Cellular stress in *Xenopus* kidney cells enhances the phosphorylation of eukaryotic translation initiation factor (eIF)4E and the association of eIF4F with poly(A)-binding protein

Christopher S. FRASER, Virginia M. PAIN and Simon J. MORLEY¹

Biochemistry Laboratory, School of Biological Sciences, University of Sussex, Falmer, Brighton, East Sussex BN1 9QG, U.K.

Eukaryotic initiation factor (eIF) 4E binds to the 5'-cap structure of eukaryotic mRNA and has a central role in the control of cell proliferation. We have shown previously that the stimulation of cultured *Xenopus* kidney cells with serum resulted in the activation of protein synthesis, enhanced phosphorylation of eIF4E and increased binding of the adapter protein, eIF4G, and poly(A)-binding protein (PABP) to eIF4E to form the functional initiation factor complex, eIF4F/PABP. We now show that cellular stresses such as arsenite, anisomycin and heat shock also result in increased phosphorylation of eIF4E, eIF4F complex formation and the association of PABP with eIF4G, in conditions under which the rate of protein synthesis is severely inhibited. In contrast with reported effects on mammalian cells, the stress-induced increase in eIF4F complex formation occurs in the absence of changes in the association of eIF4E with its binding

proteins 4E-BP1 or 4E-BP2. The stress-induced changes in eIF4E phosphorylation were totally abrogated by the p38 mitogen-activated protein (MAP) kinase inhibitor SB203580, and were partly inhibited by the phosphoinositide 3-kinase inhibitor LY294002 and the mammalian target of rapamycin (mTOR) inhibitor rapamycin. However, eIF4E phosphorylation was unaffected by extracellular signal-regulated protein kinase (MAP kinase) inhibitor PD98059. These results indicate that cellular stresses activate multiple signalling pathways that converge at the level of eIF4F complex formation to influence the interactions between eIF4E, eIF4G and PABP.

Key words: eIF2 α , eIF4F, p38 mitogen-activated protein kinase, protein synthesis.

INTRODUCTION

The control of polypeptide synthesis is important in cell proliferation. The physiological regulation of protein synthesis is almost always exerted at the level of polypeptide chain initiation (reviewed in [1,2]) and is influenced by elements in the 5' and 3' untranslated regions of the mRNA [3,4]. The initiation phase is regulated, in part, by the phosphorylation and association of initiation factors involved in binding mRNA to the 40 S ribosomal subunit (reviewed in [1,2,5,6]). The cap structure present at the 5' end of mRNA facilitates its binding to the ribosome, a process mediated by at least three eukaryotic initiation factors (eIF4A, eIF4B and eIF4F) and ATP hydrolysis. eIF4F is a cap-binding protein complex composed of three polypeptides: eIF4E, which specifically recognizes the cap structure, eIF4A, a single-strand-RNA-binding protein with helicase activity [7] and eIF4G, which acts as a bridging molecule between eIF4E and the 40 S ribosome via eIF3 [8–10]. It is believed that eIF4F functions to unwind secondary structure in the mRNA 5' untranslated region to facilitate the binding of the 40 S ribosomal subunit [1,2,5,6,10]. Recent studies in *Saccharomyces cerevisiae* and mammalian cells have indicated that a further association occurs between eIF4G and poly(A)-binding protein (PABP), which binds to the 3' poly(A) tail of mRNA. This interaction permits the functional interaction of the 5' and 3' ends of the mRNA species that is essential for transmitting the stimulatory signal of the poly(A) tail on translation to the cap structure [3,4,10].

The activity of eIF4E can be regulated both by phosphorylation and by its availability to participate in the initiation process, mediated by its interaction with the eIF4E-binding proteins 4E-BP1 and 4E-BP2 (also known as PHAS-I, PHAS-II). In resting mammalian cells, 4E-BP1 and 4E-BP2 are hypophosphorylated and bound to eIF4E [5,11–16]. Stimulation of cells with growth factors or hormones increases the phosphorylation of these eIF4E-binding proteins to disrupt their association with eIF4E, liberating eIF4E to interact with a conserved hydrophobic region of eIF4G. A similar sequence found in 4E-BP1 is involved in binding to eIF4E and competes with eIF4G for eIF4E binding [17]. eIF4E is also a phosphoprotein; increased levels of eIF4E phosphorylation and its association with eIF4G have been directly correlated with the enhancement of translation that follows mitogenic stimulation of mammalian and amphibian cells [18–23]. Parallel increases in eIF4E phosphorylation and interaction of the factor with eIF4G have been observed in a number of cellular systems [1,5,24–26], with the phosphorylated form of eIF4E reported to exhibit an increased affinity for the cap structure *in vitro* [27]. The phosphorylation site is Ser-209 [28,29]; its location adjacent to the cap-binding pocket is consistent with an effect of phosphorylation on mRNA binding [30,31]. Multiple signal transduction pathways control the phosphorylation of eIF4E, mediated via the activation of the extracellular signal-regulated protein kinases (ERKs), the p38 family of mitogen-activated protein (MAP) kinases and MAP kinase-activated proteins (MAPKAPs; [5,32–38]). A recently discovered MAPKAP kinase, Mnk-1 [36], which has been found to be

Abbreviations used: 4E-BP1 and 4E-BP2, eIF4E-binding proteins 1 and 2; eIF, eukaryotic initiation factor; ERK, extracellular signal-regulated protein kinase; m⁷GTP, 7-methyl-GTP; MAP, mitogen-activated protein; MAPKAP, MAP kinase-activated protein; MAPKAP-K2, MAPKAP kinase 2; mTOR, mammalian target of rapamycin; PABP, poly(A)-binding protein; PI-3K, phosphoinositide 3-kinase; VSIEF, vertical-slab isoelectric focusing.

¹ To whom correspondence should be addressed (e-mail s.j.morley@sussex.ac.uk).

phosphorylated and activated by the p38 MAP kinase, is able to phosphorylate eIF4E *in vitro* at the physiological site. After its activation by cellular stresses and cytokines, Mnk1 was shown to bind directly to eIF4G and to phosphorylate associated eIF4E *in vivo*, independently of events that caused the release of eIF4E from 4E-BP1 [39,40].

Cellular stress provides a powerful tool for investigating mechanisms of translational control in mammalian cells. In HeLa cells, mild heat stress (heat shock) conditions resulted in the inhibition of translation of most cellular mRNA species without affecting the phosphorylation status of eIF4E, eIF4B or eIF2 α [41,42]. However, more severe heat shock resulted in the dephosphorylation of eIF4E and eIF4B, the phosphorylation of eIF2 α [41,42] and decreased activities of eIF4F [43,44] and eIF2B [43,45]. Recent studies with rat hepatoma cells have confirmed these findings [26,46,47].

In contrast with reports with mammalian cells, we show here that cellular stress induced by heat shock, arsenite or anisomycin increases the phosphorylation of eIF4E, eIF4F complex formation and the association of PABP with eIF4G in *Xenopus* kidney cells. Under these severe stress conditions, translation is strongly inhibited, possibly mediated by the increased phosphorylation of eIF2 α [26,43,45,47]. Although these stresses also increase the phosphorylation of p70^{S6K}, only heat shock resulted in the activation of ERK. The stress-induced changes in eIF4E phosphorylation were totally abrogated by the p38 MAP kinase inhibitor SB203580 and were partly inhibited by the phosphoinositide 3-kinase (PI-3K) inhibitor LY294002 or by the mammalian target of rapamycin (mTOR) inhibitor rapamycin. These results suggest that the mTOR and PI-3K signalling pathways are involved, in part, in the stress-promoted phosphorylation of eIF4E and its association with eIF4G.

EXPERIMENTAL

Chemicals and biochemicals

Materials for tissue culture were from Life Technologies; [³⁵S]-methionine was from ICN; Immobilon PVDF was from Millipore; 7-methyl-GTP (m⁷GTP)-Sephacrose was from Pharmacia Biotech; Microcystin, PD98059 and SB203580 were from Alexis Corporation; rapamycin was a gift from Dr. J. Kay (University of Sussex, Brighton, East Sussex, U.K.). Antibodies against MAPKAP kinase 2 (MAPKAP-K2) were from TCS Biologicals; recombinant hsp25 was a gift from Dr. M. Gaestel (Berlin, Germany). Unless stated otherwise, all other chemicals were from Sigma.

Cell culture

Xenopus laevis kidney B 3.2 cells were grown at room temperature (20–24 °C) in 10 cm dishes containing 60 % (v/v) Leibovitz L-15 medium, 30 % (v/v) water and 10 % (v/v) foetal calf serum, supplemented with 2 mM glutamine, 50 i.u./ml penicillin and 50 μ g/ml streptomycin, as described previously [23]. For serum starvation, cultures were incubated with 0.5 % (v/v) foetal calf serum for 24 h before treatment; when used, inhibitors were added 1 h before treatment of the cells, as described in the Figure legends.

Preparation of cell extracts

Cultures were placed on ice and cells were scraped into 1 ml of buffer A [80 mM β -glycerophosphate (pH 7.2)/2 mM benzamidine], isolated by centrifugation, washed in the same buffer and lysed by the addition of 200 μ l of buffer B [50 mM Mops/KOH (pH 7.4)/100 mM NaCl/1 μ M microcystin/1 % (v/v)

Igepal (Nonidet P-40)/1 % (v/v) sodium deoxycholate/50 mM β -glycerophosphate/50 mM NaF/2 mM EGTA/2 mM EDTA/2 mM benzamidine/7 mM 2-mercaptoethanol]. After incubation for 5 min on ice with occasional vortex-mixing, extracts were centrifuged for 5 min at 18300 *g* (15000 rev./min) in a cooled Microfuge. The supernatant was frozen in liquid nitrogen and stored at –70 °C.

Measurement of protein synthesis

Serum-starved cells were incubated with or without anisomycin or arsenite, or subjected to heat shock for 15 min, before the addition of 10 μ Ci/ml [³⁵S]methionine into the complete medium for a further 15 min. Cells were recovered and washed twice in buffer A before lysis in 0.3 M NaOH. The incorporation of radioactivity into protein was determined by precipitation with trichloroacetic acid.

SDS/PAGE, vertical-slab isoelectric focusing (VSIEF) and immunoblotting

One-dimensional polyacrylamide gels and VSIEF gel electrophoresis were performed as described previously [19,23,33]. After the transfer of proteins to PVDF membrane, the presence of eIF4E, eIF4G, eIF4A, PABP, eIF2 α , 4E-BP1 and 4E-BP2 was detected with specific antisera, as described in the Figure legends.

m⁷GTP–Sephacrose chromatography

For the isolation of eIF4E and associated proteins, cell extracts of equal protein concentration were subjected to m⁷GTP–Sephacrose chromatography, the resin was washed three times with buffer C [50 mM Mops/KOH (pH 7.4)/100 mM NaCl/0.5 mM EDTA/0.5 mM EGTA/1 μ M microcystin/50 mM β -glycerophosphate/50 mM NaF/2 mM benzamidine/7 mM 2-mercaptoethanol/0.1 mM GTP] and recovered protein was eluted directly into sample buffer for either SDS/PAGE or VSIEF before analysis [19,23,33]. The association of eIF4E with 4E-BP1, 4E-BP2, eIF4G, eIF4A and PABP was analysed by Western blotting with specific antisera, as described [23]. In all cases, detection was within the linear response of the antiserum to the protein and immunoblots were quantified by densitometric scanning.

Assessment of p38 MAP kinase activity

p38 MAP kinase activity was assessed by monitoring the activity of the downstream kinase MAPKAP-K2 [48], with recombinant hsp25 as substrate [49]. The resulting autoradiographs were quantified with a Molecular Dynamics PhosphorImager.

RESULTS AND DISCUSSION

Arsenite- and anisomycin-stimulated phosphorylation of eIF4E requires the p38 MAP kinase pathway

In several mammalian cell types, arsenite and anisomycin can result in increased phosphorylation of eIF4E [1,5,33,38] and eIF2 α [26]. Consistent with this is our finding that exposure of serum-starved *Xenopus* kidney cells to arsenite or anisomycin results in a rapid increase in the phosphorylation of eIF4E and in the activation of the p70/p85 S6 kinases (Figure 1A). As described previously, *Xenopus* kidney cells [23] and oocytes [19,50] contain two isoforms of eIF4E, both of which are phosphorylated in response to serum [23] or cell stress (Figure 1A). The shift of eIF4E into the phosphorylated form was maximal within 30 min of stress induction with either agent

Figure 1 Cellular stress enhances the phosphorylation of eIF4E and eIF2 α in *Xenopus* kidney cells

(A) *Xenopus* kidney B3.2 cells were serum-starved for 24 h and incubated in the absence or presence of 10 μ M SB203580 before the addition of 5 mM arsenite or 10 μ g/ml anisomycin for 30 min. Cells were harvested and aliquots of extracts containing equal quantities of protein were subjected to m⁷GTP-Sepharose affinity chromatography to isolate total eIF4E. The two forms of eIF4E present in *Xenopus* cells were resolved by VSIEF and detected by immunoblotting with antiserum specific for total eIF4E. The migration of the more phosphorylated variant of each form of eIF4E resolved by VSIEF is indicated by P. In addition, aliquots of extracts containing equal quantities of protein were resolved directly by SDS/PAGE, followed by immunoblotting with antiserum specific for p70/85 S6 kinase. (B) Cells were incubated as in (A) for the durations indicated and the percentage of total eIF4E in the phosphorylated form was quantified by densitometric scanning of the immunoblots: $\blacktriangle$, arsenite; $\blacktriangledown$, anisomycin. The experiment was performed three times, each in triplicate; the error bars indicate the S.E.M. (C) Cells were incubated as in (A); aliquots of extracts containing equal quantities of protein were resolved directly by SDS/PAGE, followed by immunoblotting with antiserum specific to total eIF2 α or to the phosphorylated form of the protein, as indicated. These results were used to estimate the percentage of total eIF2 α in the phosphorylated form, expressed as the percentage of levels found in control cells (lane 1, 100 $\pm$ 5%): lane 2, 250 $\pm$ 20%; lane 3, 275 $\pm$ 15% (means $\pm$ S.D., n = 3). The rate of translation was measured in these cells as described above and is shown in the histogram as the percentage of the control rate, which was set at 100%. The experiment was performed four times; the error bars indicate the S.E.M.

(Figure 1B). At the same time, the overall rate of translation decreased by more than 95% during the 30 min after treatment with either anisomycin or arsenite (Figure 1C, lower panel). Although anisomycin is a known inhibitor of the elongation phase of protein synthesis, stress-induced shut-off of translation has long been associated with increased phosphorylation of eIF2 α and inhibition of eIF2B activity in a wide variety of cell

Figure 2 Arsenite- and anisomycin-stimulated phosphorylation of eIF4E requires the p38 MAP kinase pathway

Upper panel: *Xenopus* kidney B3.2 cells were serum-starved for 24 h before the addition of SB203580 (10 μ M), PD98059 (50 μ M) or rapamycin (50 nM) for 1 h, or LY294002 (50 μ M) for 15 min, as indicated. Cells were then incubated in the absence or presence of arsenite or anisomycin for 30 min, extracts were prepared and equal quantities of protein were subjected to m⁷GTP-Sepharose affinity chromatography to isolate total eIF4E. The two forms of eIF4E present were resolved by VSIEF and detected by immunoblotting with antiserum specific for total eIF4E. The migration of the more phosphorylated variant of each form of eIF4E resolved by VSIEF is indicated (%4E (P)). Lower panel: aliquots of extract containing equal quantities of protein were assayed for MAPKAP-K2 activity by using hsp25 as substrate, as described in the Experimental section. An autoradiograph of the SDS/polyacrylamide gel is shown; the phosphorylation of hsp25 in the immunocomplex assays was quantified by PhosphorImager analysis. The results are representative of those obtained in three separate experiments.

types [26,41–43,45–47]. In agreement with this we have shown, with the use of SDS/PAGE and immunoblotting with antiserum specific for phosphorylated eIF2 α (Figure 1C), that arsenite or anisomycin led to a marked increase in the phosphorylation of eIF2 α (see the Figure legend for quantification). On the basis of studies with mammalian cells, it is therefore likely that the arsenite- or anisomycin-induced inhibition of general translation in *Xenopus* kidney cells is a consequence of the increase in eIF2 α phosphorylation [26,43].

In *Xenopus* kidney cells, multiple signalling pathways can be used to modulate the phosphorylation of eIF4E [23]. To determine whether the increased phosphorylation of eIF4E seen in these experiments required the p38 MAP kinase pathway, we tested the effect of the specific inhibitor SB203580 [51] on the phosphorylation of eIF4E and S6 kinase. As shown in Figure 1(A), inhibition of p38 MAP kinase resulted in the total inhibition of the phosphorylation of eIF4E induced by arsenite (compare lane 3 with lane 1) or anisomycin (compare lane 6 with lane 4). These results are consistent with published results on stress-induced phosphorylation of eIF4E reported for NIH 3T3 cells [33], 293 cells and Chinese hamster ovary K1 cells [38]. As expected, SB203580 had little effect on the induction of JNK activity by either arsenite or anisomycin; however, in these studies (discussed below) and others [23,34,38], in the unstimulated cells SB203580 decreased the level of eIF4E phosphorylation below basal levels. In addition, whereas the arsenite-induced activation of the p70/p85 S6 kinases was not influenced by SB203580, a partial inhibition of the stimulation of S6 kinase by anisomycin was consistently observed (Figure 1A, and results not shown). Other workers have shown a partial inhibition of induction of S6 kinase activity by SB203580 in arsenite-stimulated cardiomyocytes [52]. These effects are consistent with reports of cross-talk between signalling pathways [21,26] and suggest that p38 MAP kinase might be involved in the stress-induced activation of S6 kinases, possibly via the phosphorylation and

Figure 3 Arsenite and anisomycin promote the co-isolation of PABP with eIF4F

(A) Serum-starved cells were incubated in the absence or presence of rapamycin or SB203580 for 1 h as indicated, before the addition of anisomycin for 30 min. Cell extracts were prepared as in Figure 1(A) and adjusted to equal protein concentration; eIF4E and associated proteins were isolated by m⁷GTP–Sepharose chromatography. Recovered proteins were resolved by SDS/PAGE and detected by immunoblotting with the specific antiserum indicated. The results are from a single experiment but are representative of those obtained in five separate experiments. Quantification (by densitometric scanning) of the eIF4G, PABP and eIF4A recovered is shown in the lower panel. For each, the quantity of protein was normalized for the recovery of eIF4E and is expressed as a percentage of control. The experiment was performed five times; the error bars indicate the S.E.M. (B) Cells were incubated as in (A) except that they were stimulated with arsenite; eIF4F/PABP complex formation was analysed as described. These experiments were performed five times; the error bars indicate the S.E.M. (C) Cell extracts from (A) were adjusted to equal protein concentration; eIF4E, 4E-BP1 and 4E-BP2 were isolated by m⁷GTP–Sepharose chromatography. Recovered proteins were resolved by SDS/PAGE and detected by immunoblotting with the specific antiserum indicated. (D) Cell extracts described in (B) were analysed as described for (C).

activation of protein kinase B [53]. However, we have been unable to address this because the currently available antiserum specific for mammalian protein kinase B in the phosphorylated form has failed to recognize the *Xenopus* kidney cell homologue (results not shown).

Stress-induced phosphorylation of eIF4E is partly inhibited by rapamycin and LY294002

The above results prompted us to examine which potential signalling pathways were used in the stress-induced phosphoryl-

ation of eIF4E. Both arsenite and anisomycin potently activate p38 MAP kinase in *Xenopus* kidney cells (Figure 2, lower panel; compare lanes 2 and 8 with lane 1), concomitant with increased levels of eIF4E phosphorylation (Figure 2, upper panel). These events were unaffected by PD98059, which inhibits the ERK signalling pathway (Figure 2, lanes 3 and 9), but were abrogated almost completely by the p38 MAP kinase inhibitor SB203580 by itself (lanes 4 and 10) or in combination with PD98059 (lanes 5 and 11). Because both stresses also activate p70/p85 S6 kinases (Figure 1), we used rapamycin and LY294002 (at levels that completely prevented that activation of p70/p85 S6 kinases in

Figure 4 Heat shock enhances the phosphorylation of eIF4E and the activity of MAPKAP-K2 in *Xenopus* kidney cells

(A) Serum-starved B3.2 cells were placed in a water bath at 37 °C for the times indicated. Extracts were prepared and equal quantities of protein were subjected to m⁷GTP-Sepharose affinity chromatography to isolate total eIF4E (upper panel). The two forms of eIF4E were resolved by VSIEF and detected by immunoblotting with antiserum specific for total eIF4E. The migration of the more phosphorylated variant of each form of eIF4E resolved by VSIEF is indicated (eIF4EP). In the lower panel, aliquots of total cell extracts containing equal quantities of protein were resolved directly by SDS/PAGE, followed by immunoblotting with antiserum specific for ERK. (B) Cells were heat-shocked for 30 min; aliquots of extracts containing equal quantities of protein were resolved directly by SDS/PAGE, followed by immunoblotting with antiserum specific for total eIF2α or to the phosphorylated form of the protein, as indicated. The proportion of total eIF2α in the phosphorylated form, expressed as the percentage found in control cells (set at 100%), was quantified by densitometric scanning of the immunoblots: lane 1, 100 ± 5%; lane 2, 350 ± 25% (means ± S.D., *n* = 3). The rate of total protein synthesis was measured as in Figure 1 and is expressed as the percentage of the control rate: lane 1, 100 ± 7%; lane 2, 20 ± 8% (means ± S.D., *n* = 3). (C) Cells were incubated in the absence or presence of inhibitors as described in the legend to Figure 2, before heat shock for 30 min. The phosphorylation status of eIF4E (left panel) and MAPKAP-K2 activity (right panel) from a single experiment is shown (upper panels) and quantified from three separate experiments in each instance (error bars indicate the S.E.M.).

these cells; results not shown) to address the role of the mTOR and PI-3K signalling pathways respectively in this response. With either arsenite (Figure 2; compare lanes 6 and 7 with lane 2) or anisomycin (Figure 2; compare lanes 12 and 13 with lane 2), the inhibition of mTOR or PI-3K partly inhibited the phosphorylation of eIF4E without affecting p38 MAP kinase activity. These results are consistent with our recent findings suggesting that *Xenopus* kidney cells differ from most mammalian cells in that signalling via the mTOR and/or PI-3K pathways has a direct, pivotal role in the phosphorylation of eIF4E [23].

Arsenite and anisomycin increase the level of eIF4F complex formation by a mechanism that requires p38 MAP kinase activity

We have shown previously that eIF4E phosphorylation in response to conditions that activate protein synthesis is often accompanied by an increase in the binding of the adapter protein eIF4G to eIF4E, to form the functional initiation factor complex

eIF4F [19–21,54]. Stimulation of cultured *Xenopus* kidney cells with serum promoted eIF4E phosphorylation, eIF4F complex formation and the co-isolation of PABP with the eIF4F complex [23]. To examine whether cell stress has any influence on eIF4F complex formation, serum-starved cells were incubated in the absence or presence of anisomycin (Figure 3A) or arsenite (Figure 3B). Extracts were then prepared and eIF4E and associated proteins were isolated by m⁷GTP-Sepharose chromatography. Recovered proteins were then detected by immunoblotting. Surprisingly, in spite of the finding that arsenite and anisomycin inhibit total protein synthesis, they each promoted a 2.5–3-fold increase in the association of eIF4G with eIF4E (Figures 3A and 3B; compare lane 2 with lane 1). These results are in contrast with those reported for 293 cells, in which arsenite had no effect on eIF4G binding to eIF4E [52]. In our cells, the anisomycin-stimulated increase in eIF4F complex formation and the parallel increase in the co-isolation of eIF4A and PABP with eIF4E were totally abrogated by either rapamycin (Figure 3A;

compare lane 3 with lane 2) or SB203580 (lane 4). With arsenite, eIF4F/PABP complex formation was inhibited to a smaller extent by rapamycin or SB203580 (Figure 3B; compare lanes 3 and 4 with lane 2). In either case, inhibition of the PI-3K pathway with LY294002 had no inhibitory effect on stress-induced eIF4F complex formation in spite of the partial inhibition of eIF4E phosphorylation (Figure 2, and results not shown).

In mammalian cells an important mechanism for regulating the ability of eIF4E to participate in the initiation process is by modulation of its availability, mediated by its interaction with 4E-BP1 and 4E-BP2. These proteins have been identified as downstream signalling targets of rapamycin-sensitive pathways [5,21,26]. In several cell types the phosphorylation of either 4E-BP1 or 4E-BP2 has been shown to result in a characteristic mobility shift on SDS/PAGE analysis together with their total release from eIF4E [5,11–16,33]. However, we have shown previously, in the *Xenopus* kidney cell system, that serum-stimulated increases in eIF4F/PABP complex formation could not be correlated with the phosphorylation and dissociation of 4E-BP1 or 4E-BP2 from eIF4E [23]. We have therefore examined the effect of cell stress on the association of these proteins with eIF4E. Figure 3 indicates that neither anisomycin (Figure 3C) nor arsenite (Figure 3D) influenced the association of 4E-BP1 or 4E-BP2 with eIF4E. In addition, although we have used this gel system previously to demonstrate the characteristic rapamycin-sensitive phosphorylation of 4E-BP1 during stress of NIH 3T3 cells [33], we observe no effect of cell stress in promoting the phosphorylation of either 4E-BP1 or 4E-BP2 in *Xenopus* kidney cells (Figure 3, and results not shown). Moreover, the interaction of eIF4E with its inhibitory binding proteins was not further influenced by either rapamycin or SB203580 (Figure 3). These results suggest that stress stimulates the interaction between eIF4E, eIF4A, eIF4G and PABP by a distinct mechanism that is independent of the association of eIF4E with 4E-BP1 or 4E-BP2. A potential role for 4E-BP3 in this response cannot be discounted [55]; however, this has yet to be addressed because the only available antiserum failed to detect the *Xenopus* protein (results not shown).

Heat shock increases eIF4E phosphorylation and eIF4F complex formation

We have also analysed the effect of heat stress (heat shock) on the serum-starved *Xenopus* kidney cells. In mammalian and *Drosophila* cells, heat shock has been associated with the inhibition of eIF4F complex formation, the dephosphorylation of eIF4E, increased phosphorylation of eIF2 α and alterations in eIF2B activity [26,41–47]. In our cells, heat shock at 37 °C resulted in the phosphorylation of eIF4E and, as reported for another *Xenopus* cell line [56], the activation of the ERK pathway (Figure 4A). The increase in ERK activity after heat shock was confirmed by immunoprecipitation and immunocomplex kinase assays with myelin basic protein as substrate (results not shown). Heat shock for 30 min resulted in a marked inhibition of general translation and disaggregation of polysomes (Figure 4B, and results not shown) and was accompanied by a 3–4-fold increase in the phosphorylation of eIF2 α (see the Figure legend for quantification), a 6-fold activation of p38 MAP kinase (Figure 4C; compare lane 2 with lane 1; quantified in the lower panel) and the activation of p70/85 S6 kinases (results not shown). We have used PD98059, SB203580, rapamycin and LY294002 to examine which signalling pathways are important for the phosphorylation of eIF4E under these conditions. PD98059 prevented the activation of ERK (results not shown) but did not appreciably

Figure 5 Enhanced eIF4F complex formation during heat shock requires the mTOR and p38 MAP kinase pathways

(A) Serum-starved cells were incubated in the absence or presence of rapamycin or SB203580 for 1 h before heat shock for 30 min. Cell extracts were prepared and analysed as described in the legend to Figure 3. (B) eIF4E was isolated by m⁷GTP-Sepharose affinity chromatography and its association with 4E-BP1 and 4E-BP2 was monitored as described in the legend to Figure 3.

attenuate the heat-shock-induced phosphorylation of eIF4E (Figure 4C; compare lane 3 with lane 2; quantified in the lower panel), suggesting that the activation of the ERK pathway after heat shock has little or no role in this event. However, SB203580 by itself (Figure 4C, lane 4) or in combination with PD98059 (lane 5) totally prevented the activation of p38 MAP kinase and eIF4E phosphorylation (quantified in the lower panel). As found with arsenite or anisomycin, rapamycin (Figure 4C, lane 6) or LY294002 (lane 7) had a reproducible but incomplete inhibitory effect on eIF4E phosphorylation, without influencing p38 MAP kinase activation. Surprisingly, heat shock promoted a 2–2.5-fold increase in the association of eIF4G, eIF4A and PABP with eIF4E (Figure 5A; compare lane 2 with lane 1; quantified in the

Figure 6 Heat shock increases eIF4E phosphorylation, but not eIF4F complex formation, in serum-fed cells

(A) *Xenopus* kidney cells were either serum-starved (lanes 1–4) or incubated in the presence of 10% (v/v) fresh serum for 24 h (lanes 5–8), before incubation in the absence or presence of SB203580 for 1 h followed by no further treatment or heat shock for 30 min, as indicated. Extracts were prepared and equal quantities of protein were subjected to m⁷GTP–Sepharose affinity chromatography to isolate total eIF4E. The two forms of eIF4E were resolved by VSIEF and detected by immunoblotting with antiserum specific for total eIF4E. The migration of the more phosphorylated variant of each form of eIF4E resolved by VSIEF is indicated (eIF4EP). The results from a single experiment are shown (upper panel) and quantified from three separate experiments (lower panel; error bars indicate the S.E.M.). (B) eIF4E and associated proteins from extracts described for (A) were isolated by m⁷GTP–Sepharose chromatography. Recovered proteins were resolved by SDS/PAGE and detected by immunoblotting with the specific antiserum indicated. The results presented are from a single experiment but are representative of those obtained in three separate experiments.

lower panel). As with anisomycin, this was sensitive to the inhibition of either the mTOR (Figure 5A, lane 3) or p38 MAP kinase (lane 4) signalling pathways. Under any of these conditions, eIF4F complex formation could not be attributed to any competition between 4E-BP1 or 4E-BP2 with eIF4G for the common site of interaction with eIF4E (Figure 5B).

Heat shock increases eIF4E phosphorylation but not eIF4F complex formation in the presence of serum

The observed increase in eIF4F complex formation in response to heat shock in these cells was rather surprising in view of earlier reports with mammalian cells [26,41–47]. As most of the latter studies were performed in non-starved cells, we analysed the effect of heat shock on the phosphorylation of eIF4E in serum-supplemented *Xenopus* kidney cells. Figure 6(A) shows that,

whereas the basal level of phosphorylation of eIF4E was increased in serum-fed cells (compare lane 5 with lane 1), the phosphorylation of eIF4E was still increased after heat shock, although to a smaller extent than observed with starved cells (Figure 6A; compare lane 6 with lane 5, and lane 2 with lane 1; quantified in the lower panel). Under either growth condition, the enhanced phosphorylation of eIF4E required signalling through the p38 MAP kinase pathway (Figure 6A; compare lanes 3 and 7 with lanes 1 and 5). However, as discussed above and in previous papers [23,38,52], SB203580 alone decreased the level of eIF4E phosphorylation to below basal levels (Figure 6A; compare lanes 4 and 8 with lanes 8 and 5), suggesting that signalling through this pathway is obligatory for the maintenance of eIF4E phosphorylation.

As described previously [23], stimulation of *Xenopus* kidney cells with serum promotes eIF4F complex formation, which is still evident even at 24 h after addition (Figure 6B; compare lane 5 with lane 1). Although eIF4F/PABP complex formation was stimulated by heat shock in starved cells (Figures 5 and 6B; compare lane 2 with lane 1), levels of the eIF4F complex were decreased slightly by heat shock of cells in the fed state (Figure 6B; compare lane 6 with lane 5), as reported for other cell systems [38,41]. The explanation that decreased eIF4F complex formation during heat shock reflects increased competition of 4E-BP1 binding for eIF4E over that of eIF4G in 293 cells [38] cannot apply to our cells because heat shock did not influence the phosphorylation or recovery of 4E-BP1 associated with eIF4E in either growth state (Figure 5B, and results not shown). For either nutritional state, the maintenance of eIF4F/PABP complex levels required signalling through the p38 MAP kinase pathway (Figure 6B; compare lane 3 with lane 2, and lane 7 with lane 6). As described for eIF4E phosphorylation above, studies with SB203580 alone indicate that signalling through the p38 MAP kinase pathway is obligatory for the maintenance of eIF4F complexes (Figure 6B; compare lanes 4 and 8 with lanes 1 and 5). Experiments are currently under way to address the role of this signalling pathway in the control of protein synthesis.

In summary, our results show that in spite of the inhibition of general translation, cell stress promotes the phosphorylation of eIF4E and eIF4F/PABP complex formation in serum-starved *Xenopus* kidney cells. Studies with specific inhibitors have indicated that these events require signalling through the p38 MAP kinase pathway, with some possible input via the PI-3K/mTOR pathways. However, they also describe an obligatory role for signalling through the p38 MAP kinase pathway in the maintenance of levels of eIF4E phosphorylation and eIF4F complexes in untreated cells. Previous studies have not shown enhanced eIF4F complex formation in response to cellular stress. It is therefore a novel finding that, in starved cells, cellular stress recruits eIF4E into the eIF4F complex together with PABP, without a change in the association of eIF4E with 4E-BP1 or 4E-BP2. Because these effects are normally observed under conditions that stimulate the total rate of protein synthesis, these results suggest that cellular stress regulates translation by an alternative mechanism to that of the activity of the cap-binding complex. Two possible candidates for this regulation are the eIF2 and eIF2B protein complexes. Regulation at the level of eIF2 seems very likely because we have shown that eIF2 α is highly phosphorylated in response to stress in *Xenopus* kidney cells. Studies with mammalian cells have suggested that cellular stress promotes the phosphorylation of eIF2 α and/or inactivates eIF2B [26], either of which can result in the inhibition of translation. Unfortunately, our attempts to investigate the changes in the activity of eIF2B in extracts prepared from *Xenopus* kidney cells have been unsuccessful. At present the role

of the eIF4E kinase Mnk1 in the phosphorylation of eIF4E in these cells is also unclear, although the sensitivity to SB203580 strongly suggests that it has a central role [36,39,40]. Although eIF4E kinase activity is increased in response to stress (results not shown), currently available antisera against Mnk1, which are not of sufficiently high titre to recognize the endogenous levels of this kinase in mammalian cells [39], have failed to recognize a homologue in our cells. Further work is currently under way to address this problem.

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