Heat shock causes a decrease in polysomes and the appearance of stress granules in trypanosomes independently of eIF2α phosphorylation at Thr169

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Summary

In trypanosomes there is an almost total reliance on post-transcriptional mechanisms to alter gene expression; here, heat shock was used to investigate the response to an environmental signal. Heat shock rapidly and reversibly induced a decrease in polysome abundance, and the consequent changes in mRNA metabolism were studied. Both heat shock and polysome dissociation were necessary for (1) a reduction in mRNA levels that was more rapid than normal turnover, (2) an increased number of P-body-like granules that contained DHH1, SCD6 and XRNA, (3) the formation of stress granules that remained largely separate from the P-body-like granules and localise to the periphery of the cell and, (4) an increase in the size of a novel focus located at the posterior pole of the cell that contain XRNA, but neither DHH1 nor SCD6. The response differed from mammalian cells in that neither the decrease in polysomes nor stress-granule formation required phosphorylation of eIF2α at the position homologous to that of serine 51 in mammalian eIF2α and in the occurrence of a novel XRNA-focus.

Supplementary material available online at
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Key words: Heat shock, *Trypanosoma brucei*, Stress granules, eIF2 alpha, P-bodies

Introduction

When cells are subjected to stress, such as heat shock, genes associated with growth are downregulated and expression of proteins believed to increase cellular survival, such as HSP70, are selectively upregulated. In eukaryotes, this regulation operates at multiple levels: transcription initiation may be enhanced via factors specific for heat shock-genes, whereas for other genes there is selective inhibition of splicing and nuclear export of mRNAs (Bond, 2006).

Stress also influences the fate of cytoplasmic mRNA in eukaryotes. Treatment of mammalian cells with a variety of stresses, such as arsenite or heat shock, results in the relocation of most mRNAs from polysomes to cytoplasmic foci called stress granules (Kedersha et al., 1999). These contain the small ribosomal subunit (Kedersha et al., 2002), the translation-initiation factors eIF2, eIF3, eIF4E and eIF4G (Kedersha et al., 2002; Kimball et al., 2003), the poly(A)-binding protein (Kedersha et al., 1999), DHH1 (also known as DDX6, Rck, ATP-dependent RNA helicase p54) (Wilczynska et al., 2005), the 5′-3′ exonuclease XRN1 (Kedersha et al., 2005) and RNA-binding proteins, including SCD6 (also known as LSM14 and RAP55) (Yang et al., 2006; Kedersha and Anderson, 2007).

Formation of stress granules in response to heat shock and metabolic stress is dependent on, and might be initiated by, the phosphorylation of eukaryotic translation-initiation factor eIF2α on a conserved serine residue (S51 in mammals and yeast) by a family of stress-activated kinases (Kedersha et al., 1999; McEwen et al., 2005). The modification inhibits initiation of translation by increasing the affinity of eIF2α for the guanine nucleotide exchange factor eIF2B, resulting in its sequestration. Stress-granule formation can also be induced independently of eIF2α phosphorylation by pateamine or hippuristanol, which interfere with the action of eIF4A, an RNA helicase necessary for translation initiation (Dang et al., 2006; Mazroui et al., 2006).

The fate of mRNAs in stress granules remains unclear; mRNAs may be degraded or stored until the stress is over. Inhibitors of translation elongation that stabilise polysomes cause the dissociation of stress granules, whereas inhibitors that destabilise polysomes promote the assembly of stress granules, suggesting that the mRNA in stress granules is in equilibrium with polysomes (Kedersha et al., 2000).

Stress granules have been shown to interact with processing bodies (P-bodies) (Kedersha et al., 2005; Wilczynska et al., 2005). P-bodies are cytoplasmic granules thought to be sites of mRNA degradation (Parker and Sheth, 2007). P-bodies share some, but not all, components with stress granules, for instance XRN1, DHH1 and SCD6. The decapping enzyme (a complex of DCP1 and DCP2) is specific to P-bodies while the small ribosomal subunit, eIF2, eIF3 and poly(A)-binding protein are absent (Bashkirov et al., 1997; Cougot et al., 2004; Ingelfinger et al., 2002; Kedersha and Anderson, 2007; Sheth and Parker, 2003). It is believed that XRN1 is the major degradative enzyme in P-bodies but the precise function of other components has yet to be fully characterised.

Trypanosoma brucei is a unicellular flagellate that causes African trypanosomiasis (sleeping sickness) in humans and a range of diseases in animals. Synthesis of mRNA in trypanosomes is atypical; tandem arrays of genes (Berriman et al., 2005; El Sayed et al., 2003; Hall et al., 2003; McDonagh et al., 2000) are transcribed

from a single promoter (Johnson et al., 1987; Kooter et al., 1987; Martinez-Calvillo et al., 2004; Martinez-Calvillo et al., 2003), and monocistronic mRNAs result from *trans*-splicing of a short, capped leader to the 5′ end and linked 3′ cleavage and polyadenylation of the upstream mRNA (Campbell et al., 1984; LeBowitz et al., 1993; Liang et al., 2003; Matthews et al., 1994; Schürch et al., 1994; Ullu et al., 1993). Consequently, the regulation of gene expression in trypanosomes is predominantly post-transcriptional (Clayton and Shapira, 2007). There is evidence for the presence of P-bodies: DHH1, XRNA and one Pumilio-family protein are located in cytoplasmic granules in normally growing cells (Caro et al., 2006; Cassola et al., 2007; Dallagiovanna et al., 2007; Dallagiovanna et al., 2005; Holetz et al., 2007), although any role in the regulation of gene expression has yet to be determined. In contrast to mRNA synthesis, the mechanisms of translation initiation and elongation appear to be typical for a eukaryote; all the factors identified in metazoa and yeast are present in the trypanosome genome (Ivens et al., 2005), although a functional analysis has only been performed on a small number (Dhalia et al., 2006; Dhalia et al., 2005) and very little is known about regulation of translation (Clayton and Shapira, 2007).

Little is known about how the overall rate of gene expression is regulated, for example when trypanosomes stop growth and enter stationary phase. The only such phenomenon that has been investigated in any detail is the response to heat shock (Lee, 1998; Lee and Van der Ploeg, 1990; Muhich and Boothroyd, 1988; Muhich and Boothroyd, 1989; Muhich et al., 1989). There is a reduction of up to 50% in the rate of transcription and a selective block in *trans*-splicing of tubulin mRNAs, whereas *HSP70* and *HSP85* mRNA maturation continues efficiently. There is a reduction in steady-state levels of tubulin and several other mRNAs, but the steady-state levels of *HSP70* and *HSP85* mRNAs are either stable or perhaps slightly increased.

Recent work has described the appearance of stress granules in trypanosomes in response to carbon-source starvation. The granules contained polyadenylated mRNA, poly(A)-binding protein, one isoform of eIF4E, the XRN1 homologue XRNA, DHH1 and several proteins with RNA recognition motifs (Cassola et al., 2007). However, unlike in mammalian cells, the stress granules did not contain the ribosomal small subunit, arguing against the presence of arrested initiation complexes. The RNA in the granules was stable and it has been suggested that the granules are involved in mRNA storage and stabilisation during stress (Cassola et al., 2007).

Here, the behaviour of cytoplasmic mRNA during heat shock and recovery in *T. brucei* has been characterised. The onset of heat shock caused a reduction in polysomes and a decrease in mRNA levels; the majority of mRNAs decreased at a rate greater than could be explained by a block in synthesis. These changes in mRNA levels were associated with alterations in cytoplasmic granules. Heat-shock stress granules of similar composition to mammalian stress granules appeared, the number of P-body-like granules increased and XRNA accumulated in a novel granule at the posterior pole of the cell. However, unlike in mammalian cells these changes were not dependent on the phosphorylation of eIF2α at the position homologous to S51.

Results

Heat shock reversibly reduces translation of most proteins

Procyclic form *Trypanosoma brucei* are normally cultured at 27°C but some procyclic cell lines can grow indefinitely at 37°C (Guttinger et al., 2007). The *T. brucei* Lister 427 procyclic cell line used in our experiments was cultured at 27°C and was able to proliferate for at least 1 day at 37°C. At 41°C, the trypanosomes underwent a heat-shock response that resulted in a cessation of proliferation. The response was reversible for at least 2 hours at 41°C and, after a lag, normal growth resumed on return to 27°C; a typical growth curve is shown in Fig. 1. The heat-shock treatments above did not cause significant cell death.

The effect of heat shock on gene expression was investigated by metabolic labelling with [^{35}S]methionine. Cells were labelled for 20-minute windows over a time course of heat shock (41°C for 60 minutes) and recovery (27°C for 5 hours; Fig. 2A). There was a decrease in the synthesis of most polypeptides within the first 30 minutes. Some polypeptides were unaffected, most noticeably two with the molecular mass of 55 kDa and 70 kDa (solid arrows in Fig. 2A). On return to 27°C, the incorporation of [^{35}S]methionine into most polypeptides returned to the level before the heat shock. However, even after 5 hours recovery some polypeptides were still synthesised at a lower rate (dashed arrows in Fig. 2A) and the 55 kDa protein was still synthesised at an increased rate. Reduced protein synthesis during heat shock could result from an inhibition of translation initiation or elongation, or from increased proteolysis of newly synthesised proteins. Polysome analysis of cells after 15 or 30 minutes at 41°C showed a large decrease in the polysome fraction, consistent with a reduced rate of initiation of translation (Fig. 2B).

Heat shock reversibly reduces steady-state levels of most mRNAs

The rapid reduction in number of polysomes during heat shock must increase the pool of non-polysome-associated mRNAs. To investigate the fate of mRNA during heat shock, RNA was prepared and northern blots were probed for the 5′-spliced leader that is present on all cytoplasmic mRNAs (De Lange et al., 1984). At 41°C, steady-state mRNA was reduced to 51% after 60 minutes and to 28% after 120 minutes. At 37°C, mRNA levels remained unaffected for the first 60 minutes but began to decrease slightly between 60 and 120 minutes (Fig. 3A).

To determine global effects of heat shock on steady-state levels of mRNA in more detail, a microarray was performed to compare the mRNAs in cells incubated at 41°C for 60 minutes with those in cells at 27°C (ArrayExpress submission accession number: E-MEXP-1476). The data were normalised and filtered for quality and reproducibility. The data are shown as a plot of the intensities for each spot in each condition for the total (Fig. 3B) and filtered

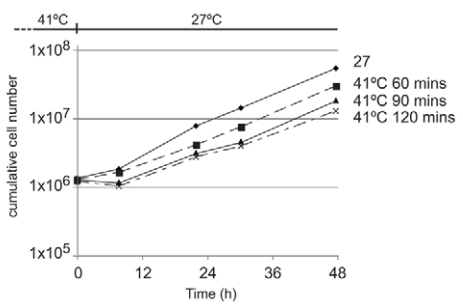

Fig. 1. Growth recovery following heat shock. Trypanosome cultures were incubated a 41°C for 60, 90 or 120 minutes. Growth was monitored after cultures were returned to 27°C. One representative growth curve from three experiments is shown.

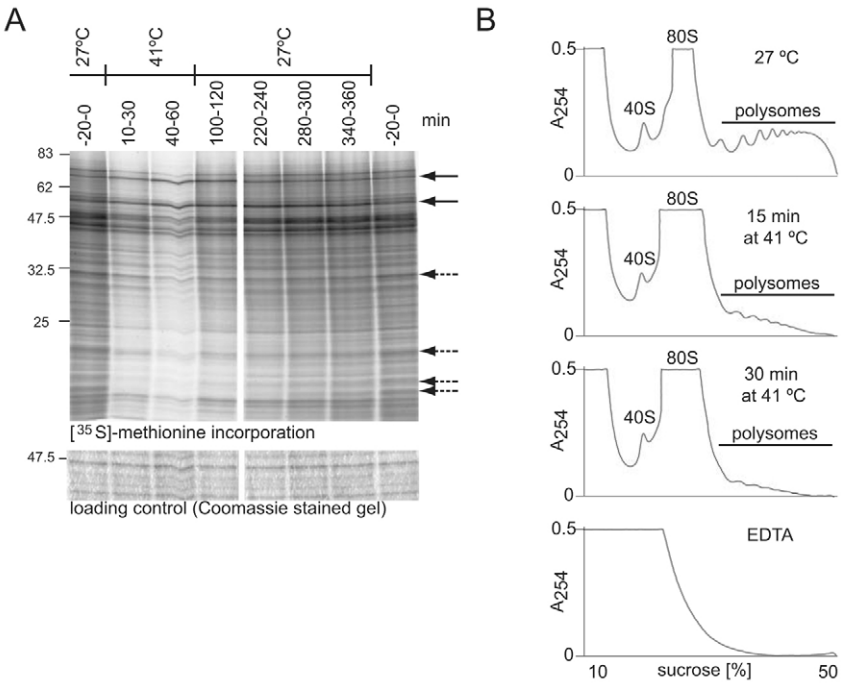

Fig. 2. Effect of heat shock on translation and polysomes. (A) Changes in translation during heat-shock treatment. Cells were labelled for 20-minute windows at 27°C and during a heat-shock time course followed by recovery at 27°C. An autoradiograph of the SDS-PAGE analysis is shown. Two polypeptides with unchanged synthesis are marked with solid arrows. Examples of proteins that have reduced synthesis after 5 hours recovery are marked with dashed arrows. The stained gel was used to control for equal loading (bottom). One lane of the gel has been removed. (B) Changes in polysomes during heat shock treatment. Absorbance profiles at 254 nm of sucrose density gradients of cells incubated at 41°C for 15 and 30 minutes. 50 mM EDTA was added to the cell lysate to dissociate polysomes. Representative results from several experiments are shown.

dataset (Fig. 3C). Data normalisation was based on the standard assumption that the total mRNA levels remain unchanged under the two conditions. Since, in reality, incubation at 41°C for 60 minutes resulted in a 50% loss of total mRNA (Fig. 3A), an mRNA with a ratio outcome of 1.0 (41°C/27°C) in the plot of the array data was actually reduced twofold; an mRNA with a ratio outcome of 2.0 was unaffected by heat shock and an mRNA with a ratio outcome of 0.5 was reduced fourfold. In an initial analysis we selected genes that behaved significantly differently from the average mRNA using cut-off values of $\geq$2.5 and $\leq$0.4 (supplementary material Table S1). The heat-shock-regulated genes were randomly distributed throughout the genome.

The list of genes with a ratio outcome of $\geq$2.5 represents the mRNAs that escaped the general reduction in mRNA levels upon heat shock. There were 371 genes in this category (supplementary material Table S2). 230 genes had no assigned function (half of all open reading frames in the genome are unassigned). The annotated genes included several stress-response genes including one HSP100, four E2 ubiquitin ligases, two proteosome subunits, and two DnaJ-domain-containing chaperones. Genes involved in mRNA metabolism included a polyU polymerase (Stagno et al., 2007), an RNA helicase and three mRNA-binding proteins (RBP6, RBP10 and hnRNP/F). The 317 genes with a ratio outcome of $\leq$0.4 included both mRNAs that were downregulated after heat shock and those which normally have a high turnover rate. More than half of these were functionally annotated (supplementary material Tables S1 and S2). Core metabolic functions were strongly represented. There were eleven amino acyl tRNA synthases (three more have ratio outcomes between 0.41 and 0.45) and five translation initiation factors. In addition, there were three subunits of the t-complex chaperone (three further subunits have ratio outcomes between 0.41 and 0.45).

Response of individual mRNAs to heat shock

The kinetics of the response of individual mRNAs was investigated in further detail. Four genes associated with cell growth were used: actin, α-tubulin and β-tubulin (probed together), and protein disulphide isomerase 2 (*PDI-2*) (Rubotham et al., 2005). After 60 minutes of heat shock, steady-state levels of these mRNAs were reduced to between 50% and 25% (Fig. 3D).

Next, the expression of putative heat shock protein genes was investigated. The *T. brucei* genome encodes a range of different *HSP70* genes (Folgueira and Requena, 2007), and a tandem array of identical *HSP83* genes (Mottram et al., 1989). Specific probes were used to screen northern blots of RNA prepared over a time course of heat shock. The steady-state levels of *HSP70* (Tb11.01.3110) and *HSP83* (Tb10.26.1080) mRNAs were maintained during heat shock (Fig. 3E). *HSP70* and *HSP83* mRNAs increased slightly at 37°C, whereas the housekeeping genes tested did not. The results with *HSP70* and *HSP83* mRNAs confirmed earlier observations (Lee, 1998; Muhich et al., 1989). Other HSP70 isoforms were investigated: mitochondrial HSP70s (Tb927.6.3740, Tb927.6.3750 and Tb927.6.3800) and cytoplasmic *HSP70.4* (Tb927.7.710) (Searle and Smith, 1993), which all decreased in response to heat shock, similar to the majority of mRNAs (supplementary material Fig. S1). The changes in mRNA levels determined by northern blot for actin and *PDI-2* were very similar to those determined by in the microarray experiment. The tubulin, *HSP70* (Tb11.01.3110) and *HSP83* could not be accurately measured in the microarray experiments because the mRNAs are very abundant and saturated the spots (Fig. 3C and ArrayExpress submission accession number: E-MEXP-1476).

The decrease in mRNA steady-state levels during heat shock was fully reversible. Total mRNA returned to the pre-heat shock levels after ~3 hours of recovery (Fig. 4). Individual mRNAs had variable kinetics of return with tubulin being noticeably slower than the mean. Together, the data from metabolic labelling, microarray experiments and northern blots show that trypanosomes undergo a defined and reversible change in gene expression in response to heat shock.

Bulk mRNA decay rate is increased during heat shock

The decrease in steady-state levels of mRNAs during heat shock could have resulted from decreased synthesis and/or increased

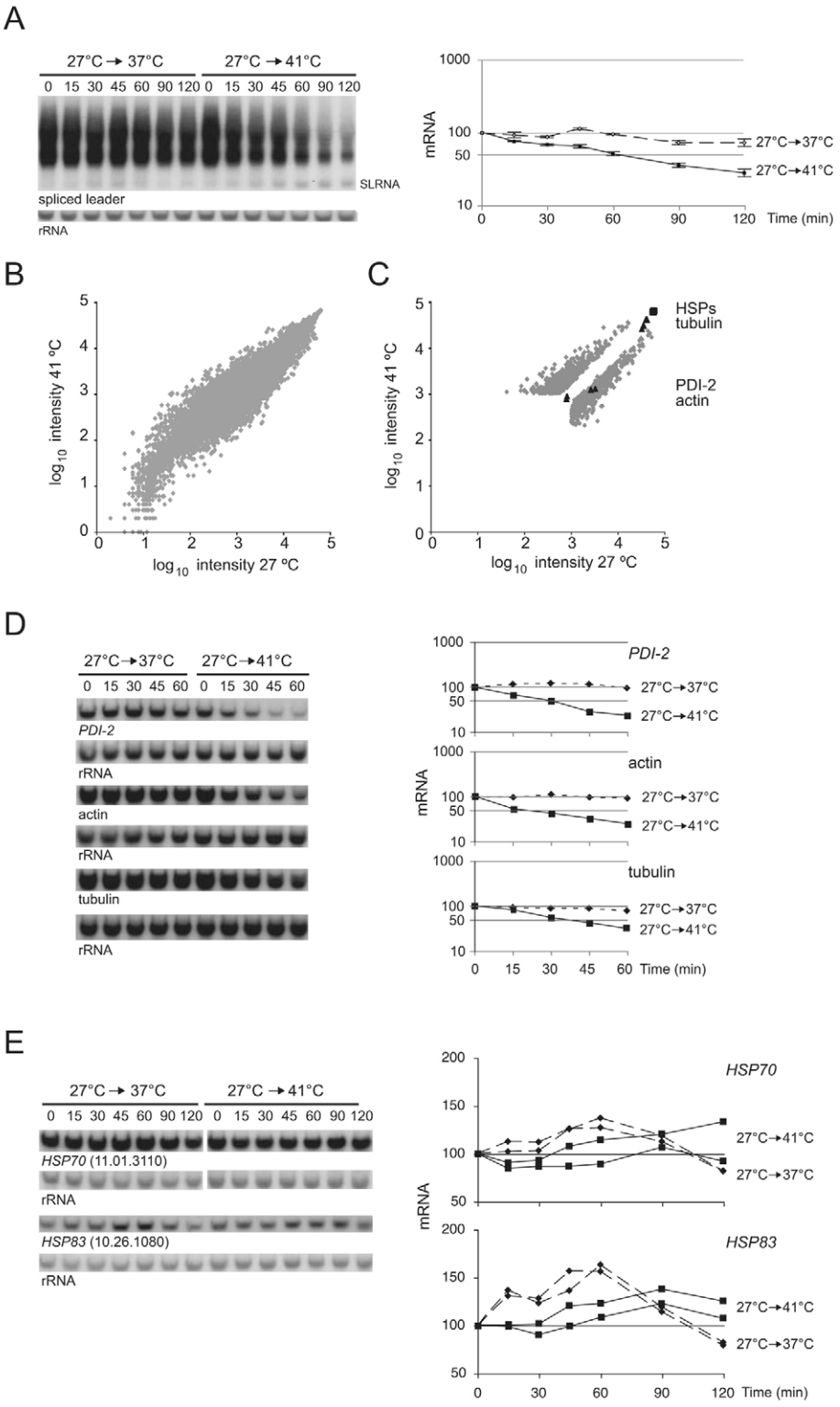

Fig. 3. Changes in mRNA steady-state levels following heat shock. Cell cultures were transferred from 27°C to either 37°C or 41°C over a time course, and RNA was analysed by northern blotting and microarrays. All probes used in this work were specific (supplementary material Fig. S2A). (A) Northern blot probed for total mRNA using an antisense oligonucleotide recognising the spliced leader; the spliced leader RNA (SLRNA) is indicated. One of three replica gels is shown, all three were used for quantification; error bars indicate ± s.d. (B) Oligonucleotide microarray slides were probed with RNA from cells incubated at either 27°C or 41°C. The total dataset is shown as a plot of the intensities for each spot. (C) The filtered dataset from B. (D) Northern blots probed for *PDI-2*, actin and α- and β-tubulin. Data from one representative experiment out of several repeats are shown. (E) Northern blots probed for HSP70 (Tb11.01.3110) and HSP83 (Tb10.26.1080). One out of two replica gels is shown. Quantification from both replica gels is shown.

decay. Two experiments were performed to distinguish between the two possibilities. First, mRNA synthesis was inhibited by sinefungin, an inhibitor of *trans*-splicing (McNally and Agabian, 1992; Ullu et al., 1993; Ullu and Tschudi, 1991), and the rate of mRNA decay was compared in cells grown without heat shock at either 27°C or 37°C, and in cells heat-shocked at 41°C. The rate of decay was significantly higher in the heat-shocked cells, both for bulk mRNA (Fig. 5A) and for tubulin, *PDI-2* and actin mRNAs (Fig. 5B). A similar result was obtained when transcription was inhibited with actinomycin D (data not shown). The results provide strong evidence for an increased rate of turnover of mRNA during heat shock. Second, the rate of mRNA decrease was measured in heat-shocked cells with or without sinefungin. For both bulk mRNA (Fig. 5A) and individual mRNAs (Fig. 5B), the

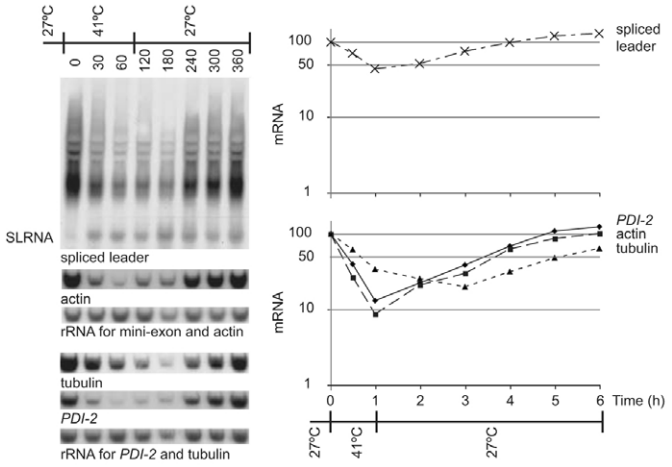

Fig. 4. The decrease in mRNA steady-state levels is reversible. Trypanosome cultures were transferred from 27°C to 41°C and returned to 27°C after 1 hour. Northern blots were probed for total mRNA, actin, tubulin and *PDI-2*.

rate of decrease was more rapid in the presence of sinefungin than with heat shock alone (Fig. 5) indicating that synthesis of mRNA was still occurring at 41°C. This is consistent with our microarray results, which indicate that some mRNAs are elevated after heat shock (supplementary material Tables S1 and S2).

Heat shock causes reversible formation of cytoplasmic granules that contain components typical of mammalian stress granules

At 41°C, the decrease in polysomes was more rapid than the decrease in mRNA (Fig. 2B and Fig. 3). Thus, non-polysome associated mRNA in the cell must increase during the onset of heat shock. To investigate the fate of the mRNAs released from polysomes, a screen for the presence of heat-shock-inducible stress granules was performed. Homologues of known stress-granule components were expressed as enhanced yellow fluorescent protein (eYFP)-fusion proteins. Poly(A)-binding proteins, PABP1 (Tb09.211.0930) (De Gaudenzi et al., 2005) and PABP2 (Tb09.211.2150) (Hotchkiss et al., 1999), and translation initiation factors eIF4E1 to 4 (Tb11.18.0004, Tb10.61.0210, Tb11.01.3630, Tb927.6.1870) (Dhalia et al., 2005), eIF2A (Tb927.3.2900) (Moraes et al., 2007) and eIF3B (Tb11.01.1370) (De Gaudenzi et al., 2005) were tagged. The expression of all fusion proteins was confirmed by western blotting using anti-GFP (supplementary material Fig. S2B) and all were the expected molecular mass.

At 27°C, PABP1 and PABP2 were predominantly in the cytoplasm, eIF2A, eIF3B, eIF4E3 and eIF4E4 were present mainly in the cytoplasm and in lower concentrations in the nucleus, eIF4E1 and eIF4E2 were about equally distributed between nucleus and cytoplasm (Fig. 6A,B). One hour after transfer to 41°C, all of the above eYFP-fusion proteins had concentrated in distinct cytoplasmic foci of heterogenous size and shape, whereas the localisation of the eYFP

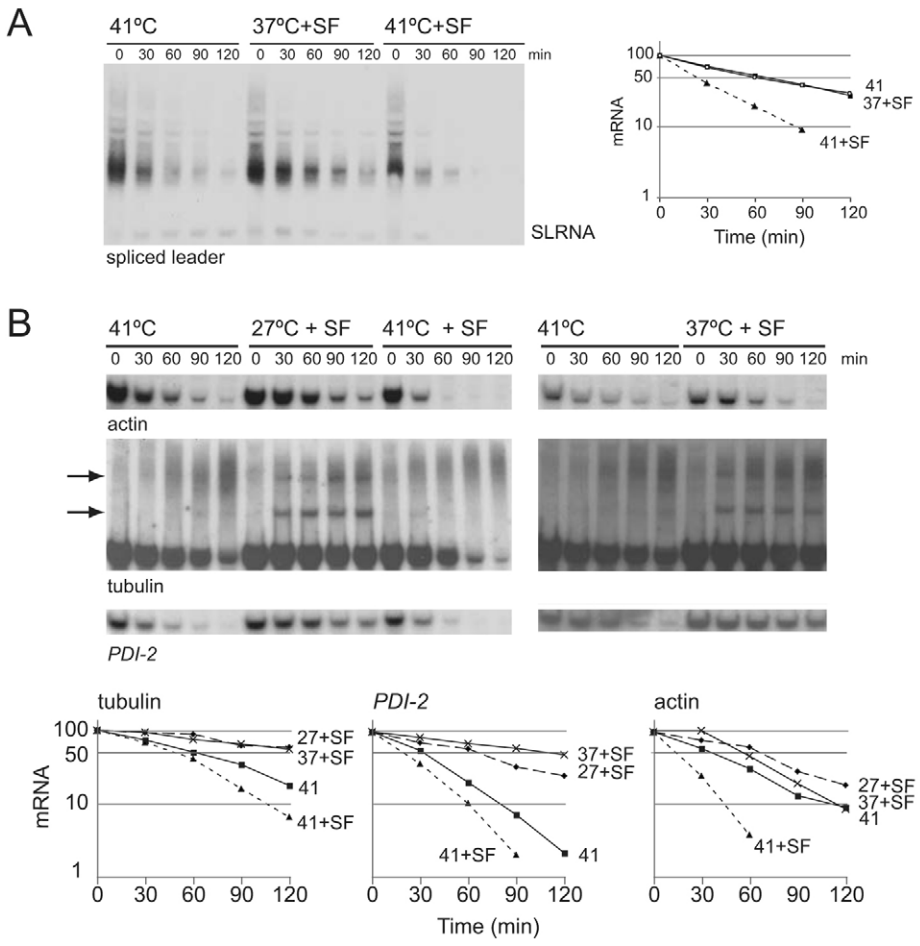

Fig. 5. Effect of heat shock on transcription and mRNA processing. (A,B) Trypanosome cultures were heat-shocked at 41°C or treated with 2 μg/ml sinefungin at 27°C, 37°C or 41°C. The decrease in mRNAs caused by the different treatments was analysed by northern blots probed for total mRNA (A), actin, tubulin and *PDI-2* (B). Tubulin mRNAs aberrantly spliced by sinefungin (McNally and Agabian, 1992) are indicated by arrows.

control was unaffected (Fig. 6A,B). Granule formation occurred within 30 minutes, increased up to 120 minutes (Fig. 6B) and required a minimum temperature of 40°C (data not shown). Inhibition of new mRNA synthesis using actinomycin D for up to 2 hours did not prevent the formation of heat-shock stress granules (Fig. 6B), indicating that granule formation does not require newly synthesised mRNAs.

The formation of granules was fully reversible and they disappeared within 5-6 hours after return to 27°C (Fig. 6C). Optical sections through a heat-shocked cell that expressed either eIF4E3-eYFP or PABP1-eYFP revealed that the granules were concentrated towards the cell periphery (Fig. 6D). eIF4E3 and eIF3B were tagged in the same cell line and colocalised in granules (Fig. 6E).

Cycloheximide prevents the formation of heat-shock granules and can reverse the decline in the polysome pool during heat shock

Cycloheximide was added before and during heat shock to investigate the relationship between polysome dissociation and stress-granule formation. Polysome analyses were performed on cells incubated at: (1) 27°C, (2) 41°C, (3) 27°C with cycloheximide, (4) 27°C with cycloheximide followed by an increase in temperature to 41°C, (5) 41°C with cycloheximide (6) 27°C with puromycin (Fig. 7A). Stress-granule formation was monitored in similar conditions using cells expressing PABP1-eYFP (Fig. 7B). Cycloheximide stabilised and puromycin dissociated polysomes as expected, and neither caused stress-granule formation. Thus, polysome dissociation by puromycin is not sufficient for granule formation. Addition of cycloheximide prior to heat shock largely prevented polysome dissociation and granule formation was blocked providing evidence that polysome dissociation is necessary for stress granule formation.

Cycloheximide addition during heat shock caused an increase in polysomes. The increase could result from newly synthesised mRNAs becoming trapped and/or 'old' mRNAs returning to polysomes. However, the rapid increase in polysomes after cycloheximide addition favours a return of old mRNA. Cycloheximide also inhibited heat-shock-induced decrease in *PDI-2* and tubulin mRNAs (Fig. 7C) providing evidence that polysome dissociation is required for mRNA decay during heat shock.

Heat shock increases the number of P-bodies and causes relocalisation of XRNA, but not of DHH1 and SCD6, to a focus at the posterior pole of the cell

Homologues of P-body components were screened by expression of eYFP fusions from extragenic loci. At 27°C, three proteins localised to discrete foci within the cytoplasm: DHH1 (Tb10.70.3290), SCD6 (Tb11.03.0530) and XRNA (Li et al., 2006). The putative P-bodies differed from the stress granules induced by heat shock in that they were constitutively present and more defined with a clearly spherical shape.

The localisation of DHH1 and SCD6 to cytoplasmic foci was confirmed using antisera raised against recombinant proteins (for details, see supplementary material Fig. S3). The foci visualised using antibodies were less apparent against the background signal from the cytoplasm than the foci visualised using fluoresecence from the cognate fusion protein. The same was true when anti-eYFP antibody was used. The most possible explanation for this discrepancy is that the antibodies only labelled the surface of the foci, whereas the fluorescent proteins labelled the foci uniformly, giving a stronger signal relative to the cytoplasm.

For further analysis cell lines were made with one endogenous allele of either DHH1 or SCD6 that had been modified to express a fluorescent protein fusion. The expression levels from the modified allele were either similar (SCD6) or less (DHH1) than from the remaining wild-type allele (supplementary material Fig. S3A). DHH1 was expressed with eYFP at the N-terminus (eYFP-DHH1) and this fusion protein was shown to be functional as cells grew at the normal rate after deletion of the second allele whereas knockdown of DHH1 by RNA interference was lethal (Schwede et al., 2008). SCD6 was expressed as either an N- or C-terminal eYFP-fusion protein (eYFP-SCD6 or SCD6-eYFP) and both localised into discrete foci in the cytoplasm. There was an average of 2.9 foci per cell (Fig. 8A; supplementary material Fig. S4A). DHH1 and SCD6 were expressed as eYFP and mCherry fluorescent protein fusions (eYFP-DHH1 and SCD6-mChFP) in the same cell line (Fig. 8B, left panel). Both proteins colocalised to the same cytoplasmic foci and no foci were found that did not contain both fluorescent proteins. In response to protein synthesis inhibitors, the foci behaved in a similar manner to P-bodies in yeast and cultured mammalian cells. Puromycin caused an increase in size of the foci but not in the number, and cycloheximide caused the foci to fully disappear. Heat shock at 41°C caused an increase in the number and the size of the foci (Fig. 8B).

XRNA was expressed as either an N- or C-terminal eYFP-fusion protein by modification of one endogenous allele (eYFP-XRNA, XRNA-eYFP; supplementary material Fig. S5); both localised to foci distributed throughout the cytoplasm but, in contrast to DHH1 and SCD6, one focus was always located at the posterior pole of the cell. A cell line expressing XRNA-eYFP and mChFP-DHH1 (Fig. 8B, right panel; supplementary material Fig. S5) confirmed that the two proteins localised to the same foci in the cytoplasm (dashed arrows in Fig. 8B) but only XRNA-eYFP localised to the focus present at the posterior pole of cells (solid arrows in Fig. 8B). Cycloheximide caused all XRNA-containing foci to disappear (Fig. 8B right panel), whereas puromycin caused an increase in size of all (Fig. 8B, right panel; supplementary material Fig. S4B). By contrast, heat shock caused a preferential increase in the size of the XRNA-containing focus at the posterior pole, which always became the brightest focus in the cell (Fig. 8B, right panel; supplementary material Fig. S4B).

Heat-shock stress granules do not colocalise with P-bodies

Any interaction between P-bodies and heat-shock stress granules was investigated using cell lines expressing PABP1-eYFP and either mChFP-DHH1 or SCD6-mChFP (supplementary material Fig. S5). At 27°C, PABP1-eYFP was uniformly distributed throughout the cytoplasm, whereas mChFP-DHH1 and SCD6-mChFP were concentrated in cytoplasmic foci (Fig. 8C). At 41°C, PABP1 localised to heat-shock stress granules, and the DHH1 and SCD6 foci increased in size and number (Fig. 8C). The majority of the PABP1 foci did not colocalise with the DHH1 and SCD6 foci. Only a small number of heat-shock stress granules (PABP1-foci) and P-bodies (DHH1-SCD6 foci) were found to either colocalise or lie adjacent to each other (dashed arrows in Fig. 8C).

Formation of heat-shock granules is independent of eIF2A phosphorylation at T169, the equivalent of S51 in mammalian eIF2α

One of the first steps in the heat shock response in mammalian cells is the inhibition of the initiation of translation after phosphorylation

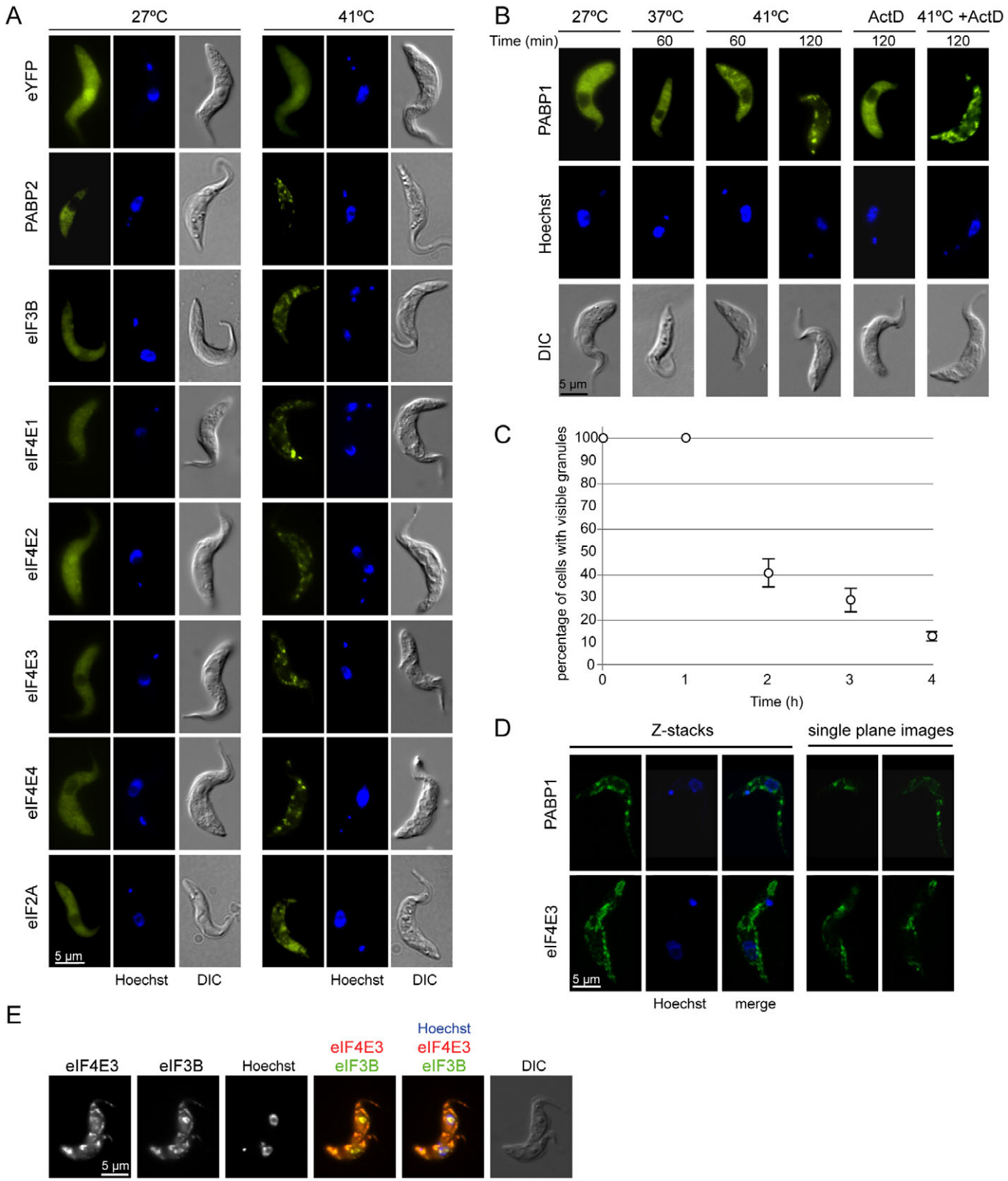

Fig. 6. Characterisation of *T. brucei* heat-shock stress granules. (A) Homologues of stress granule markers were expressed as eYFP fusion proteins. Fluorescent images of unstressed (27°C) and heat-shocked (1 hour 41°C) cells are shown. (B) The effect of heat shock on cells that express PABP1-eYFP from a modified endogenous locus; treatments are indicated. (C) Disappearance of heat-shock stress granules after a return to 27°C in cells that express PABP1-eYFP from a modified endogenous locus. The percentage of cells with visible granules was determined during recovery and the average values (n=100) from three slides is shown, error bars indicate ± s.d. (D) Optical sections through *T. brucei* cells that express PAPB1-eYFP or eIF4E3-eYFP after 60 minutes at 41°C are shown as projection (left) and some selected single plane images (right). (E) Colocalisation of eIF3B-eYFP and eIF4E3-mChFP to heat-shock stress granules.

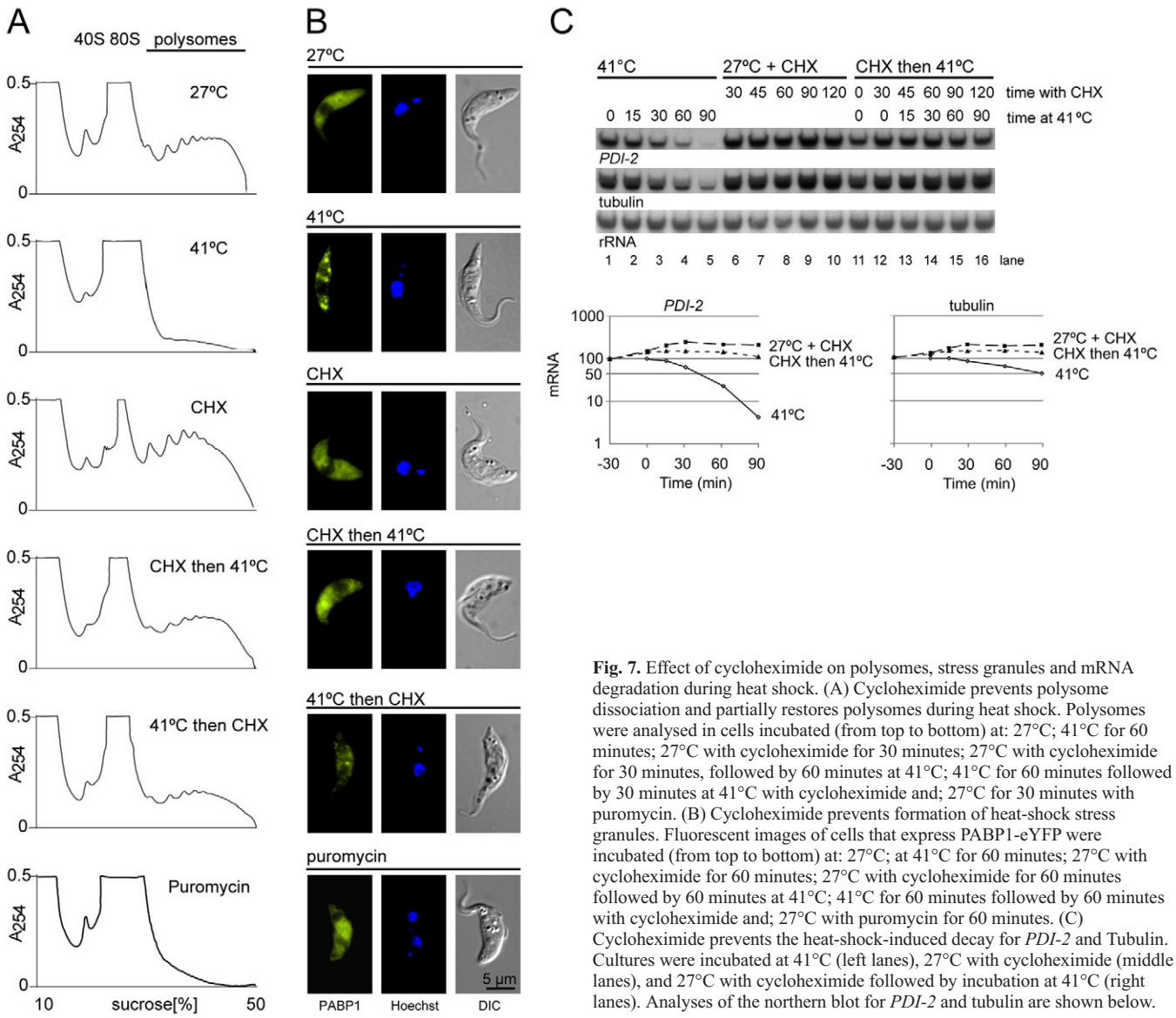

Fig. 7. Effect of cycloheximide on polysomes, stress granules and mRNA degradation during heat shock. (A) Cycloheximide prevents polysome dissociation and partially restores polysomes during heat shock. Polysomes were analysed in cells incubated (from top to bottom) at: 27°C; 41°C for 60 minutes; 27°C with cycloheximide for 30 minutes; 27°C with cycloheximide for 30 minutes, followed by 60 minutes at 41°C; 41°C for 60 minutes followed by 30 minutes at 41°C with cycloheximide and; 27°C for 30 minutes with puromycin. (B) Cycloheximide prevents formation of heat-shock stress granules. Fluorescent images of cells that express PABP1-eYFP were incubated (from top to bottom) at: 27°C; at 41°C for 60 minutes; 27°C with cycloheximide for 60 minutes; 27°C with cycloheximide for 60 minutes followed by 60 minutes at 41°C; 41°C for 60 minutes followed by 60 minutes with cycloheximide and; 27°C with puromycin for 60 minutes. (C) Cycloheximide prevents the heat-shock-induced decay for *PDI-2* and Tubulin. Cultures were incubated at 41°C (left lanes), 27°C with cycloheximide (middle lanes), and 27°C with cycloheximide followed by incubation at 41°C (right lanes). Analyses of the northern blot for *PDI-2* and tubulin are shown below.

of serine 51 (S51) of eIF2α (Kedersha et al., 1999; McEwen et al., 2005). eIF2A, the *T. brucei* homologue of eIF2α, has an N-terminal extension and the residue aligned with mammalian S51 is threonine 169 (T169) (Moraes et al., 2007). To test the role of T169 phosphorylation in the heat shock response, a cell line expressing PABP1-eYFP was used as the starting cell line (*eIF2A*+/+). First, one eIF2A allele was replaced with a blasticidin resistance gene (*eIF2A*+/–), then the remaining wild type allele was modified so that a T169A mutation was introduced (*eIF2A* T169A/+). This second manipulation altered the 3′ untranslated region of the eIF2A gene and a control cell line was made with the same change in the 3′ untranslated region but with unaltered eIF2A sequence (*eIF2A* T169T/+; supplementary material Fig. S6).

The cell lines T169A/– and T169T/– were incubated at 41°C and in both cases PABP1-eYFP was localised to multiple cytoplasmic granules on induction of heat shock (Fig. 9A) and polysome abundance decreased (Fig. 9B). Thus, in trypanosomes neither heat shock granule formation nor translational exit requires phosphorylation of T169 in eIF2A.

Discussion

The changes in gene expression that occur to favour cellular survival during heat shock are a universal phenomenon and minimally comprise two components, a decrease in expression of genes associated with cell growth and an increase in expression of genes associated with maintaining the integrity of protein structure. Procyclic form trypanosomes are no exception. However, the mode of gene expression in trypanosomes precludes transcriptional regulation of individual genes in response to heat shock and the regulation must occur post-transcriptionally. Previously, it was shown that heat shock causes the overall transcription by RNA polymerase II to halve (Lee, 1995; Muhich and Boothroyd, 1988) and that *HSP70* mRNA maturation continues during heat shock, whereas tubulin mRNA maturation is blocked (Muhich and Boothroyd, 1988; Muhich and Boothroyd, 1989). Here, the effect of heat shock on mRNA metabolism in trypanosomes has been investigated. The major findings are that heat shock caused: (1) a rapid and large decrease in polysomes, (2) a decrease in the levels of many mRNAs that is caused, in part, by increased decay, (3)

increased levels of some mRNAs, (4) the formation of heat-shock stress granules at the cell periphery, (5) an increase in the number and size of P-bodies and, (6) an increase in size of a novel XRNA focus at the posterior pole of the cell. The decrease in polysomes and appearence of stress granules were not dependent on phosphorylation of eIF2A at T169 (equivalent to S51 in mammalian eIF2α). The inhibition of stress-granule formation by cycloheximide indicates that polysome-free mRNA is required for their formation. The P-bodies contained XRNA, DHH1 and SCD6; by contrast, a novel focus at the posterior pole of the cell contained XRNA but neither DHH1 nor SCD6. Importantly, the changes on heat shock were fully reversible and the kinetics of return to proliferative growth, restoration of starting mRNA levels, and disappearance of heat shock stress granules were similar.

Changes in mRNA metabolism following heat shock

Within 15 minutes of heat shock the polysome pool and the translation of most proteins had decreased. Heat shock also reduced steady-state levels of most mRNAs but with slower kinetics than the decrease in polysomes. Thus, the dissociation of polysomes on heat shock must result in increased levels of non-polysome-associated mRNA in the cell. The appearance of heat-shock stress granules and the increase in the number of P-bodies following heat shock occurred with similar kinetics to polysome dissociation. One interpretation of the kinetics is that the mRNAs released from polysomes were directed to either or both of these cytoplasmic mRNP particles, but this remains to be proven.

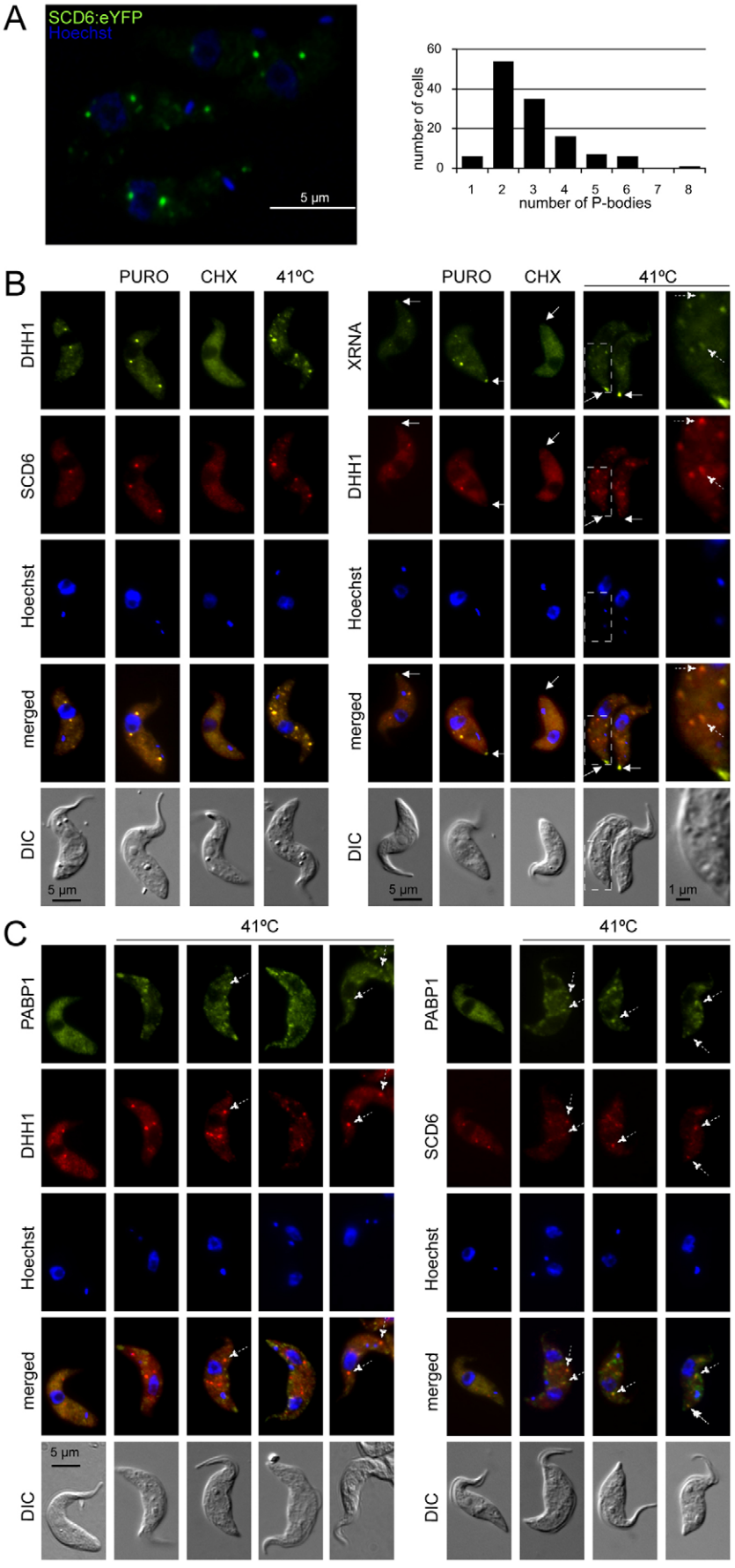

Fig. 8. *T. brucei* P-bodies. (A) *T. brucei* cells have in average 2.9 P-bodies. Projection of optical sections through puromycin-treated cells that express SCD6-eYFP. Similar *Z*-stacks (supplementary material Fig. S5A) were used to determine the number of P-bodies per cell: the average was 2.9 per cell (n=125) with little variation in different cell-cycle stages: 2.7 in 1K1N (n=95), 3.3 in 2K1N (n=17) and 3.3 in 2K2N (n=12) cells. (B) P-body components DHH1, SCD6 and XRNA. eYFP-DHH1 and SCD6-mChFP (left panel) or mChFP-DHH1 and XRNA-eYFP (right panel) were expressed in the same cell line from endogenous loci. Representative fluorescent images of untreated, and cells after 1 hour with puromycin, cycloheximide or heat shock (41°C) are shown. Solid arrows indicate the posterior pole of the cell. An enlargement of a heat-shocked cells expressing mChFP-DHH1 and XRNA-eYFP is shown to indicate the colocalisation of DHH1 and XRNA in all spots (dashed arrows), except at the posterior pole. (C) P-bodies and heat-shock stress granules. mChFP-DHH1 and PABP1-eYFP (left) or mChFP-SCD6 and PABP1-eYFP (right) were expressed in the same cell line from their endogenous loci. Representative fluorescent images of one untreated cell and several images of cells that have been heat shocked (1 hour 41°C) are shown. Dashed arrows point at granules that contain both a P-body marker (DHH1 or SCD6) and the stress granule marker PABP1, either colocalised or close to each other.

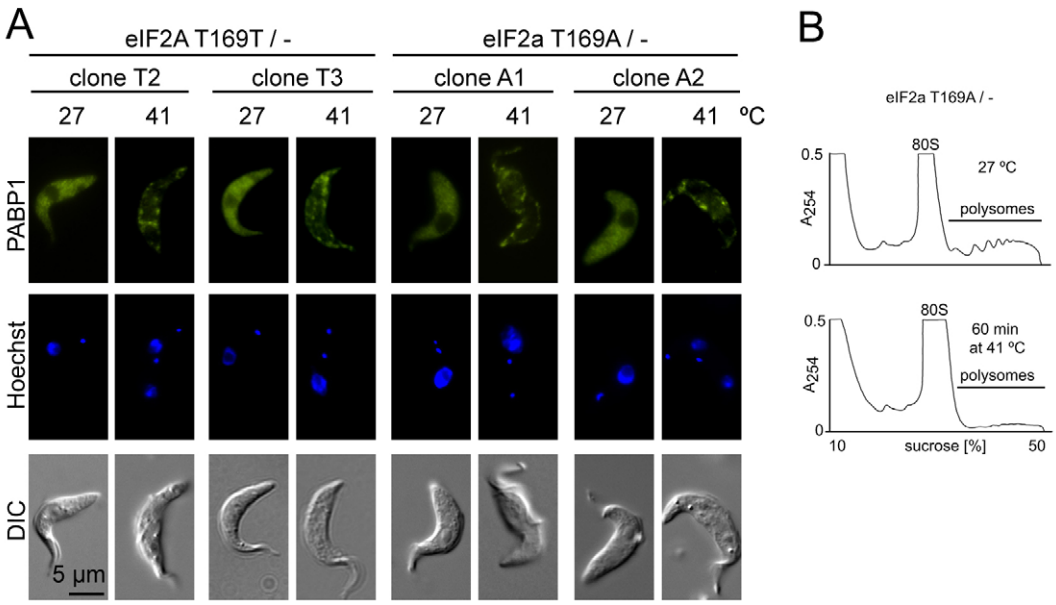

Fig. 9. Formation of heat-shock stress granules is independent on eIF2α phosphorylation at T169. Heat shock treatment (1 hour 41°C) of cells expressing PABP1-eYFP from the endogenous locus and with either a single wild-type (T169T) or mutated (T169A) *eIF2A* gene (supplementary material Fig. S6). (A) Fluorescent images of two clonal cell populations expressing wild-type eIF2A (T2 and T3) or mutant eIF2A (A1 and A2) are shown. (B) Polysome analysis of the eIF2A T169A/– cells incubated at either 27°C or 41°C for 60 minutes.

What causes the decrease in mRNA steady-state levels following heat shock? The microarray experiment showed a wide range of responses for individual mRNA steady-state levels; some were unaffected or even increased, most decreased and some decreased very rapidly. Experiments measuring the rate of decay strongly suggested that the decrease in mRNAs at 41°C was due to both increased degradation, and reduced transcription and maturation.

Inhibition of polysome dissociation by cycloheximide blocked both the decrease in *PDI-2* and tubulin mRNAs and heat shock stress granule formation. Dissociation of polysomes by puromycin was not sufficient to induce either heat shock stress granule formation or a decrease in mRNAs. These results provide evidence that dissociation of polysomes is necessary but not sufficient for the heat-shock response. The addition of cycloheximide during heat shock resulted in an increase in polysomes (Fig. 7A); one explanation for this is that mRNAs are in equilibrium between polysomes and stress granules as found in mammals (Kedersha et al., 2000). If this is correct, exit from the polysome pool was not irreversible and did not inevitably lead to degradation.

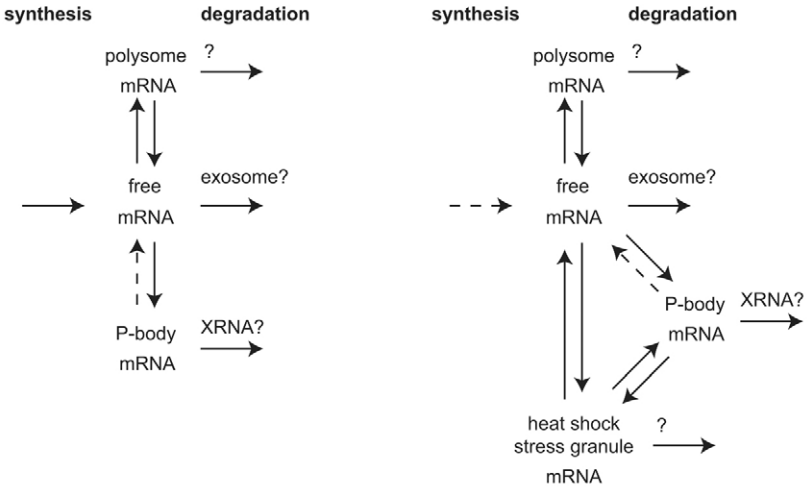

Fig. 10. Model of compartments that may determine mRNA fate in growing cells (left) and in heat-shocked cells (right).

P-bodies

In yeast and mammalian cells, P-bodies contain the activities involved in 5′-3′ exonucleolytic digestion of mRNA: the decapping enzyme components DCP1 and DCP2, XRN1 exonuclease, and accessory proteins including DHH1 and SCD6 (reviewed in Parker and Sheth, 2007). Decapping activity has been detected in trypanosomatid cell lysates (Milone et al., 2002) but the genes encoding this activity have not been identified as there are no readily identifiable orthologues of DCP1 or DCP2 in the trypanosome genome (Berriman et al., 2005). P-body components identified in trypanosomes include: DHH1 (Cassola et al., 2007; Holetz et al., 2007), SCD6 (this work), XRNA (Cassola et al., 2007), one member of the PUF protein family (Caro et al., 2006; Dallagiovanna et al., 2007; Dallagiovanna et al., 2005) and the RNA binding protein RBP3 (A. Robles, D. Gudjonsdottir-Planck, C. Hartmann and C.E.C., unpublished). eIF4E is found in P-bodies in yeast and mammalian cells but has not been reported to be present in P-bodies in trypanosomes (Cassola et al., 2007). There is no LSM1 gene in the genome, and the LSM1 to LSM7 complex is absent whereas the LSM2 to LSM8 splicing factor is present in the nucleus (Liu et al., 2004).

The most unexpected result from this work was the localisation of XRNA, but not DHH1 or SCD6, to a spot at the posterior cell pole in addition to the cytoplasm and P-bodies. This focus shared properties with P-bodies: cycloheximide caused the focus to dissolve and puromycin caused it to increase in size. In response to heat shock, this focus increased in size to become the largest focus of XRNA in the cell. It is not clear why XRNA is found there, nor why heat shock induces XRNA to further accumulate there. The posterior pole contains the

plus end of the cortical microtubules (Robinson et al., 1995) that define the trypanosome shape (Ngo et al., 1998) and comprise the vast majority of cytoplasmic microtubules (reviewed in Gull, 1999). In *Saccharomyces cerevisiae*, XRN1 can promote microtubule assembly in vitro and XRN1 mutants are hypersensitive to benomyl (Johnson, 1997; Page et al., 1998; Solinger et al., 1999); the location of XRNA at the posterior pole may reflect a distinct function to its role as an exoribonuclease (Li et al., 2006).

Heat-shock stress granules

In response to heat shock, all translation initiation factors tested (eIF4E1, eIF4E2, eIF4E3 and eIF4E4, eIF3B and eIF2A) and both poly(A)-binding proteins relocalised into cytoplasmic granules of uneven shape and size that remained largely separate from P-bodies (Fig. 8B). Orthologues of these components are also present in cytoplasmic stress granules described in mammals (Kedersha and Anderson, 2007). The heat-shock granules were preferentially localised to the periphery of the cell, close to the subpellicular array of microtubules (Sherwin and Gull, 1989; Vickerman and Preston, 1976); whether there is an interaction is not yet known.

Formation of stress granules also occurs in trypanosomes in response to metabolic stress brought by either azide/2-deoxyglucose treatment (Marchetti et al., 2000) or by starving cells in phosphate-buffered saline (Cassola et al., 2007). The granules resulting from metabolic stress and heat shock share components that are involved in initiation of translation but are different in two important ways: first, during carbon-source starvation, granules contained both translation initiation factors and the P-body components DHH1 and XRNA (Cassola et al., 2007), whereas upon heat shock they remained largely distinct. Second, metabolic stress granules contained mRNA that was stabilised (Cassola et al., 2007) in contrast to the decay that occurred over a shorter time scale on heat shock. The metabolic stress granules in trypanosomes resemble EGP-bodies (named for their protein constituents eIF4E, eIF4G and Pab1p) that appear in yeast in response to glucose starvation (Hoyle et al., 2007), with the exception that there was only partial colocalisation of EGP-body components and a P-body marker.

Induction of events

The earliest event detected in the response to heat shock was a decrease in polysomes, probably caused by a reduction in initiation of translation. Regulation of translation initiation by phosphorylation of eIF2α on a conserved serine residue (S51) is essential for most stress responses (Wek et al., 2006), including heat-shock response in mammalian cells (Doerwald et al., 2006; McEwen et al., 2005), and stress granules do not form in eIF2α/S51A mutants (McEwen et al., 2005). However, formation of stress granules can also be induced in mammalian cells by manipulation of the RNA helicase eIF4A (Dang et al., 2006; Mazroui et al., 2006). In yeast, the reduction in translation in response to amino-acid starvation is dependent on eIF2α phosphorylation, whereas the response to glucose starvation is not (Hoyle et al., 2007).

Trypanosome eIF2A has an N-terminal extension; T169 aligns with S51 and is phosphorylated by TbeIF2K2 in vitro (Moraes et al., 2007). A cell line dependent on a single eIF2A gene containing a T169A mutation was made and exhibited a normal heat-shock response. The pathway that causes the translational arrest required for the formation of heat-shock granules remains unknown and might be novel.

The heat-shock response in procyclic form trypanosomes

The effect of heat shock can thus be summarised as follows: the response was initiated at ~41°C, and within 15 minutes there was a decrease in polysomes and a reduction in the translation of most but not all mRNAs independent of phosphorylation of eIF2A on T169. The mechanism of suppressing translation during heat shock remains unclear. Within 30 minutes, heat-shock stress granules appear in the cytoplasm at the cell periphery, the number of P-bodies increases and the XRNA-containing focus at the posterior pole of the cell increases in size. The total mRNA in the cell decreases, so that by 1 hour the amount per cell has been halved. The loss is selective; some mRNAs are not affected. The decrease in mRNA is caused by both reduced synthesis and accelerated turnover. The appearance of heat-shock stress granules and increase in P-bodies are coincident with the alterations in mRNA metabolism, and it can be speculated that they provide different compartments (Fig. 10), each of which can act as a gateway for a particular fate. The mechanism that regulates mRNA partitioning is central to the understanding of how mRNA levels are regulated.

Materials and Methods

Trypanosomes

Trypanosoma brucei Lister 427 procyclic cells were used throughout. Either *T. brucei* Lister 427 pLEW29:pLEW13 (Wirtz et al., 1999), or *T. brucei* Lister 427 SIMP (Bill Wickstead, Sir William Dunn School of Pathology, Oxford, UK) or PTT (Philippe Bastin, Institute Pasteur Paris, France) were used for tetracycline-inducible expression. Cells were cultured in SDM-79 (Brun and Schonenberger, 1979). Transgenic trypanosomes were generated using standard procedures (McCulloch et al., 2004). All experiments used logarithmically growing trypanosomes.

Expression of eYFP- and mChFP-fusion proteins

Previously described methods and vectors were used (Kelly et al., 2007), details of constructs are shown in supplementary material Tables S3 and S4. Where appropriate, expression of transgenes was induced using 1 μg/ml tetracycline for 12-16 hours.

eIF2A deletion and mutation

A blasticidin-resistance ORF flanked by 316 nucleotides upstream and 975 nucleotides downstream of the *eIF2A* open reading frame was used to delete one *eIF2A* allele. The second allele was replaced by either mutated (T169A; ACG to GCG) or wild-type *eIF2A* by targeting the locus with a linearised plasmid consisting of the *eIF2A* open reading frame followed by a hybrid intergenic region derived from the pC-PTP-NEO plasmid (Schimanski et al., 2005) followed by a neomycin resistance cassette, followed by 975 nucleotides of the eIF2A 3′ UTR. Successful targeted insertion resulted in the *eIF2A* mRNA having a 3′ UTR derived from the *RPA1* gene.

Quantitative RNA analysis by northern blotting

RNA was prepared with the RNAeasy kit (Quiagen). Northern blotting was done as described (Webb et al., 2005). Probes for *PDI-2*, actin, mitochondrial *HSP70* isoforms (927.6.3740, 927.6.3750, 927.6.3800), *HSP70* isoform 927.7.710, *HSP83/90* (Tb10.26.1080) were full-length open reading frames. The *HSP70* isoform 11.01.3110 probe contained nucleotides 1140-2073 of the open reading frame. The tubulin probe contained one α-β repeat from the locus. Quantification was performed using a Molecular Dynamics Storm 840 and ImageQuant software. Individual bands were measured and the background (an equal area either above or below each band) substracted. Ribosomal RNA was used to measure loading. Total mRNA was measured using an oligonucleotide complementary to the mini-exon (5′-CAATATAGTACAGAAACTGTTCTAATAATAGCGTT-3′). Hybridisation was performed at 42°C and the blot was washed at room temperature in 4×SSC, 0.1% SDS. Experiments were performed at least twice and representative results from individual experiments are shown.

RNA extraction throughout this work is based on guanidinium-isothiocyanate solubilisation of the cells. In yeast, it has been reported that mRNAs become resistant to some RNA extraction methods when stationary-phase cells are exposed to stress (Aragon et al., 2006). RNA was prepared from heat-shocked trypanosomes by using either a monophasic solution of 1:1 (v/v) phenol and buffer containing 1% SDS at 80°C (Carrington, 1993) or the standard method. The two methods gave identical results.

Polysome analysis

5×10^8 trypanosomes (<1×10^7 cells/ml) were harvested at room temperature, washed in SDM79 without serum, resuspended in 360 μl polysome buffer [120 mM KCl, 2 mM $MgCl_2$, 20 mM Tris pH 7.5, 1 mM DTT, protease inhibitors (complete protease

inhibitor cocktail, Roche)] and lysed by adding 40 µl 10% n-octyl glycoside. The lysate was cleared by centrifugation at 16,000 ***g*** (4°C, 10 min), layered onto a 10-50% sucrose gradient (12 ml) prepared in polysome buffer, and centrifuged at 4°C for 2 hours at 36,000 rpm in a Beckman SW 40 rotor. Absorption at 254 nm was monitored using the UVICORD SII system. Both cycloheximide and puromycin were used at 50 µg/ml.

Metabolic labelling

5×10^6 cells were transferred into 500 µl methionine-free RPMI-1640 (R7513, Sigma) with 3.7 kBq/ml [^{35}S]methionine (>37 TBq/mmol) for 20 minutes. Cells were pelleted, washed and lysed in SDS-PAGE sample buffer. Samples were analysed by SDS-PAGE and autoradiography.

Microscopic Imaging

Cells were washed with SDM79 without serum and fixed at a density of 1×10^7 cells/ml with 2.4% paraformaldehyde overnight, washed once in PBS and stained with Hoechst H33258. Fluorescence microscopy was carried out using a Zeiss Axioskop microscope equipped with a Plan-Apochromat 100×/1.4 Oil DIC objective. Images were taken with the monochrome CCD camera AxioCam MR using AxioVision software (Zeiss). Confocal images were made with a BioRad Radiance 2100 on a Nikon Eclipse E800 upright microscope using a 100×/1.4 Oil DIC objective.

Microarrays

Procyclic cultures (~5×10^6 cells/ml) at 27°C were added to one volume SDM79 at 53°C and incubated at 41°C in a waterbath in a closed tube for 60 minutes. The control cells were added to one volume medium at 27°C and also incubated for 60 minutes in a closed tube at 27°C. Total RNA was prepared using the Qiagen RNAeasy Midi kit, including the DNAse digestion on the column. Two biological replicates were performed on different days. 10 µg of total RNA of each condition (41°C and 27°C) were labelled as previously described (Brems et al., 2005; Diehl et al., 2002; Luu et al., 2006). After checking dye incorporation using a fluorimeter (Nanodrop ND-1000 3.3), samples were mixed and hybridised onto the array (JCVI PFGRC *T. brucei* 37K v2) according to the M008 TIGR protocol. Five technical replicates were performed. Image acquisition and data analysis were performed as previously described (Diehl et al., 2002). The MCHiPS software package (Fellenberg et al., 2002; Fellenberg et al., 2001) was used for data normalisation and analysis. After normalising the original signal intensities by logarithmic regression, each individual signal was filtered for quality and reproducibility. Only spots with a fitted intensity of at least twice the standard deviation above background, a *P*-value $\leq$0.05 and a minimum-maximum separation value of 0.1 survived the filtering.

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