Heat Shock Increases the Association of Binding Protein-1 with Initiation Factor 4E*

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The effects of heat shock on the regulation of the cap-binding initiation factor 4E (eIF4E) and its inhibitory binding protein, 4E-BP1, have been examined in Chinese hamster ovary cells and in cardiac myocytes. Heat shock increased the association between eIF4E and 4E-BP1, and this was associated with a dephosphorylation of 4E-BP1. These effects did not appear to be due wholly to decreased activity of the p70 S6 kinase pathway, which is implicated in the control of 4E-BP1, and they were not mediated by the stress-activated p38 microtubule-associated protein kinase pathway. Increased binding of 4E-BP1 to eIF4E correlated with a decrease in the amount of eIF4G which co-purified with the latter. This could account for the previously observed impairment of eIF4F function during heat shock, and, since heat shock protein mRNAs are believed to be relatively cap-independent, could provide a mechanism for the selective up-regulation of the synthesis of heat shock proteins and other stress proteins during heat shock.

Initiation factor 4E (eIF4E) plays a key role in mRNA translation in eukaryotic cells. It binds the 5'-cap structure (7-methylguanosine), which is present at the 5'-end of all cellular cytoplasmic mRNAs (1–3). eIF4E undergoes phosphorylation at a single major site (Ser209) (4, 5), and the level of phosphorylation of eIF4E is increased under a variety of conditions in which rates of translation are enhanced (reviewed in Refs. 6 and 7).

eIF4E forms a complex termed eIF4F that also contains the translation factors eIF4G (also called p220) and eIF4A, and phosphorylation may enhance its ability to form such complexes (8, 9). eIF4G appears to act as a "molecular bridge"; distinct domains of this large polypeptide bind to eIF4E and to eIF4A (10). eIF4A has ATP-dependent RNA helicase activity which is thought to play a key role in "unwinding" regions of self-complementary secondary structure in the 5'-untranslated regions of certain mRNAs (2, 11). Such secondary structure inhibits mRNA translation, and mRNAs rich in secondary structure are often poorly translated. Their translation may be enhanced by treatment of cells with insulin or other agents that increase the phosphorylation of eIF4E (8, 9). Overexpression of eIF4E also causes increased translation of such mRNAs (12–15), and in some cells this is associated with acquisition of a malignant phenotype (16, 17). These data indicate that eIF4E may regulate mRNA translation in a selective manner and contribute to the control of cell proliferation. They also suggest that the availability of eIF4E may be limiting at least for this stage (mRNA binding) of translation initiation. In contrast to many other cellular mRNAs, the translation of heat shock protein mRNAs appears to be relatively cap-independent (for reviews, see Refs. 18–20).

Recently, two additional proteins that interact with eIF4E were discovered (21). They are termed 4E-BP1 and 4E-BP2 (eIF4E binding proteins 1 and 2) and each of them inhibits cap-dependent mRNA translation, implying that they block the function of eIF4E in peptide-chain initiation (22). They do not block the translation of mRNAs containing features that allow cap-independent initiation to occur, e.g. elements derived from picornaviral mRNAs (22, 23). 4E-BP1 (also known as PHAS-I) is a phosphoprotein whose state of phosphorylation increases in response to insulin (22, 24–27), insulin-like growth factor-1 (28) or angiotensin II (29). This causes its dissociation from eIF4E and should result in the alleviation of the inhibition of eIF4E. 4E-BP1 competes with eIF4G for binding to eIF4E, preventing formation of the eIF4F complex and thus inhibiting the recruitment of eIF4A to the initiation complex on the 5'-end of the mRNA (30, 31). Studies on 4E-BP2 (PHAS-II) show that its phosphorylation is also enhanced by insulin and that this also causes it to dissociate from eIF4E (32).

The insulin- or insulin-like growth factor-1-induced phosphorylation of 4E-BP1 has been shown, in several cell types, to be inhibited by the immunosuppressant rapamycin, which is a specific inhibitor of the signaling pathway that leads to activation of the p70 ribosomal protein S6 kinase (p70 S6 kinase) (23, 26–28, 33). Recent findings (26) indicate that there are additional phosphorylation site(s) in 4E-BP1, which undergo phosphorylation in response to insulin but which do not have a pronounced effect on its association with eIF4E and whose phosphorylation is not blocked by rapamycin. Consistent with its effect on 4E-BP1, rapamycin blocks translation in mammalian cells and in Saccharomyces cerevisiae (23, 34).

Since 4E-BP1 inhibits cap-dependent translation, we have investigated the possibility that the proportion of eIF4E which is bound to 4E-BP1 (and is therefore "inactive") rises under conditions where mRNA translation is inhibited, i.e. under conditions of heat shock. We find that heat shock markedly increases the amount of 4E-BP1 bound to eIF4E and, conversely, reduces the association of eIF4E with eIF4G, as expected from the competition between these proteins for a com-

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mon binding site in eIF4E (30). This would be expected to impair cap-dependent translation (22). Previous data from mammals and from Drosophila indicate that heat shock causes inactivation of eIF4E and dissociation of complexes between eIF4E and other translation factors (8, 35–37). Heat shock proteins (hsp) continue to be made under these conditions and the available evidence suggests that hsp mRNAs have a low requirement for eIF4E for their translation (18). For example, hsp mRNAs continue to be translated in cells in which the level of eIF4E is reduced using an antisense approach (38). The increased association of 4E-BP1 with eIF4E appears to be associated with decreases in its phosphorylation. Although heat shock does stimulate the stress-activated p38 MAP kinase, several criteria indicate that this pathway does not appear to be involved in the effects of heat shock on the phosphorylation of 4E-BP1. Heat shock has only small effects on the activity of p70 S6 kinase and, although this pathway is implicated in the phosphorylation of 4E-BP1 (23, 26–28, 32, 33), changes in its activity seem unlikely to be important in the effects of heat shock reported here.

MATERIALS AND METHODS

Chemicals and Biochemicals—mGTP-Sepharose was from Pharmacia Biotech Inc. γ-[32P]-Labeled ATP and [35S]-labeled methionine/cysteine were purchased from Amersham Corp. Chinese hamster ovary (CHO.K1) cells were kindly provided by Dr. L. Ellis (Houston, TX). Materials for tissue culture were obtained from Life Technologies, Inc. Microcystin-LR and rapamycin were from Calbiochem. Recombinant mouse hsp25 was kindly provided by Dr. M. Gaestel (Berlin, Germany), and recombinant c-Jun-glutathione S-transferase was generously donated by Ian Baines (Kent).

Cell Culture and Stress Treatment—CHO.K1 cells were grown and maintained in culture as described previously (39). Cells were grown to near-confluence in 90-mm dishes prior to exposure to heat shock (44 °C, 20 min unless otherwise stated) or fetal calf serum (10% (v/v), 20 min). Where applicable, cells were preincubated with rapamycin at 2 nM for 30 min prior to heat shock treatment of the cells. Control cells (i.e. those not exposed to either inhibitor) were treated with dimethyl sulfoxide that had been dried down and resuspended in the same way. Tumor necrosis factor-α (TNF-α) was used at 10 ng/ml and lipopolysaccharide (LPS) at 10 μg/ml (final concentrations). In all cases, cell extracts were prepared as described earlier (39) and clarified by centrifugation at 4 °C (13,000 g, 10 min). Where used, the p38 MAP kinase inhibitor SB203,580 (20 μM) was added 30 min prior to heat shock treatment of the cells.

Ventricular myocytes were isolated from adult male rat hearts by the collagenase perfusion technique as described previously (40). Cell treatments and experiments were as for CHO cells (see above).

Analysis of eIF4E and 4E-BP1—eIF4E was isolated from cell extracts by affinity chromatography on mGTP-Sepharose and subjected to SDS-PAGE and Western blotting as described previously (26, 41).

Gel Electrophoresis, Isoelectric Focusing, and Immunoblotting—For analysis of the 4E-BP1 bandshift or the interaction between 4E-BP1 and eIF4E, samples were subjected to electrophoresis on SDS-polyacrylamide gels containing 15% acrylamide, 0.4% bis-acrylamide (42). Isoelectric focusing of eIF4E was performed as described in Flynn and Proud (5). In all cases, gels were transferred to polyvinylidene difluoride membrane (Millipore), and Western blotting was performed as described earlier (43) using the enhanced chemiluminescence system (Amersham plc).

Assays of Protein Synthesis—Rates of protein synthesis were assayed in near-confluent CHO.K1 cells by measuring the incorporation of [35S]methionine/cysteine into acid-insoluble protein as described earlier (44). In all cases, cells were radiolabeled (1000 Ci/mmol) and correspondingly used to 60-mm dishes of cells. Where used, rapamycin (2000 nM) was added to the cells 20 min prior to the addition of radiolabeled amino acid.

Protein Kinase Assays—p70 S6 kinase activity was assessed by direct kinase assay (45). MAPKAPK2 (which is activated by p38 MAP kinase) and JNK (c-Jun N-terminal kinase) were assayed by their abilities to phosphorylate recombinant hsp25 (46) and recombinant c-Jun(1–169)-glutathione S-transferase, respectively (47).

RESULTS

Heat Shock Leads to Increased Association of 4E-BP1 with eIF4E—Fig. 1A shows that heat shock led to a rapid inhibition of protein synthesis in CHO cells. To study whether heat shock increased the amount of 4E-BP1 associated with eIF4E, cells were exposed to elevated temperature, lysed, and extracted, and eIF4E and associated proteins were isolated using the m7GTP-Sepharose affinity matrix that is widely employed for this purpose. The effect of heat shock on the binding of 4E-BP1 to eIF4E was examined over a time course (Fig. 1B). The amount of 4E-BP1 recovered in association with eIF4E rose quickly after the transfer of the cells to the higher temperature and this effect was maximal by 30 min.

Effects of Heat Shock on the Phosphorylation State of 4E-BP1—As discussed in the Introduction, the hormone- or growth factor-induced dissociation of 4E-BP1 from eIF4E is associated with increased phosphorylation of 4E-BP1, and this is manifested as a decrease in its mobility on SDS-PAGE (22, 28, 48). To examine whether or not the converse is true in the case of heat shock-induced association of 4E-BP1, cell extracts were analyzed by SDS-PAGE under conditions where the phosphorylation state of the 4E-BP1 altered its migration (such that more highly phosphorylated species migrate more slowly) (22, 23, 48). Fig. 1C shows that a total of three species of 4E-BP1 (α–γ) can be resolved on SDS-PAGE. As expected, treatment of the cells caused a shift in the pattern, with increased amounts of the most slowly migrating (γ) and correspondingly less of the other two being observed. In contrast, heat shock resulted in a shift in the other direction, such that the most slowly migrating form virtually disappeared, and more of the other two was generated. The most slowly migrating (γ) form of 4E-BP1 was not found to associate with eIF4E when extracts were subjected to affinity chromatography on m7GTP-Sepharose, while the β and α forms did bind eIF4E. These data from CHO cells were entirely reproducible (in >50 experiments) but differ from those published for some other cell types, where only the most rapidly migrating form was found to associate with eIF4E. Since 4E-BP1 contains at least five phosphorylation sites (49), giving rise to 5′ (120) different phosphorylated forms, each band on SDS-PAGE must be a mixture of several species. This apparent discrepancy is therefore likely to be due to alterations in the relative abundance of different phosphorylated isoforms of eIF4E, which differ in their abilities to bind eIF4E, but which co-migrate on SDS-PAGE. These data suggest that the enhanced association of 4E-BP1 with eIF4E in response to heat shock is the consequence of dephosphorylation of a proportion of the cellular 4E-BP1 yielding increased levels of the less phosphorylated species which bind to eIF4E.

Heat Shock-induced Dephosphorylation of 4E-BP1 Is Independent of the p70 S6 Kinase Pathway—Studies with insulin and growth factors have revealed that the activity of p70 S6 kinase is important for the regulation of 4E-BP1 phosphorylation and its association with eIF4E (23, 26–28, 32, 33). We have therefore investigated the role of this kinase in the action of heat shock on 4E-BP1 using the macrolide immunosuppressant rapamycin, which blocks the activation of p70 S6 kinase. Fig. 2A shows that preincubation of the cells with rapamycin increased the amount of 4E-BP1 which co-purified with eIF4E in control cells. When heat-shocked cells were also treated with rapamycin, an additive effect was observed such that rapamycin increased further both the amount of 4E-BP1 bound to eIF4E and the downward band shift caused by heat shock. In addition, in our cells heat shock did not significantly affect the activity of p70 S6 kinase when compared with untreated cells (110 ± 15% of control activity; n = 5).
The Effect of Heat Shock on 4E-BP1 Is Independent of the p38 MAP Kinase Pathway—Stresses such as heat shock are now known to activate at least two stress-activated kinase cascades involving enzymes which are related to MAP kinase (50, 51). Since a specific inhibitor of one of these is available (SB203,580, which inhibits p38 MAP kinase) (46), we used it to assess whether this pathway mediates the effect of heat shock on 4E-BP1 phosphorylation. As shown in Fig. 2B, heat shock activated this pathway (as assessed by the activity of MAPKAPK-2, a kinase which is activated by p38 MAP kinase (52), and this was completely blocked by preincubation of the cells with SB203,580 prior to the heat shock treatment. However, preincubation with this compound had no discernible effect on the phosphorylation of 4E-BP1. CHO.K1 cells were exposed to heat shock and labeled with [35S]methionine/cysteine by adding ~10 µCi of the radiisotope for a 10-min period ("window"). The graph shows the incorporation of [35S] radioactivity into acid-insoluble material, with the data expressed as a percentage of the control (normothermic) cells. Each time on the x axis represents the midpoint of each 10-min radiolabeling window. The graph shows data typical of those obtained from three independent experiments. Panel B, heat shock promotes increased binding of 4E-BP1 to eIF4E. CHO.K1 cells were exposed to heat shock, extracts were prepared, and eIF4E was isolated as described under "Materials and Methods." Shown are Western blots of a typical time course, with the positions of eIF4E and the isoforms of 4E-BP1 indicated. Panel C, effect of heat shock or serum treatment on the phosphorylation of 4E-BP1. CHO.K1 cells were extracted following no treatment (control, lane 1) or exposure to 10% (v/v) fetal calf serum (lane 2) or heat shock (lanes 3 and 4). 4E-BP1 was analyzed either by subjecting cell extracts directly to SDS-PAGE (lanes 1–3) or first subjecting the cell extract to affinity chromatography on m'GTP-Sepharose prior to applying the bound material to the gel (lane 4). Subsequent Western blotting analysis employed a polyclonal anti-4E-BP1 antiserum. The positions of the three species of 4E-BP1 resolved on SDS-PAGE are indicated.

The second pathway referred to above is the JNK pathway. Heat Shock Decreases the Association of eIF4G with eIF4E—It is now apparent that 4E-BP1 inhibits the function of eIF4E in cap-dependent translation by blocking the binding of eIF4E to eIF4G, another component of the eIF4F complex. Mader et al. (31) have reported that 4E-BP1 and eIF4G share a common eIF4E-binding motif, and mutually exclusive binding of eIF4E to 4E-BP1 or eIF4G has been demonstrated (9, 30). Fig. 2G shows that under conditions of heat shock which cause increased binding of 4E-BP1 to eIF4E, a much lower degree of co-purification of eIF4G is seen, consistent with the idea that binding of eIF4G to 4E-BP1 is mutually exclusive. The samples analyzed in Fig. 2G contained equivalent amounts of eIF4E (as assessed by Western blotting) and similar data were obtained in six separate experiments. Thus, heat shock reduces the binding of eIF4E to eIF4G, an effect which presumably underlies the previously reported impairment of eIF4F function in heat-shocked cells (8, 35, 53, 54). The two types of complex thus behaved in a reciprocal fashion, consistent with current models of how 4E-BP1 regulates eIF4F assembly. Consistent with the findings discussed above (Fig. 2C), the p38 MAP kinase inhibitor SB203,580 had no effect on the heat shock-induced decrease in the association of eIF4G with eIF4E (Fig. 2G).
Effect of Heat Shock on the Association of 4E-BP1 with eIF4E in Heart Cells—To examine whether heat shock affected the association of eIF4E with 4E-BP1 in other cell types, we examined its effect on this interaction in primary cardiac ventricular myocytes, to extend the study to a truly “physiological” cell type. As shown in Fig. 3, heat shock resulted in a marked increase in the amount of 4E-BP1 bound to eIF4E in myocytes. Heat shock had a much larger effect on the binding of 4E-BP1 to eIF4E than rapamycin did, which is expected from the fact that rapamycin has little effect on p70 S6 kinase activity in unstimulated myocytes (control activity, 100%; after rapamycin pretreatment, 105 ± 12%). These data add force to the argument, developed above, that the increased binding and decreased phosphorylation of 4E-BP1 seen in response to heat shock are not due solely to reduced activity of the p70 S6 kinase (FRAP/TOR) signaling pathway, since in this case p70 S6 kinase activity is very low in cardiac myocytes.

**DISCUSSION**

The findings presented here show that heat shock results in increased association of eIF4E with 4E-BP1. While this work was in progress, similar findings were reported by Feigenblum and Schneider (55) for human embryonic kidney (293) cells, although no analysis of the effect on eIF4G association or of the signaling pathways involved was provided, and the events described here in response to heat shock are much more rapid.
than in 293 cells (3–4 h). The present study shows that heat shock also causes increased the association of 4E-BP1 with eIF4E in CHO cells and in primary cardiac ventricular myocytes. Given that 4E-BP1 inhibits the function of eIF4E in cap-mediated translation initiation (22, 23, 48), this could contribute to the shut-off of most cellular mRNA translation as a consequence of inhibition of eIF4E, which is required for the translation of “normal” cellular mRNAs. Our data clearly show that heat shock decreases the association of eIF4E with eIF4G, which is consistent with the current idea that 4E-BP1 and eIF4G compete with one another for binding to a common structural element in eIF4E, such that 4E-BP1 acts as an inhibitor of the assembly of the eIF4F complex (30, 31). The finding that heat shock reduces the binding of eIF4F to eIF4G is also consistent with earlier studies which showed that heat shock impaired eIF4F function in mammalian cells and caused dissociation of the eIF4F complex (8, 35, 53). Similar results have also been obtained in Drosophila cells (36, 37).

Our data now provide a molecular mechanism by which heat shock brings about impaired assembly and activity of eIF4F which is consistent with our current knowledge of the interactions between eIF4E, 4E-BP1, and eIF4G. A number of diverse lines of evidence suggest that the translation of hsp mRNAs is cap-independent in mammalian cells (18–20). These include the continued translation of hsp mRNAs in cells where cap-dependent translation has been shut off by picornavirus infection (56) or removal of eIF4E and eIF4G using an antisense approach (38, 57). The enhanced association of 4E-BP1 with eIF4E may therefore play a key role in favoring translation of hsp mRNAs over other, cap-dependent, cellular mRNAs under heat shock conditions (although it may not itself be responsible for the overall inhibition of translation in heat-shocked cells, see below). Hence, while increased association of eIF4E with 4E-BP1 would contribute to the inhibition of overall mRNA translation, the translation of the cap-independent hsp mRNAs would be expected to continue under such conditions. Indeed, given that the cell’s ribosomes would not now be engaged in translating other mRNAs, one would expect to see increased translation of hsp mRNAs. This could therefore provide a mechanism for the selective enhancement of hsp synthesis. The observation referred to above that, when cellular levels of eIF4E and eIF4G were reduced using an antisense method, total protein synthesis was, as expected, decreased, while hsp synthesis actually rose (38), is consistent with the operation of the type of mechanism discussed above. Translation of the mRNA encoding grp78 (also called BiP, the immunoglobulin heavy chain binding protein) is also cap-independent, in this case because its 5′-untranslated region contains a region allowing internal ribosome entry (58, 59). Since 4E-BP1 does not inhibit translation of such mRNAs (22, 23), the enhanced binding of 4E-BP1 to eIF4E could also provide a mechanism for favoring translation of this and similar mRNAs in heat-shocked cells.

The increased binding of 4E-BP1 to eIF4E correlates with a heat shock-induced dephosphorylation of 4E-BP1. This suggests that heat shock results either in the activation of a phosphatase which dephosphorylates 4E-BP1 or inactivation of a kinase which phosphorylates it. Neither the phosphatases nor the kinases acting on 4E-BP1 in vivo have yet been identified; although phosphorylation of 4E-BP1 is linked to the pathway which leads to activation of p70 S6 kinase, 4E-BP1 is not itself a substrate for this kinase (4E-BP1 has been reported to be phosphorylated directly by RAFT, the target of rapamycin) (60). However, heat shock caused only a small decrease in p70 S6 kinase activity. Given the large change in 4E-BP1 binding, this small change in p70 S6 kinase may not be the only mechanism underlying the effect of heat shock. It might involve activation of the protein phosphatase acting on 4E-BP1, but it is not currently possible to test this.

There are two well established stress-activated kinase cascades in mammalian cells. One of these, the p38 MAP kinase pathway, seems not to play a role in the dephosphorylation of 4E-BP1 observed in response to heat shock or in the dissociation of eIF4E from eIF4F, as judged by the inability of other stimuli which activate this pathway to bring about the dephosphorylation of 4E-BP1 and the use of a specific and effective inhibitor of this pathway, SB203,580. The absence of a specific inhibitor for the other (JNK) pathway has precluded our assessing its role in modulating 4E-BP1 phosphorylation in this study. Nonetheless, since activation of JNK correlates with dephosphorylation of 4E-BP1 (both occur in response to heat shock, neither with LPS or TNF-α), the possibility remains open that JNK is involved in the effect of heat shock on 4E-BP1 phosphorylation.

It is not clear whether the effects observed here and elsewhere on the assembly of the eIF4F complex in response to heat shock can entirely account for the overall inhibition of protein synthesis seen during heat shock. For example, rapamycin, which also causes increased binding of 4E-BP1 to eIF4E, has only a small effect on total protein synthesis in many types of cells (23, 61), and this is probably due mainly to marked inhibition of the translation of certain mRNAs that have a high requirement for eIF4E (e.g. Myc) (61). The overall inhibition of translation during heat shock may largely be due to increased phosphorylation of eIF2α (reviewed in Refs. 6 and 20) and consequent impairment of the eIF2B-mediated regeneration of the active form of eIF2, which is likely to be required for the translation of all mRNAs. Heat shock increases the state of phosphorylation of eIF2α in CHO cells as in many other cell types.2 The observation that overexpression of a phospho-rylation-resistant mutant of eIF2α reduces the extent of inhibition of protein synthesis seen during heat shock supports this idea (62, 63), although the fact that such protection was only partial is consistent with the hypothesis that other distinct mechanisms (e.g. the enhanced binding of 4E-BP1 to eIF4E) may also operate to reduce translation rates under this condition. The effect observed here probably serves the function of directing the residual translation activity toward relatively cap-independent mRNAs such as those encoding hsps or BiP.

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