cells to synthesize the HSPs, or by overexpression of one of the HSPs [15–19].

To elucidate the possible mechanism underlying the inactivation of eIF2B in heat-shocked cells we have performed various experiments in which cell extracts were exposed to elevated temperatures, to inactivate eIF2B *in vitro*. These experiments demonstrated that heat-inactivation of eIF2B in cell extracts was not due to phosphorylation of the α-subunit of eIF2 but was paralleled by aggregation of eIF2B. Furthermore these results suggested that the activity of eIF2B is regulated by other factors, which might be chaperones. To corroborate this hypothesis, eIF2B activity was also determined in thermotolerant cells.

EXPERIMENTAL

Tissue culture

Rat hepatoma Reuber H35 cells were grown in Leibowitz (L15) medium (Flow/ICN Laboratories) containing potassium penicillin G (100 i.u./ml), streptomycin sulphate (100 μg/ml), and 10 % (v/v) fetal calf serum (Gibco).

Preparation of cell extracts

Cells were harvested in 20 mM Tris/HCl (pH 7.6)/100 mM KCl/1 % (v/v) Triton X-100/0.2 mM EDTA/50 mM β-glycerophosphate/1 mM sodium molybdate/10 % (v/v) glycerol/4 μg/ml leupeptin/0.2 mM benzamidine/0.2 mM sodium vanadate/7 mM 2-mercaptoethanol. For experiments *in vitro*, cells from 175 cm^2 flasks were harvested in 400 μl of buffer. The

Abbreviations used: eIF, eukaryotic initiation factor; HSP, heat-shock protein.

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protein concentration of these extracts was approx. 4 mg/ml. For experiments *in vivo*, with untreated and thermotolerant cells, cells from 25 cm² flasks were harvested in 75 μl of buffer.

Heat treatment

Heat shocks were applied by incubating aliquots of the cell extracts in a water-bath providing a temperature stable within $\pm$0.1 °C (S.E.M.).

eIF2B assay

eIF2–[^{3}H]GDP complexes were made as described [20]. In brief, 1 pmol of eIF2 was incubated with 0.2 μCi of [^{3}H]GDP (approx. 15 pmol; 30000 d.p.m./pmol) in 20 mM Tris/HCl (pH 7.6)/120 mM KCl/1 % (w/v) BSA/1 mM dithiothreitol. After incubation at 30 °C for 15 min, 5 mM $MgCl_2$, 1 mM GTP and approx. 10 μg of cell extract were added to eIF2–[^{3}H]GDP complexes and incubated for 15 min at 30 °C. The GDP/GTP exchange reaction was stopped by adding 1 ml of a cold wash buffer [50 mM Tris/HCl (pH 7.6)/5 mM $MgCl_2$/100 mM KCl/7 mM 2-mercaptoethanol]. The mixture was filtered through nitrocellulose and washed three times with the same buffer. The activity of eIF2B was determined by quantification of the amount of eIF2–[^{3}H]GDP retained on the filter. No activity (0 %) corresponded to approx. 15000 c.p.m. retained on the filter, 100 % activity corresponded to 1000 c.p.m., the value obtained with 10 μl of active extract.

Phosphorylation of eIF2α

Samples of the heat-treated extracts were separated by SDS/PAGE and analysed by Western blotting. Monoclonal antibodies against eIF2α were used to determine the total amount of eIF2α, whereas the amount of phosphorylated eIF2α was determined with a polyclonal antibody raised against an eIF2α peptide containing a phosphorylated serine residue [21].

Glycerol gradients

Isokinetic glycerol gradients (15–40.5 %) were made in a buffer containing 20 mM Tris/HCl, pH 7.6, 100 mM KCl, 1 mM magnesium acetate and 0.1 mM EDTA. After being layered with 200 μl of cell extract, the gradients were centrifuged in a SW50.1 rotor at 30000 rev./min (g_{av} 84200) for 16 h at 4 °C. After centrifugation, 14 fractions of approx. 300 μl were collected per gradient. One-quarter of each fraction was analysed by SDS/PAGE and Western blotting with antibodies against eIF2α and eIF2Bϵ (a gift from Dr. Proud).

RESULTS AND DISCUSSION

Inactivation of eIF2B in cell extracts

Heat-shock treatment of Reuber H35 rat hepatoma cells results in a very rapid and severe inhibition of protein synthesis. In a search for the cause for this inhibition we reported previously that the activity of several eIFs was changed at temperatures of 42 °C or higher. Under mild heat-shock conditions the main cause of decreased protein synthesis was shown to be the inactivation of eIF2B [7]. To investigate this inhibition of eIF2B activity we explored the inactivation of eIF2B by heat shock *in vitro* (Figure 1).

All four heat-shock temperatures resulted in a decrease in eIF2B activity, whereas incubation at 37 °C did not affect eIF2B activity. Inactivation of eIF2B *in vitro* occurred in a temperature range at which eIF2B activity was also decreased *in vivo*. Moderate heat-shock temperatures, such as 43 and 44 °C, had more severe effects than the milder heat shocks, again similar to the results *in vivo* [7].

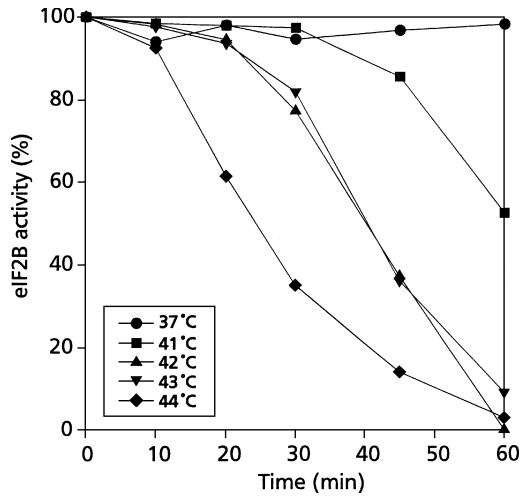

Figure 1 Inactivation of eIF2B *in vitro*

Cell extract was prepared from H35 cells grown at 37 °C as described in the Experimental section. Samples (10 μl) were incubated at the temperatures and times indicated. After the heat-treatment eIF2B assays were performed. The eIF2B activity of an untreated sample was set at 100 %.

Thermal inactivation kinetics *in vitro* was characterized by a temperature-dependent shoulder, whereas in intact cells these temperatures caused a decrease in eIF2B activity of approx. 60 % within 15 min [7]. Although inactivation of eIF2B by heat shock *in vitro* and *in vivo* occurred at comparable temperatures, the kinetics of inactivation *in vitro* differed from the kinetics of the previously reported eIF2B inhibition in H35 cells during heat shock. Differences between the effects of these heat shock temperatures in cells became evident only during recovery at 37 °C. Then the activity of eIF2B regained control levels within 1 h when cells were treated at 41 °C, whereas the reactivation of eIF2B in cells preincubated at 44 °C required more than 6 h of recovery at 37 °C [7].

Inactivation of eIF2B *in vitro* is not caused by formation of an inhibitor

The kinetics of the thermal inactivation of eIF2B in cell extracts could be explained by the induction of an inhibitor. The best studied mechanism for inhibiting eIF2B activity is phosphorylation of the α-subunit of eIF2. This phosphorylation leads to sequestering of eIF2B in an inactive state. It has been shown that denatured proteins could activate an eIF2α kinase in rabbit reticulocyte lysates [22], and a similar mechanism could explain the eIF2B inactivation as found in Figure 1. Therefore we determined the phosphorylation of eIF2α by SDS/PAGE and Western blotting with an antibody specific for phosphorylated eIF2α [7,21] (Figure 2).

Hardly any change occurred in the phosphorylation state of eIF2α after incubation at 44 °C for 60 min. This reflects the situation in cells that were shocked at 41 °C, in which eIF2B was inactivated in the absence of increased eIF2α phosphorylation [7]. Apparently the activities of the kinases or phosphatases that regulate phosphorylation of eIF2α on exposure of cells to 42 °C or higher were not affected in the extracts *in vitro*, and it is

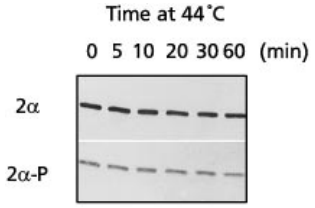

Figure 2 Heat shock of cell extracts does not lead to eIF2α phosphorylation

Extract of H35 cells was prepared as described in the Experimental section. The extract was incubated at 44 °C and at the indicated times, aliquots of 2 μl were diluted with Laemmli sample buffer, and analysed by SDS/PAGE and Western blotting.

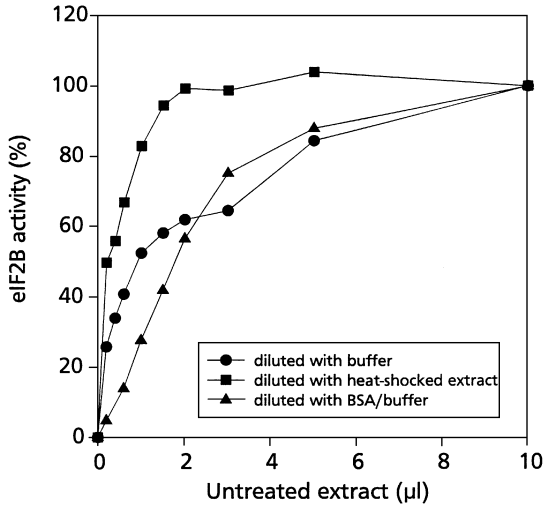

Figure 3 Untreated extracts do not lose eIF2B activity in heat-shocked extracts

Extract from H35 cells was prepared as described in the Experimental section. Increasing amounts of untreated extract (0–10 μl) were diluted in harvest buffer alone, in harvest buffer containing 5 mg/ml BSA or in heat-shocked extract (pretreated for 60 min at 44 °C). The final volume of each mixture was 10 μl. eIF2B activity was determined as described. The activity of 10 μl of untreated and undiluted extract was set at 100%.

concluded that phosphorylation of eIF2α was not involved in the inactivation of eIF2B *in vitro* (Figure 1). The heat-shock-activated eIF2α kinase is still unknown, although under some stressful conditions protein kinase regulated by double-stranded RNA (PKR) might be involved [23]. PKR is a thermolabile protein: heat shock of cells leads to aggregation of the protein [24]. Little is known about the thermostability of other eIF2α kinases or eIF2α phosphatases. HSP70 and HSP90 have been shown to be involved in the regulation of the activity of the eIF2α kinase, haemin-regulated protein kinase [25,26].

In a further attempt to investigate thermal inactivation *in vitro* and to examine whether inhibitors other than phosphorylated eIF2α were induced by the heat treatment of cell extracts, we tried to restore the eIF2B activity of heat-shocked extracts with untreated extracts. Therefore the activity of increasing amounts of untreated extract was determined either alone, or in the presence of BSA as a non-specific protein source, or in the presence of inactive heat-shocked extract. eIF2B activity in the last extracts was completely abolished by incubation at 44 °C for 1 h (see Figure 1). This would show whether inhibitory factors are present in the heat-shocked extract (Figure 3).

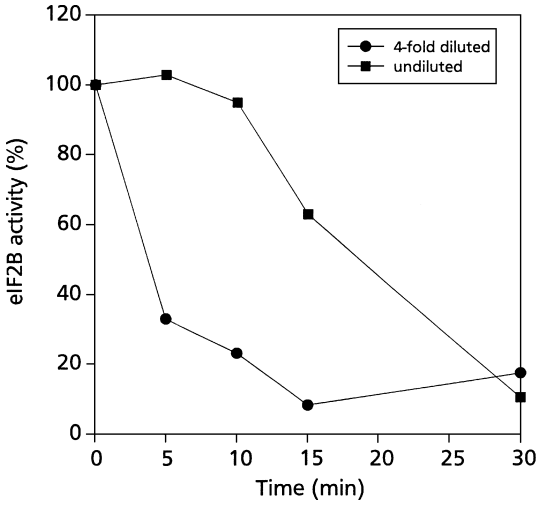

Figure 4 Dilution increases the sensitivity of eIF2B to heat shock

H35 cell extract, either undiluted or diluted 1:4 with the harvest buffer, was incubated at 44 °C for the indicated durations. Subsequently, the undiluted extracts were also diluted 1:4 and eIF2B assays were performed as described. The activities of samples not exposed to heat shock were set at 100%.

eIF2B in untreated extract was not inactivated when heat-shocked extract was added. Furthermore, higher values of eIF2B activity were found in these assays than in either buffer or BSA in buffer. Several similar experiments were performed and in all cases we found high eIF2B activity when a small aliquot of untreated extract was added to heat-shocked extract. Amounts of heat-treated extract that had only approx. 60% activity in buffer alone always had close to 100% activity in combination with untreated extracts. These results not only show that a heat shock leads to a decrease in eIF2B activity but also reveals the presence of various stimulatory factors in the heat-shocked extract that were not present in the harvest buffer. The nature of these factors is not yet clear. They might be proteins, such as denatured proteins that could activate or protect eIF2B, or smaller molecules such as ATP, GTP, NADPH or other allosteric compounds [27,28]. Passing heat-shocked extracts through a Sephadex G50 column, separating the large molecules (e.g. proteins) from the small molecules (e.g. ATP, GTP and NADPH), showed that the stimulatory effect was present in the void volume of the column, well separated from low-molecular-mass material (results not shown), indicating that large molecules, probably proteins in the inactivated extract, had a positive influence on eIF2B activity in the untreated extract.

From the results in Figures 2 and 3 it is evident that the decrease in eIF2B activity *in vitro* was not mediated or dominated by an inhibitory activity, as untreated extract was fully active in heat-shocked extracts.

Control of eIF2B activity by other factors

Another explanation for the kinetics of eIF2B inactivation found in Figure 1 could be that factors protecting eIF2B become inactive in a temperature-dependent manner. Therefore the extract was first diluted and then incubated at 44 °C (Figure 4). This method indicates whether the inactivation of eIF2B occurred via a monomolecular reaction or whether *trans*-acting factors might be involved. In the diluted extract the interaction between

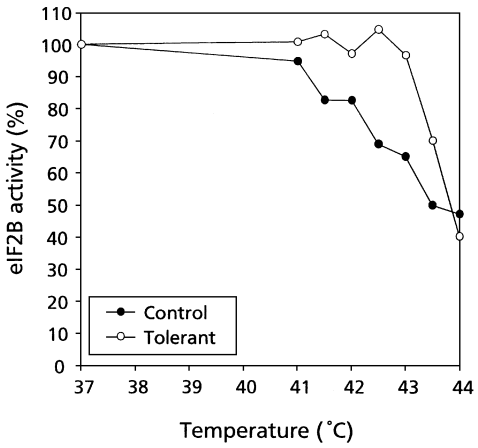

Figure 5 Protection of eIF2B activity in thermotolerant cells

H35 cells were made thermotolerant by incubation for 30 min at 42.5 °C followed by recovery at 37 °C for 5 h. Untreated control cells and thermotolerant cells were incubated at various temperatures for 30 min and extracts were prepared as described in the Experimental section. The activity of eIF2B in these extracts was determined as described. The eIF2B activity of either control or thermotolerant cells that were kept at 37 °C was set at 100%.

eIF2B and these factors would be decreased, whereby the sensitivity of eIF2B for heat treatment would be increased.

Dilution of the extract rendered eIF2B in the extract more thermosensitive. So, direct inactivation of eIF2B, for example by denaturation, did not seem to be the major cause for eIF2B inactivation because such a process would be independent of dilution of the extract. Instead, this characteristic of thermal eIF2B inactivation *in vitro* is evidence of a process in which thermostabilizing factors protected eIF2B against the harmful effect of heat shock.

Summarizing the complexity of the regulation of heat-inactivation of eIF2B in crude extracts, the results demonstrate that: (1) heat shock decreases eIF2B activity; (2) this heat-shock effect is dependent on factors that protect eIF2B activity; and (3) heat shock is able to produce activation of eIF2B activity.

eIF2B activity is less sensitive to heat shock in thermotolerant cells

The results of Figure 4, showing increased sensitivity of eIF2B to heat shock in diluted extracts, could be explained by the dilution of a thermoprotective factor, rendering eIF2B more susceptible to inactivation. A good candidate for such a protective factor would be one of the HSPs. Under heat-shock conditions the HSPs would be titrated out by denatured or unfolded proteins. To test the hypothesis that HSPs are involved we measured the effects of heat shock on eIF2B activity in thermotolerant cells. In such cells increased amounts of the HSPs are present, owing to a previous heat shock [1,2,15]. The sensitivity of eIF2B to heat shock in normal and tolerant cells was determined (Figure 5).

In control cells, heat shock resulted in a rapid decrease in eIF2B activity. At 41.5 °C a 20% inhibition was detected, and further elevation of the temperature led to a decrease of more than 50% at 44 °C. In the tolerant cells thermal inactivation of eIF2B was not detected until temperatures of 43.5 °C or higher were employed. There was therefore a shift of approx. 1.5 °C in thermosensitivity. The tolerance of eIF2B activity coincided with a decreased inhibition of protein synthesis by heat shock, as determined by [^{35}S]methionine/cysteine incorporation (results not shown). These findings strongly support the possibility that HSPs are involved in the regulation of the heat sensitivity of eIF2B activity.

Aggregation of eIF2B in heat-shocked extracts

The decrease in enzyme activity after heat shock has been studied in parallel with structural changes, even aggregation of a specific protein, whereas in several cases similar to the results of Figure

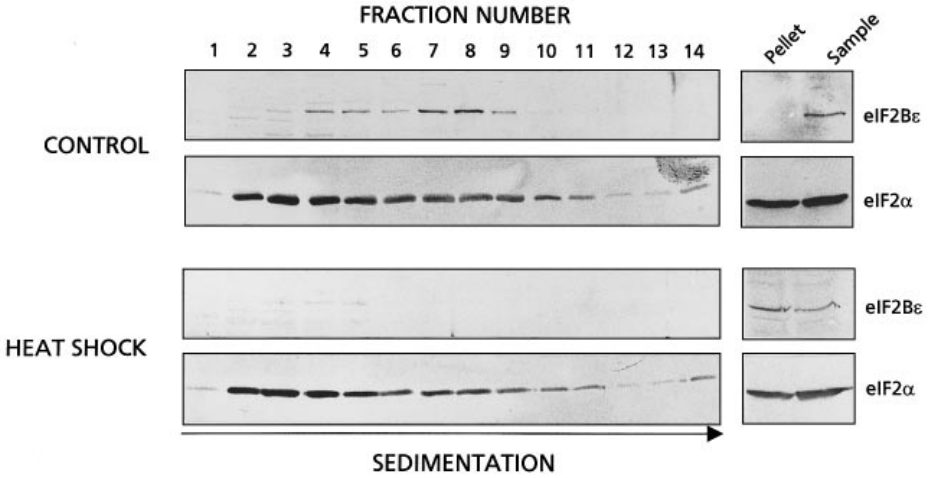

Figure 6 Heat shock leads to aggregation of eIF2B

Extract was prepared from H35 cells, grown at 37 °C as described in the Experimental section. Extracts were split into two parts: one part was untreated, the other part was incubated at 44 °C for 30 min. Both parts were sedimented in 15–40.5% isokinetic glycerol gradients. Fractions were collected and the presence of either eIF2α (as a measure of the amount of eIF2) or eIF2Bϵ (as a measure of the amount of eIF2B) was determined by Western blotting. The top of the gradient is on the left side and the bottom on the right side, as indicated by the sedimentation arrow. The pellet in the tube was resuspended and analysed (pellet). An aliquot of the sample applied to the gradient was analysed directly (sample).

5, attenuation of inactivation and aggregation in thermotolerant cells has been reported [13,24,29–31]. To test whether heat-shock-induced inhibition of eIF2B activity paralleled aggregation, eIF2 and eIF2B from either untreated or heat-shocked cell extracts were separated on glycerol gradients and the positions of these two proteins within these gradients were determined (Figure 6).

Heat treatment of H35 cell extracts resulted in the aggregation of eIF2B into heavy complexes. Hardly any eIF2B was detected in the gradient fractions of heat-shocked extracts, whereas in the untreated (37 °C) extract eIF2Bϵ was clearly present in the gradient. The pellet of the heat-treated extract contained eIF2B, whereas this protein was totally absent from the pellet of the untreated sample. As a control we looked for the presence of eIF2α in the gradient. Aggregation of this protein apparently did not occur, as it was still present in the gradient fractions of the extract that was exposed to 44 °C. The band seen in the bottom fraction was probably derived from ribosome-bound eIF2 [32]. The presence of eIF2α from heat-shocked extracts in the gradient showed that neither general denaturation of all proteins nor complex formation between eIF2 and eIF2B was the cause of the sedimentation in heavy complexes of eIF2B.

A similar experiment was performed to examine whether aggregation of eIF2B also occurred *in vivo* when H35 cells were shocked at 44 °C. However, in extracts made from heat-shocked cells we could not detect the aggregation of eIF2Bϵ (results not shown). This discrepancy with the results *in vitro* could be explained by the conditions under which the heat shock was applied. In the cell extracts all components, such as chaperone proteins, but also ATP, which is needed for the HSPs to function, will be diluted many-fold compared with the physiological conditions in the cells. Therefore in the extracts the heat shock could exert a much more drastic effect on the integrity of eIF2B than *in vivo*.

In summary, we have studied heat-shock-induced inactivation of the guanine nucleotide exchange factor eIF2B in extracts from H35 Reuber hepatoma cells. Our results indicate that this inactivation is not due to the phosphorylation of eIF2α. We suggest two possible mechanisms for inactivation: a direct effect of heat on eIF2B, or an indirect effect on the integrity of this protein. The first mechanism proposes that heat directly inactivates eIF2B. Its net effect is dependent on thermoprotective factors in the extract (Figure 4), as well as on activators that are induced during heat shock (Figure 3).

The second mechanism proposes that eIF2B is inactivated indirectly by thermally denatured proteins that are able to aggregate with eIF2B, thereby decreasing eIF2B activity. By this mechanism thermoprotective factors (Figure 4), presumably HSPs (Figure 5), could protect the thermal denaturation of proteins and consequently their aggregation with and inactivation of eIF2B. The mixing experiments (Figure 3) suggest that heat-induced factors, perhaps denatured proteins, stimulate eIF2B activity before complexation. To discriminate between these two possibilities further studies require the use of purified eIF2B and HSPs.

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