The Four Trypanosomatid eIF4E Homologues Fall into Two Separate Groups, with Distinct Features in Primary Sequence and Biological Properties

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Abstract

Translation initiation in eukaryotes requires eIF4E, the cap binding protein, which mediates its function through an interaction with the scaffolding protein eIF4G, as part of the eIF4F complex. In trypanosomatids, four eIF4E homologues have been described but the specific function of each is not well characterized. Here, we report a study of these proteins in *Trypanosoma brucei* (*Tb*EIF4E1 through 4). At the sequence level, they can be assigned to two groups; *Tb*EIF4E1 and 2, similar in size to metazoan eIF4E1; and *Tb*EIF4E3 and 4, with long N-terminal extensions. All are constitutively expressed, but whilst *Tb*EIF4E1 and 2 localise to both the nucleus and cytoplasm, *Tb*EIF4E3 and 4 are strictly cytoplasmic and are also more abundant. After knockdown through RNAi, *Tb*EIF4E3 was the only homologue confirmed to be essential for viability of the insect procyclic form. In contrast, *Tb*EIF4E1, 3 and 4 were all essential for the mammalian bloodstream form. Simultaneous RNAi knockdown of *Tb*EIF4E1 and 2 caused cessation of growth and death in procyclics, but with a delayed impact on translation, whilst knockdown of *Tb*EIF4E3 alone or a combined *Tb*EIF4E1 and 4 knockdown led to substantial translation inhibition which preceded cellular death by several days, at least. Only *Tb*EIF4E3 and 4 were found to interact with *T. brucei* eIF4G homologues; *Tb*EIF4E3 bound both *Tb*EIF4G3 and 4 whilst *Tb*EIF4E4 bound only to *Tb*EIF4G3. These results are consistent with *Tb*EIF4E3 and 4 having distinct but relevant roles in initiation of protein synthesis.

Keywords

translation; eIF4E; eIF4G; Trypanosomatid; *Leishmania*

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1   Introduction

The trypanosomatids, including the various *Leishmania* and *Trypanosoma* species, are pathogenic protozoans well known not only for the diseases they cause, but also for their complex biology and the unusual molecular mechanisms required for their gene expression. Transcription of protein coding genes occurs polycistronically [1, 2] and processing to monocistronic mRNAs occurs through coupled *trans*-splicing and polyadenylation (reviewed in [3]). The result is that mRNAs have a common 39nt long spliced-leader (or mini-exon) sequence at the distal end of the 5' UTR, which is identical for all mRNAs of a given species. At the 5' end of the spliced-leader sequence lies the 7-methyl-GTP cap nucleotide (m^7G), followed by four methylated nucleotides in the spliced-leader sequence, the cap4 structure [4]. Regulation of gene expression in trypanosomatids is accomplished mainly through post-transcriptional mechanisms such as control of mRNA stability and possibly translation (for reviews see [5–7]). However, in contrast to the considerable characterization of their mechanisms of mRNA synthesis and processing, relatively little is understood regarding protein synthesis and how it can be regulated.

Translation initiation is the most complex stage of protein synthesis and the one which can vary more significantly between different taxonomic groups [8]. A major player in this process in eukaryotes, and also a major target for translation control, is eIF4E (eukaryotic initiation factor 4E), the m^7G cap binding protein. eIF4E is a small polypeptide (24-25 kDa in mammals) which, apart from protein synthesis, has been implicated in a number of processes involved in mRNA metabolism such as transport and control of its stability (reviewed in [9–12]). In translation it is part of the heterotrimeric complex eIF4F, which also includes the RNA helicase eIF4A and the large scaffolding protein eIF4G [13]. eIF4F facilitates the recruitment of the small ribosomal subunit to the mRNA, which is accomplished with the help of another translation initiation complex, eIF3. eIF4E mediates the binding of the complex to the mRNA and, as part of eIF4F, can bind simultaneously to both the cap and eIF4G, which can then mediate interactions with other translation factors [14–16]. Despite its well described function in translation in the cytoplasm, substantial levels of eIF4E localize to the nucleus (reviewed in [17]), where it promotes the nuclear export of selected mRNAs [10, 18, 19].

The structure of eIF4E bound to 7-methyl-GDP [20, 21] is shaped like a cupped hand, with the cap analogue located in a narrow cap-binding slot on the concave side of the protein. eIF4E is characterized by eight tryptophan residues located at conserved positions along the protein. Cap recognition is mediated by, among other interactions, base sandwich-stacking between W56 and W102 (mammalian eIF4E-1 numbering) while W73 is involved in the interaction with eIF4G [9, 13, 20]. The activity of eIF4E can be regulated in a number of ways such as through the action of eIF4E interacting proteins, eIF4E-BPs, or directly through phosphorylation (reviewed in [22–25]).

In the yeast *Saccharomyces cerevisae* only one eIF4E homologue, essential for viability, is found [26]. More usually, multiple forms of this protein are present in any specific organism (reviewed by [27]). In mammals, three different classes of eIF4E have been described: eIF4E-1, the prototypical cap-binding protein; eIF4E-2 (also known as 4EHP), which binds

the cap less well, does not interact with eIF4G and has a regulatory role [28, 29]; and eIF4E-3, which binds both cap and eIF4G but has a restricted, tissue specific, expression pattern [30]. An extensive analysis of hundreds of eIF4E-related sequences has indicated that different events of duplication of the eIF4E gene may have occurred in distinct eukaryotic groups after divergence, resulting in new forms of the protein with novel or modified functions [31].

In trypanosomatids, the initial description of a single putative eIF4E homologue [32] was followed by the identification of four homologues in genome sequences from *L. major*, all of which are also conserved in *Trypanosoma* species [33]. The four *Leishmania* eIF4E homologues (*Lm*EIF4E1 through 4, LeishIF4E1 through 4 or simply EIF4E1 through 4) were shown to diverge in binding affinity for different synthetic cap analogues, in expression levels and in polysomal association in sucrose sedimentation gradients. None could rescue the growth of a yeast strain lacking a functional eIF4E and none could confidently be implicated as having a predominant role in translation or its control [33, 34].

Here we describe work focused on the four *T. brucei* proteins (here named *Tb*EIF4E1 through 4). First, sequence analysis identified features which distinguish *Tb*EIF4E1 and 2 from *Tb*EIF4E3 and 4. For all four homologues, the cap-binding affinities, subcellular localization, and the expression in two stages of the *T. brucei* life cycle were characterised. The effect of depletion, through RNAi, on cellular growth and translation was determined and the interaction between putative eIF4E/eIF4G homologues was also analysed. The results presented allow the separation of the four proteins into two distinct groups. The first comprises *Tb*EIF4E1 and 2, which localise both to the nucleus and the cytoplasm, do not seem to be directly involved in translation but perform functions essential for cellular viability. The second group is formed by the *Tb*EIF4E3 and 4 homologues, more abundant, strictly cytoplasmic proteins which seem to be required for translation and take part in the formation of distinct eIF4F-like complexes.

2   Materials and methods

2.1   Sequence analysis

BLAST searches were carried out with the *T. brucei* genome sequences available at the Gene DB website of the Sanger Institute Pathogen Sequencing Unit (www.genedb.org). Further sequence searches and Clustal W alignments were done as previously described [33]. Relevant trypanosomatid GeneDB accessions: *Tb*EIF4E1 – **Tb11.18.0004**; *Tb*EIF4E2 – **Tb927.10.16070**; *Tb*EIF4E3 – **Tb11.01.3630**; *Tb*EIF4E4 –**Tb927.6.1870**; *Lm* (*L. major*) EIF4E1 – **LmjF27.1620**; *Lm*EIF4E2 – **LmjF19.1500**; *Lm*EIF4E3 – **LmjF28.2500**; *Lm*EIF4E4 – **LmjF30.0450** (the annotated sequence for *Lm*EIF4E4 is missing 139 residues from what we believe is the N-terminus of the protein – they are encoded upstream of the annotated open reading frames and are homologous to the equivalent segment in the *T. brucei* orthologue).

2.2 PCR and cloning methods

All *T. brucei* eIF4E and eIF4G coding sequences were amplified from Lister 427 total genomic DNA and first cloned into the pGEM-T Easy vector (Promega). All amplified fragments were first sequenced, and the resulting sequences compared with those from the *T. brucei* genome sequencing project, prior to their use in the subcloning reactions. In order to express N-terminal His-tagged fusion proteins the *Tb*EIF4E1 through 4 full length sequences were subcloned into the modified pET15b vector as described previously for the *T. brucei* eIF4A homologues [35]. Alternatively, to generate the eYFP and HA-tagged fusions and for the RNAi experiments, the same fragments were cloned respectively into the transfection vectors p2216 [35,36], p2477 [36] or p2T7-177 [37]. To generate the plasmids used in the transcription and translation reactions they were also subcloned into the pGEM3zf+ plasmid (Promega). To express the full length proteins as N-terminal GST tagged fusions, the *Tb*EIF4G3 and 4 sequences were subcloned into the pGEX4T3 plasmid (GE Healthcare). His-tagged *Tb*EIF4G3 and 4 were also expressed, with a C-terminal tag, as fragments consisting of the proteins' N-terminus plus the central MIF4G/HEAT domain (*Tb*EIF4G3$_{1-228}$ and *Tb*EIF4G4$_{1-319}$ – numbers indicating the residues from the wild type protein remaining in the recombinant product) using the pET21a vector (Novagen). Supplementary TABLE I lists all oligonucleotides used in the amplifications reactions as well as the strategies and restrictions enzymes used in the subsequent subcloning events cited above. For the double *Tb*EIF4E1/2 construct, for the RNAi experiments, the *Tb*EIF4E2 gene was reamplified flanked by sites for *Bgl* II and *Bam*H I and cloned into the dephosphorylated *Bam*H I site of the p2T7-177-*Tb*EIF4E1 construct. For the double *Tb*EIF4E1/4 construct, first the p2T7-177-*Tb*EIF4E1 was digested with *Bam*H I, which cuts internally to the *Tb*EIFE1 gene and at its 3' end at the site introduced after the original PCR reaction, releasing a ~360bp fragment consisting of the second half of the target gene. This fragment was then cloned into the linearised *Bam*H I site of the p2T7-177-*Tb*EIF4E4 construct in a similar procedure as described for *Tb*EIF4E1/2. Recombinant protein expression was performed according to standard procedures using the pET15, pET21 or pGEX4T3 derived constructs after transformation into *Escherichia coli*.

2.3 Cap binding assay

The four [^{35}S]- methionine labeled *Tb*EIF4Es were synthesized after *in vitro* transcription of their respective genes cloned in the pGEM3zf+ vector, following linearization with *Xba* I (*Tb*EIF4E1 and 3) or *Bam*H I (*Tb*EIF4E2 and 4), with T7 RNA polymerase followed by translation with the nuclease treated Rabbit Reticulocyte Lysate System (Promega). For the *Lm*EIF4E4 homologue, its gene was amplified from *L. major* (MHOM/IL/81/Friedlin) genomic DNA flanked by sites for *Afl* III and *Not* I (5' primer - CT GAC ATG TCT ACC CCT CTC GAT GTG; 3' primer - TA TGC GGC CGC GTA GCG ACG ACG GTT CTT TTT C) and cloned into the *Nco* I/*Not* I sites of pET21D (Novagen). The resulting plasmid was sequenced and linearized with *Not* I prior to transcription and translation, as described above. Assays were performed essentially as described previously [33], with non-specific binding removed by washes with 0.1 mM GTP and specific elution achieved with 50 µM cap analogue. As positive control, the labeled *Xenopus laevis* eIF4E cDNA was used.

2.4 Parasite growth, transfection and RNA interference

Cultivation of procyclic and bloodstream forms of *T. brucei*, Lister 427, as well as the procyclic cell line used for the ectopic expression and RNAi experiments, Lister 427 29-13 [38], were performed as previously described [35]. Cultures grown to mid-log phase (10^6 – 10^7 cells/ml for procyclic and 10^5 – 10^6 for bloodstream forms) were harvested for the production of total protein extract. For the RNAi of the bloodstream forms, Lister 427 90-13 was used [38], grown in the presence of 5 μg/ml of G418 and hygromycin. Transfection procedures were performed using standard conditions with selection of stable DNA integration being carried out using phleomycin (2.5 μg/ml for procyclic and 0.5 μg/ml for bloodstream cells). For the RNAi experiments, and induction of eYFP and HA-tagged fusion proteins, 1 μg/ml of tetracycline was added to mid-log phase cultures of transfected cells.

For metabolic labeling, methionine-free SDM-79 medium was used. Cultures were washed once in the same medium prior to being resuspended to a concentration of $1x10^7$ cells/ml in the medium supplemented with 50 μCi/ml [^{35}S]- methionine and incubated at 28°C. Aliquots were then taken to measure the trichloroacetic acid precipitable incorporation into protein and for analysis through SDS-PAGE and autoradiography.

2.5 RNA and protein analysis

RNA extraction and Northern blots were performed using standard methods [35, 39, 40]. DNA fragments containing complete open reading frames were used as probes for *Tb*EIF4E1 through 4. Polyclonal serum against *Tb*EIF4E1 through 4 and *Tb*EIF4G3 and 4 were produced in rabbits using the corresponding recombinant His-tagged proteins. The different sets of antibodies were affinity purified and used in western blotting assays with the respective recombinant proteins and different samples of total protein extract from both procyclic and bloodstream forms of *T. brucei*. The western-blots were generally developed by ECL.

2.6 Fluorescence microscopy

For the indirect immunofluorescence assay, wild type procyclic cells grown to mid-log phase were harvested and washed with SDM-79 minus serum. The cells were fixed at a density of $5x10^6$/ml with 3% paraformaldehyde, washed once in PBS and adhered to poly-L-lysine coated slides. Permeabilization was carried out with 0.1% Triton X-100 followed by blocking with 1% BSA. Antibody detection of the eIF4E homologues followed standard procedures using the various rabbit anti-sera and goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen – Molecular Probes, USA). For the eYFP fusions, after induction with tetracycline, over-expressing transfected cells were directly observed in 35 mm culture plates. All the samples were visualized using a Leica SPII-AOBS confocal microscope.

2.7 Pull-down assays

Pull-down assays were essentially performed as described previously [33] using Glutathione-Sepharose beads (GE Healthcare). Beads alone, GST or GST-tagged *Tb*EIF4G3 and 4 were immobilized on the beads and assayed for their ability to bind to [^{35}S]-labelled

*Tb*EIF4E1 through 4, obtained after transcription and translation of the full-length genes cloned in the pGEM3zf+ plasmid.

2.8 Immunoprecipitation assays

Immunoprecipitations (IPs) were carried out with *T. brucei* cytoplasmic extracts produced from exponentially grown procyclic cells lysed in lysis buffer (20 mM Hepes-KOH, pH7.4, 75 mM potassium acetate, 4 mM magnesium acetate, 2 mM DTT) at a concentration of $2x10^8$ cells/ml. IPs were carried out at 4°C using standard procedures. For the assays with the anti-*Tb*EIF4E3 and 4 sera, protein-A Sepharose beads (GE Healthcare) were used with the affinity purified antibodies, or the corresponding total pre-immune sera as controls and cytoplasmic extracts from wild type cells. For the IPs of the HA-tagged proteins, cytoplasmic extracts from transfected cells expressing each recombinant protein were directly incubated with the Monoclonal Anti-HA Agarose Conjugate (Sigma). In both instances, proteins were eluted in SDS-PAGE samples buffer and assayed in western blots with affinity purified antibodies directed against the different *T. brucei* eIF4E, eIF4G or eIF4A homologues, as described in the text. For the Western blots of the IPs using the anti-*Tb*EIF4E3 and 4 sera, proteins transferred to the PVDF membranes were stained with Rouge Ponceau and the band corresponding to the heavy weight chain of IgG from the immunoprecipitated complexes was cut out prior to blocking and incubation with the primary and secondary antibodies.

3 Results

3.1 The Trypanosoma eIF4E homologues fall in two distinct sequence groups

Based on the alignment shown in Figure 1, which compares the *T. brucei* and *L. major* eIF4E sequences with representative homologues from different eukaryotic lineages, including metazoans, plants, fungi and unrelated protozoans, the four trypanosomatid sequences can be broadly classified into two different groups. Group 1 comprises the EIF4E1 and 2 sequences, which are more similar in size to the human and yeast sequences, whilst group 2 includes EIF4E3 and 4, which share a few common features absent from the remaining proteins. These include a long N-terminus of more than 150 amino acids which share extensive homology between different orthologues of say, the EIF4E3 sequences, and also contain short segments of limited homology which seem to be conserved between the EIF4E3 and 4 sequences. Of the eight conserved tryptophan residues typical of eIF4E sequences, most are either conserved in the various trypanosomatid homologues or are replaced by other aromatic residues. The only exception is W112 (human eIF4E numbering), present in the EIF4E1 and 2 sequences but which is replaced by non-aromatic hydrophilic residues in EIF4E3 and 4. Additional noteworthy substitutions in the trypanosomatid sequences are D104, next to the near universally conserved W102E103, involved in cap binding [20] and conserved in all sequences shown but which is replaced by a histidine in EIF4E2 and 3; and V69E70, implicated in binding to eIF4G [41] but which is missing in the EIF4E4 homologues.

3.2 *Tb*EIF4E1, 2 and 4, but not *Tb*EIF4E3, can efficiently bind the m⁷G cap

To start the study of the *Tb*EIF4E proteins, their genes were first cloned and the respective [^{35}S]-labeled proteins, synthesized *in vitro*, assayed as to their ability to bind to the commercially available m^{7}G cap bound to Sepharose beads. Despite the significant differences between the more complex cap4 from trypanosomatids and the m^{7}G cap from their metazoan hosts, this assay is useful to compare the substrate binding properties of the various homologues. *Tb*EIF4E1, 2 and 4 were all found to be able to bind to the beads, and were specifically eluted with the soluble cap analogue, whilst *Tb*EIF4E3 did not show any binding (Figure 2). A similar approach has been previously carried out for the first three *L. major* eIF4E homologues [33], where neither *Lm*EIF4E2 nor *Lm*EIF4E3 bound to the cap-beads, while *Lm*EIF4E1 bound efficiently. Here, *L. major Lm*EIF4E4 was also assayed and bound efficiently to the resin, as its *T. brucei* orthologue. The sole difference between the two organisms then lies with the EIF4E2 orthologues whose sequences diverge by the presence of a unique insertion in the *Leishmania* protein, found near its C-terminus, which is absent from its *Trypanosoma* counterpart (Figure 1).

3.3 All four eIF4E homologues are expressed in both procyclic and bloodstream forms

The four *T. brucei* eIF4Es were expressed as His-tagged recombinant proteins in *Escherichia coli* and used to raise specific polyclonal antisera in rabbits (Supplementary Figure 1). Cloned DNAs and sera were then used in Northern and Western blots to assay for expression of each gene in exponentially growing procyclic and bloodstream cells. All four mRNAs were expressed with little variation in levels between the two stages (Supplementary Figure 2) and the same applied for the corresponding proteins (Figure 3). The Western blot results were then quantitated and used to produce an estimate of the copy number of each protein (summarized in Table 1). *Tb*EIF4E3 is by far the most abundant protein, especially in procyclic cells (>5x10^4 molecules per cell), followed by *Tb*EIF4E4, which also is present at levels above 1x10^4 molecules per cell. In contrast, both *Tb*EIF4E1 and 2 are present at much lower levels (compatible with the Northern results for *Tb*EIF4E2 where its mRNA required a longer exposure than the remaining homologues in order to be detected). For most proteins the levels observed are slightly reduced in bloodstream forms, when compared with procyclics, which is consistent with the smaller cell volume. With the exception of EIF4E4, which was not previously quantified, the values observed for the *T. brucei* proteins are equivalent to those obtained for the *L. major* orthologues (Table 1 and reference [33]), presumably a reflection of conserved function.

3.4 *Tb*EIF4E1 and 2 are found in both cytoplasm and nuclei, whereas *Tb*EIF4E3 and 4 are confined to the cytoplasm

Immunofluorescence experiments were carried out for *Tb*EIF4E proteins in procyclic cells. *Tb*EIF4E3 and 4 have a very similar localization and both are found throughout the cytoplasm with very little or no localization to the nucleus (Figure 4A and Supplementary Figure 3). In contrast, *Tb*EIF4E1 and 2 can be clearly detected both in the cytoplasm as well as in the nucleus in the majority of cells. These results were confirmed in over-expression experiments using EIF4E-enhanced yellow fluorescent protein (eYFP) fusions in transgenic procyclic *T. brucei* cells (Figure 4B). *Tb*EIF4E3 and 4 again were found confined to the

cytoplasm, whilst *Tb*EIF4E1 and 2 were found in the cytoplasm and in the nucleus, in good agreement with previously reported results [42].

3.5 RNAi of *Tb*EIF4E3, but not the remaining eIF4E homologues, in procyclic forms prevents cell viability whilst RNAi of *Tb*EIF4E1, 3 and 4 in bloodstream forms impacts on cell proliferation and survival

The requirement for the various eIF4E homologues for cellular viability was then investigated in *T. brucei* procyclic and bloodstream forms by knockdown of their expression through RNA interference. Representative growth curves of the various knockdowns for procyclic cells are shown in Figure 5. The phenotype of cells after RNAi ablation of *Tb*EIF4E1, 2 and 4 (Figures 5A, 5B and 5D) was similar and no significant impact on cell survival was observed after knockdown of any of the three proteins. For *Tb*EIF4E1, a reduction in the rate of cellular growth was observed four days after addition of tetracycline, but the cells remained viable and cell numbers kept increasing. Western blotting, shown below each curve, confirmed major depletions of the respective proteins (over 90% depletion for *Tb*EIF4E1 and 4; 70 to 80% depletion for *Tb*EIF4E2) within two to three days of RNAi induction, with no effect observed on the levels of the endogenous loading control, the *T. brucei* homologue of eIF4AI, *Tb*EIF4AI. Knockdown of *Tb*EIF4E3 produced a very distinct phenotype, since proliferation stopped two days after RNAi induction, followed by subsequent cell death (Figure 5C). The western-blotting confirmed substantial reduction (over 90%) in levels of *Tb*EIF4E3 within 48 hours of tetracycline addition whilst the control, *Tb*EIF4AI, remained unaffected. For both *Tb*EIF4E3 and 4 knockdowns, western-blots were also carried out to see if depletion of *Tb*EIF4E3 had any impact on *Tb*EIF4E4 and vice-versa. Whilst no effect was observed on the levels of *Tb*EIF4E4 after depletion of *Tb*EIF4E3, knockdown of *Tb*EIF4E4 seemed to provoke a slight increase on the levels of *Tb*EIF4E3 (Figures 5C and 5D). Two double RNAi experiments were also performed to test for redundancy: *Tb*EIF4E1 and 2, as these two are resident in the nucleus, and *Tb*EIF4E1 and 4, as their *L. major* orthologues are both reported to interact with an eIF4G homologue [43]. When the simultaneous knockdown of *Tb*EIF4E1 and 2 was performed, cells ceased proliferation and died (Figure 5E), with cellular proliferation stopping within four days of tetracycline induction. A distinct phenotype was observed by the simultaneous knockdown of *Tb*EIF4E1 and 4 (Figure 5F). Although cellular proliferation stopped after RNAi induction, at about the same timeframe as the *Tb*EIF4E1/2 RNAi, cell death did not occur for more than 15 days onwards (data after day 7 not shown).

The RNAi procedure was then repeated with transfected bloodstream cells. Knockdown of *Tb*EIF4E3 led to a cessation of proliferation around 24 h after induction followed by cell death (Figure 6C). Knockdown of *Tb*EIF4E1 or 4 also caused the cells to stop growing between 24 and 48 hours after induction (Figures 6A and 6D). Cell death ensued for the *Tb*EIF4E4 RNAi cells, whilst the cells depleted of *Tb*EIF4E1 stopped growing but did not subsequently die, a phenotype which resembles what was observed for the double knockdown of *Tb*EIF4E1/4 in procyclic cells. As for *Tb*EIF4E2, no effect was observed in cellular growth after induction of the RNAi procedure (Figure 6B), as seen for procyclic cells. Western-blotting of selected samples confirmed the efficient depletion of the four proteins within their corresponding knockdown experiments (over 90% depletion for

*Tb*EIF4E1 and 4; 70 to 80% depletion for *Tb*EIF4E2 and 3). As observed for procyclic cells, knock-down of *Tb*EIF4E4 also seemed to provoke a slight increase on the levels of *Tb*EIF4E3.

3.6 *Tb*EIF4E3 and *Tb*EIF4E1/4 RNAi directly affects protein synthesis

To investigate if the no-growth or cellular death phenotypes observed after the knock-down of *Tb*EIF4E3 or the double constructs *Tb*EIF4E1/2 and *Tb*EIF4E1/4 was due to an inhibition of protein synthesis or to some unrelated process, we performed metabolic labeling with ^{35}S-methionine at selected time points after tetracycline addition for the respective RNAi curves. Incorporation of labeled methionine was monitored and the results obtained were compared with those derived from the control curves without tetracycline. These values were used to calculate the total rate of protein synthesis between the two curves (plus and minus tetracycline) and estimate the effect on protein synthesis of the knockdown of the various proteins. As shown in Figure 7A at three days after RNAi induction, no effect on protein synthesis is observed for the *Tb*EIF4E1/2 knockdown whilst depletion of *Tb*EIF4E3 and *Tb*EIF4E1/4 had significantly impaired translation. These results are consistent with both *Tb*EIF4E3 and *Tb*EIF4E1/4 knockdowns directly affecting protein synthesis since substantial translation inhibition preceded cell death for a minimum of three days for the *Tb*EIF4E3 RNAi or for much longer in the case of the *Tb*EIF4E1/4 knockdown. Nevertheless, in both cases no substantial changes in the profile of proteins synthesized were detected even in conditions of severe protein inhibition (Figure 7B). In contrast, for the *Tb*EIF4E1/2 knockdown any inhibition of translation is only observed late in the RNAi curve, very near to the time of cell death. Although this does not rule out a direct role in protein synthesis as well, the inhibition observed could more likely be an indirect consequence of the various metabolic processes associated with cell death induced by the RNAi.

3.7 *Tb*EIF4E3 interacts with *Tb*EIF4G3 and 4, while *Tb*EIF4E4 binds *Tb*EIF4G3

Since eIF4E participates in translation within the context of the eIF4F complex, bound to the eIF4G subunit, to investigate more directly the role of the four *T. brucei* eIF4Es in translation we opted to study their interaction with selected trypanosome eIF4G homologues. Of the five eIF4G homologues conserved in trypanosomatid species, only two (EIF4G3 and 4) share conserved features outside the HEAT domain, such as a short N-terminus, containing limited segments of similarity, and regions of homology on the C-terminal half. Furthermore, the *L. major* EIF4G3 orthologue has been shown to be able to bind to EIF4AI [33] as well as to orthologues of *Tb*EIF4E1 and 4 [43], therefore confirming a potential role in translation. Here, to investigate possible interactions between the eIF4G and eIF4E homologues *in vitro*, the genes encoding *Tb*EIF4G3 and 4 were cloned and expressed as N-terminal fusions with glutathione-S sepharose (GST). The resulting proteins, and GST alone used as negative control, were used in pull-down assays to analyze binding to the four [^{35}S]-labeled *T. brucei* eIF4E homologues. GST-*Tb*EIF4G3 specifically and efficiently bound to labeled *Tb*EIF4E4, whilst GST-*Tb*EIF4G4 bound to *Tb*EIF4E3 (Figure 8A). Minor bindings were also observed for both *Tb*EIF4E1 and 2 with the two eIF4G homologues and between *Tb*EIF4E3 and GST-*Tb*EIF4G3.

To investigate if the same interactions occurred *in vivo* we used *Tb*EIF4E3 and 4 antibodies in immunoprecipitation reactions with *T. brucei* cytoplasmic extracts. Immunoprecipitation of *Tb*EIF4E3 was first carried out, with its presence in the immunoprecipitate as well as its depletion from the non-bound supernatant assessed by western- blotting with the anti-*Tb*EIF4E3 antibody (Figure 8B). The same blot was then re-probed with antibodies raised against *Tb*EIF4G3 and 4. All *Tb*EIF4G4, and to a lesser extent *Tb*EIF4G3, co-precipitated with *Tb*EIF4E3. Immunoprecipitation of *Tb*EIF4E4 was performed likewise followed by western-blotting with its own antibody as well as the one directed against *Tb*EIF4G3. All *Tb*EIF4E4 was brought down by the procedure and some, but not all, *Tb*EIF4G3 co-precipitated as well (Figure 8C). To compare the binding of *Tb*EIF4E1, 3 and 4 with the same eIF4G homologues *in vivo* under equivalent conditions, we expressed HA-tagged versions of these proteins in *T. brucei* cells followed by immunoprecipitation of the resulting cellular extracts with anti-HA antibody and western blotting (Figure 8D and E). In agreement with the pull down data above, *Tb*EIF4G3 co-precipitated with both HA-tagged *Tb*EIF4E3 and 4, but no co-precipitation with *Tb*EIF4E1 was observed. In contrast, *Tb*EIF4G4 only co-precipitated with *Tb*EIF4E3. To confirm that functional eIF4F complexes were being formed, the blots were also incubated with anti-*Tb*EIF4AI anti-sera. Both *Tb*EIF4E3 and 4 were able to bring down the eIF4AI homologue, but no interaction was observed with *Tb*EIF4E1 (Figure 8D and E). These binding results then confirmed specific interactions of *Tb*EIF4E3 with both *Tb*EIF4G3 and 4 and of *Tb*EIF4E4 with *Tb*EIF4G3, and highlight their potential to reconstitute three functional trypanosomatid eIF4F complexes.

4   Discussion

In cells with multiple eIF4E homologues, it has been proposed that only one acts as a general initiation factor functioning during the translation of the majority of the mRNAs, whilst the remainder perform more specialized functions, related or not, to protein synthesis [27, 44]. Most of the evidence for this proposal comes from multicellular organisms whereas the need for distinctions in the translation apparatus between different tissues or during development may be the driving force behind the evolution of various eIF4E homologues. In unicellular eukaryotes this may not apply although one initial aspect investigated here was the possibility that different eIF4E homologues would be differentially expressed during distinct life stages of the trypanosomatid life cycle, as indeed has been proposed for *Leishmania* EIF4E1 [34]. Overall the data presented rule out large differences in expression for the four eIF4E homologues between the two stages of the *T. brucei* cycle, consistent with the different homologues acting on different aspects of mRNA metabolism or on different mRNA populations, whilst reinforcing differences in abundance previously observed for the *L. major* proteins, with EIF4E3, and also EIF4E4, being present at significantly higher levels than EIF4E1 and 2. The distinct RNAi phenotypes in procyclic and bloodstream cells might suggest differences in the translation machinery, but the differential sensibilities of the two stages to the knockdowns could also be a consequence of their different metabolic rates, with bloodstream cells, which grow much faster, being most sensitive to changes in translation rate.

The localization data suggests that *Tb*EIF4E3 and 4 do not function in the nucleus though *Tb*EIF4E1 and 2 may have nuclear roles. In mammals, at least, nuclear eIF4E localize to speckles which are also the sites where splicing factors are found [45]. This localization pattern differs from what was seen for the endogenous or the over-expressed *Tb*EIF4E1 and 2 which are spread throughout the nucleoplasm. The RNAi data does implicate both *Tb*EIF4E1 and 2 as having important and possibly redundant roles for cellular survival, which could be associated with their nuclear localization, although the possibility remains that the two proteins participate in independent processes which, upon simultaneous inhibition by the double RNAi procedures, result in sufficient overall damage to produce the phenotype observed (the same applies to the *Tb*EIF4E1/4 knockdown discussed below). So far EIF4E2 seems to be the least conserved of the four trypanosomatid eIF4E homologues studied. Although *Tb*EIF4E2 binds to the m^{7}G Sepharose in a similar manner to *Tb*EIF4E1 and 4, *Leishmania Lm*EIF4E2 does not bind this cap [33], but rather, preferentially binds the methylated cap 4 [34]. This difference, plus the existence of insertions in the *Leishmania* EIF4E2 which are missing from its *T. brucei* or *T. cruzi* orthologues, or even the human and yeast sequences, imply a divergence in function unique to the *Leishmania* protein.

In *L. major*, both EIF4E1 and 4 bind the cap4 structure, physically interact with EIF4G3 and all co-migrate with polysomes in sucrose gradients, strongly implicating these proteins in translation [34, 43]. *Leishmania* EIF4G3 also binds efficiently to the eIF4A homologue involved in translation [33, 35] and *T. brucei* EIF4G3 acts in protein synthesis, since its depletion rapidly blocks translation (D. Moura, unpublished data). Both *Tb*EIF4E1 and 4 are required for growth of the bloodstream cells although the lack of a phenotype after knockdown in procyclics does not rule out a minor requirement for them in levels below the threshold produced after RNAi (considering the efficiency of the RNAi procedure, any requirements are nevertheless well below their original intracellular levels). Here we have confirmed, by both *in vitro* and *in vivo* approaches, the interaction between the EIF4G3 and EIF4E4 in *T. brucei* which clearly implicates the latter in protein synthesis. However, contrary to a previously reported interaction [43], neither *in vitro* (with *L. major* and *T. brucei*) nor *in vivo* (*T. brucei*) assays detected any interaction between EIF4G3 and EIF4E1 (this work and unpublished data). This discrepancy remains unresolved but it is nevertheless possible that in Figure 8D minor amounts of *Tb*EIF4G3 co-precipitated with HA-tagged *Tb*EIF4E1 but was not detected through the western-blotting procedure.

The similarities observed between *Tb*EIF4E3 and 4 at the sequence level, their similar subcellular localization, abundance and their ability to bind to eIF4G partners are consistent with both performing related roles as part of distinct eIF4F complexes. So far, the existence of multiple eIF4F complexes, with distinct eIF4E and eIF4G subunits and within the same organism, has only been reported from plants where they were named eIF4F and eIF(iso)4F [46]. These two complexes can co-exist and differ in their ability to translate different classes of mRNAs [47, 48], raising the possibility that something similar may be happening with the trypanosomatid proteins. A potential role for phosphorylation in regulating their activity is also possible since *Tb*EIF4E3 has been identified as a phosphoprotein [49] and *Tb*EIF4E4 migrates in gels with a pattern indicative of post-translational modifications. Indeed the phosphorylated residues in *Tb*EIF4E3 are located next to one of the two blocs of conserved residues unique to the N-terminus of both EIF4E3 and 4 sequences (see Figure 1).

EIF4E3, the most abundant *Trypanosoma* and *Leishmania* eIF4E, is the only confirmed essential homologue in procyclic and bloodstream *T. brucei*. Nevertheless, when compared with EIF4E4 in *L. major*, it binds much less efficiently to the trypanosomatid cap4 [34]. Moreover, *L. major* EIF4E3 does not seem to associate with polysomes [34], although this does not necessarily exclude a role in translation since the majority of mammalian eIF4E is not bound to ribosomes either [50]. To date, eIF4F-like complexes participate only in translation, so it seems plausible to suggest then that both EIF4E3 and 4 participate in protein synthesis; however their distinct cap binding affinities may indicate distinct modes of mRNA recognition leading to binding to different target mRNAs. It is possible that EIF4E3 may only bind to mRNAs in the context of an eIF4F complex or when associated with some other RNA binding protein. In metazoans the eIF4E-2 homologue (4EHP), involved in the selective translation repression of target messages, is recruited to selected mRNAs by binding proteins associated with their 3'UTRs [28, 29, 51]. This protein binds very weakly to the cap [52], so it cannot compete with eIF4E-1 in the absence of its co-factors. It is plausible to suggest then that, despite its abundance, for the parasite EIF4E3 to bind to the message RNAs it needs some other protein partner which can mediate selectivity in mRNA binding with a direct consequence on translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

References

[1]. Johnson PJ, Kooter JM, Borst P. Inactivation of transcription by UV irradiation of *T. brucei* provides evidence for a multicistronic transcription unit including a VSG gene. Cell. 1987; 51:273–81. [PubMed: 3664637]

[2]. Martinez-Calvillo S, Yan S, Nguyen D, et al. Transcription of *Leishmania major* Friedlin chromosome 1 initiates in both directions within a single region. Mol Cell. 2003; 11:1291–9. [PubMed: 12769852]

[3]. Liang XH, Haritan A, Uliel S, et al. *trans* and *cis* splicing in trypanosomatids: mechanism, factors, and regulation. Eukaryot Cell. 2003; 2:830–40. [PubMed: 14555465]

[4]. Bangs JD, Crain PF, Hashizume T, et al. Mass spectrometry of mRNA cap 4 from trypanosomatids reveals two novel nucleosides. J Biol Chem. 1992; 267:9805–15. [PubMed: 1349605]

[5]. Clayton CE. Life without transcriptional control? From fly to man and back again. EMBO J. 2002; 21:1881–8. [PubMed: 11953307]

[6]. Clayton C, Shapira M. Post-transcriptional regulation of gene expression in trypanosomes and leishmanias. Mol Biochem Parasitol. 2007; 156:93–101. [PubMed: 17765983]

[7]. Haile S, Papadopoulou B. Developmental regulation of gene expression in trypanosomatid parasitic protozoa. Curr Opin Microbiol. 2007; 10:569–77. [PubMed: 18177626]

[8]. Marintchev A, Wagner G. Translation initiation: structures, mechanisms and evolution. Q Rev Biophys. 2004; 37:197–284. [PubMed: 16194295]

[9]. von der Haar T, Gross JD, Wagner G, et al. The mRNA cap-binding protein eIF4E in post-transcriptional gene expression. Nat Struct Mol Biol. 2004; 11:503–11. [PubMed: 15164008]

[10]. Culjkovic B, Topisirovic I, Borden KL. Controlling gene expression through RNA regulons: the role of the eukaryotic translation initiation factor eIF4E. Cell Cycle. 2007; 6:65–9. [PubMed: 17245113]

[11]. Sonenberg N. eIF4E, the mRNA cap-binding protein: from basic discovery to translational research. Biochem Cell Biol. 2008; 86:178–83. [PubMed: 18443631]

[12]. Goodfellow IG, Roberts LO. Eukaryotic initiation factor 4E. Int J Biochem Cell Biol. 2008; 40:2675–80. [PubMed: 18069043]

[13]. Gingras AC, Raught B, Sonenberg N. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem. 1999; 68:913–63. [PubMed: 10872469]

[14]. Hershey, JWB, Merrick, WC. Pathway and mechanism of initiation of protein synthesisTranslational control of gene expression. Sonenberg, N, Hershey, JWB, Mathews, MB, editors. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2000. 33–88.

[15]. Preiss T, Hentze MW. Starting the protein synthesis machine: eukaryotic translation initiation. Bioessays. 2003; 25:1201–11. [PubMed: 14635255]

[16]. Jackson RJ, Hellen CU, Pestova TV. The mechanism of eukaryotic translation initiation and principles of its regulation. Nat Rev Mol Cell Biol. 2010; 11:113–27. [PubMed: 20094052]

[17]. Strudwick S, Borden KL. The emerging roles of translation factor eIF4E in the nucleus. Differentiation. 2002; 70:10–22. [PubMed: 11963652]

[18]. Culjkovic B, Topisirovic I, Skrabanek L, et al. eIF4E is a central node of an RNA regulon that governs cellular proliferation. J Cell Biol. 2006; 175:415–26. [PubMed: 17074885]

[19]. Topisirovic I, Siddiqui N, Lapointe VL, et al. Molecular dissection of the eukaryotic initiation factor 4E (eIF4E) export-competent RNP. EMBO J. 2009; 28:1087–98. [PubMed: 19262567]

[20]. Marcotrigiano J, Gingras AC, Sonenberg N, et al. Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell. 1997; 89:951–61. [PubMed: 9200613]

[21]. Matsuo H, Li H, McGuire AM, et al. Structure of translation factor eIF4E bound to m^7GDP and interaction with 4E-binding protein. Nat Struct Biol. 1997; 4:717–24. [PubMed: 9302999]

[22]. Sonenberg N, Gingras AC. The mRNA 5' cap-binding protein eIF4E and control of cell growth. Curr Opin Cell Biol. 1998; 10:268–75. [PubMed: 9561852]

[23]. Raught B, Gingras AC. eIF4E activity is regulated at multiple levels. Int J Biochem Cell Biol. 1999; 31:43–57. [PubMed: 10216943]

[24]. Scheper GC, Proud CG. Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation? Eur J Biochem. 2002; 269:5350–9. [PubMed: 12423333]

[25]. Lasko P, Sonenberg N. Coordinated transcriptional and translational control in metabolic homeostasis in flies. Genes Dev. 2007; 21:235–7. [PubMed: 17289913]

[26]. Altmann M, Handschin C, Trachsel H. mRNA cap-binding protein: cloning of the gene encoding protein synthesis initiation factor eIF-4E from *Saccharomyces cerevisiae*. Mol Cell Biol. 1987; 7:998–1003. [PubMed: 3550438]

[27]. Rhoads RE. eIF4E: new family members, new binding partners, new roles. J Biol Chem. 2009; 284:16711–5. [PubMed: 19237539]

[28]. Lasko P, Cho P, Poulin F, et al. Contrasting mechanisms of regulating translation of specific *Drosophila* germline mRNAs at the level of 5'-cap structure binding. Biochem Soc Trans. 2005; 33:1544–6. [PubMed: 16246166]

[29]. Cho PF, Gamberi C, Cho-Park YA, et al. Cap-dependent translational inhibition establishes two opposing morphogen gradients in *Drosophila* embryos. Curr Biol. 2006; 16:2035–41. [PubMed: 17055983]

[30]. Joshi B, Cameron A, Jagus R. Characterization of mammalian eIF4E-family members. Eur J Biochem. 2004; 271:2189–203. [PubMed: 15153109]

[31]. Joshi B, Lee K, Maeder DL, et al. Phylogenetic analysis of eIF4E-family members. BMC Evol Biol. 2005; 5:48. [PubMed: 16191198]

[32]. Yoffe Y, Zuberek J, Lewdorowicz M, et al. Cap-binding activity of an eIF4E homolog from *Leishmania*. RNA. 2004; 10:1764–75. [PubMed: 15388875]

[33]. Dhalia R, Reis CR, Freire ER, et al. Translation initiation in *Leishmania major*: characterisation of multiple eIF4F subunit homologues. Mol Biochem Parasitol. 2005; 140:23–41. [PubMed: 15694484]

[34]. Yoffe Y, Zuberek J, Lerer A, et al. Binding specificities and potential roles of isoforms of eukaryotic initiation factor 4E in *Leishmania*. Eukaryot Cell. 2006; 5:1969–79. [PubMed: 17041189]

[35]. Dhalia R, Marinsek N, Reis CR, et al. The two eIF4A helicases in *Trypanosoma brucei* are functionally distinct. Nucleic Acids Res. 2006; 34:2495–507. [PubMed: 16687655]

[36]. Kelly S, Reed J, Kramer S, et al. Functional genomics in *Trypanosoma brucei*: a collection of vectors for the expression of tagged proteins from endogenous and ectopic gene loci. Mol Biochem Parasitol. 2007; 154:103–9. [PubMed: 17512617]

[37]. Wickstead B, Ersfeld K, Gull K. Targeting of a tetracycline-inducible expression system to the transcriptionally silent minichromosomes of *Trypanosoma brucei*. Mol Biochem Parasitol. 2002; 125:211–6. [PubMed: 12467990]

[38]. Wirtz E, Leal S, Ochatt C, et al. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Mol Biochem Parasitol. 1999; 99:89–101. [PubMed: 10215027]

[39]. Carrington M, Roditi I, Williams RO. The structure and transcription of an element interspersed between tandem arrays of mini-exon donor RNA genes in *Trypanosoma brucei*. Nucleic Acids Res. 1987; 15:10179–98. [PubMed: 2827116]

[40]. Webb H, Burns R, Ellis L, et al. Developmentally regulated instability of the GPI-PLC mRNA is dependent on a short-lived protein factor. Nucleic Acids Res. 2005; 33:1503–12. [PubMed: 15755751]

[41]. Marcotrigiano J, Gingras AC, Sonenberg N, et al. Cap-dependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G. Mol Cell. 1999; 3:707–16. [PubMed: 10394359]

[42]. Kramer S, Queiroz R, Ellis L, et al. Heat shock causes a decrease in polysomes and the appearance of stress granules in trypanosomes independently of eIF2(alpha) phosphorylation at Thr169. J Cell Sci. 2008; 121:3002–14. [PubMed: 18713834]

[43]. Yoffe Y, Leger M, Zinoviev A, et al. Evolutionary changes in the *Leishmania* eIF4F complex involve variations in the eIF4E-eIF4G interactions. Nucleic Acids Res. 2009; 37:3243–53. [PubMed: 19321500]

[44]. Hernandez G, Vazquez-Pianzola P. Functional diversity of the eukaryotic translation initiation factors belonging to eIF4 families. Mech Dev. 2005; 122:865–76. [PubMed: 15922571]

[45]. Dostie J, Lejbkowicz F, Sonenberg N. Nuclear eukaryotic initiation factor 4E (eIF4E) colocalizes with splicing factors in speckles. J Cell Biol. 2000; 148:239–47. [PubMed: 10648556]

[46]. Browning KS, Webster C, Roberts JK, et al. Identification of an isozyme form of protein synthesis initiation factor 4F in plants. J Biol Chem. 1992; 267:10096–100. [PubMed: 1577779]

[47]. Gallie DR, Browning KS. eIF4G functionally differs from eIFiso4G in promoting internal initiation, cap-independent translation, and translation of structured mRNAs. J Biol Chem. 2001; 276:36951–60. [PubMed: 11483601]

[48]. Mayberry LK, Allen ML, Dennis MD, et al. Evidence for variation in the optimal translation initiation complex: plant eIF4B, eIF4F, and eIF(iso)4F differentially promote translation of mRNAs. Plant Physiol. 2009; 150:1844–54. [PubMed: 19493973]

[49]. Nett IR, Martin DM, Miranda-Saavedra D, et al. The phosphoproteome of bloodstream form *Trypanosoma brucei*, causative agent of African sleeping sickness. Mol Cell Proteomics. 2009; 8:1527–38. [PubMed: 19346560]

[50]. Rau M, Ohlmann T, Morley SJ, et al. A reevaluation of the cap-binding protein, eIF4E, as a rate-limiting factor for initiation of translation in reticulocyte lysate. J Biol Chem. 1996; 271:8983–90. [PubMed: 8621544]

[51]. Cho PF, Poulin F, Cho-Park YA, et al. A new paradigm for translational control: inhibition via 5'-3' mRNA tethering by Bicoid and the eIF4E cognate 4EHP. Cell. 2005; 121:411–23. [PubMed: 15882623]

[52]. Zuberek J, Kubacka D, Jablonowska A, et al. Weak binding affinity of human 4EHP for mRNA cap analogs. RNA. 2007; 13:691–7. [PubMed: 17369309]
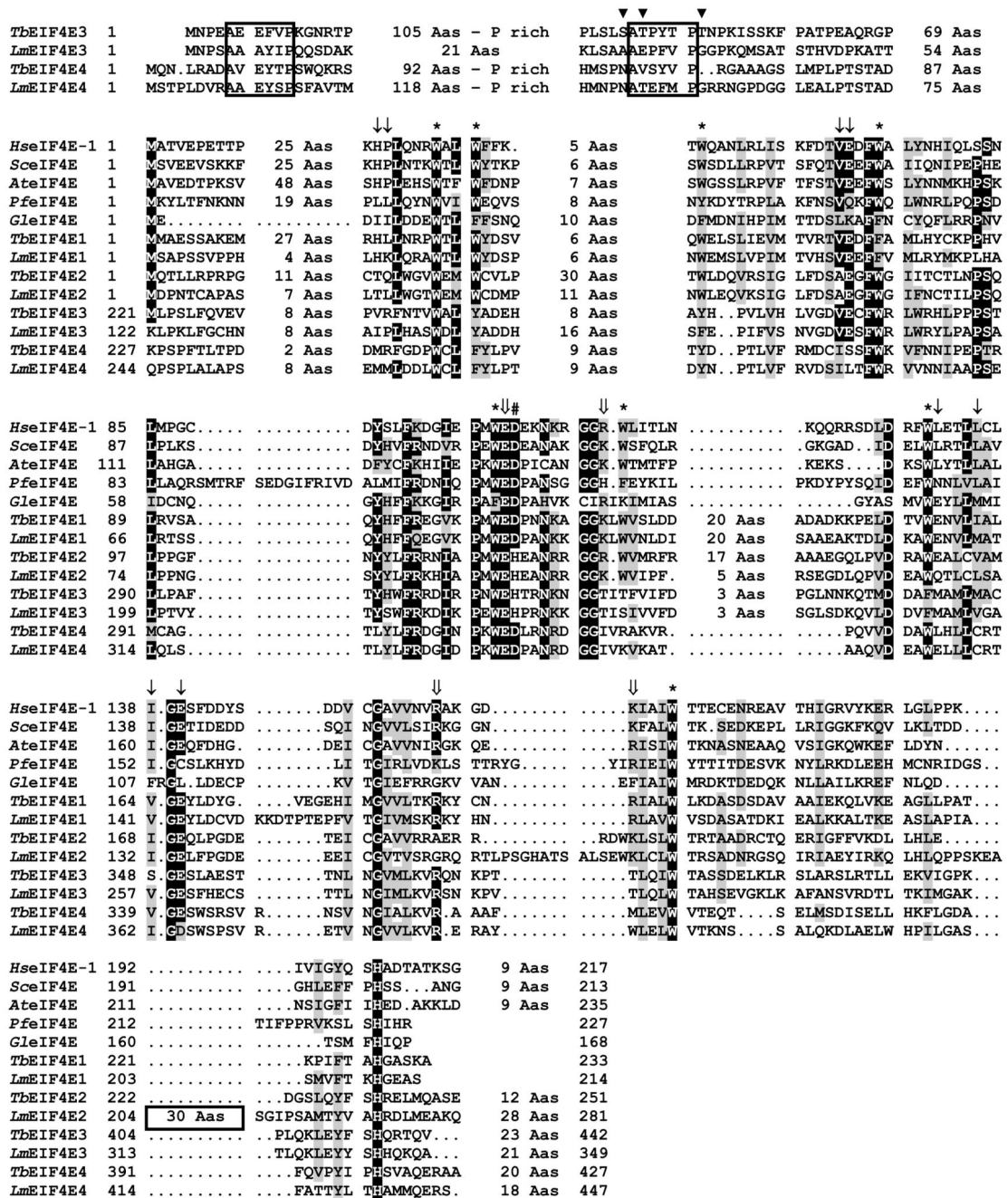
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                                                      ▼ ▼        ▼
TbEIF4E3   1        MNPEAE EFVFKGNRTP   105 Aas - P rich   PLSLSATPYT PTNPKISSKF PATPEAQRGP   69 Aas
LmEIF4E3   1        MNPSAA AYIFQQSDAK    21 Aas            KLSAAAEPFV PGGPKQMSAT STHVDPKATT   54 Aas
TbEIF4E4   1        MQN.LRADAV EYTFSWQKRS   92 Aas - P rich   HMSPNAVSYV P..RGAAAGS LMPLPTSTAD   87 Aas
LmEIF4E4   1        MSTPLDVRAA EYSFSFAVTM  118 Aas - P rich   HMNPNATEFM PGRRNGPDGG LEALPTSTAD   75 Aas


                               ⇓⇓        *        *                      *           ⇓⇓   *
HseIF4E-1  1        MATVEPETTP   25 Aas   KHPLQNRWAL WFFK.     5 Aas   TWQANLRLIS KFDTVEDFWA LYNHIQLSSN
SceIF4E    1        MSVEEVSKKF   25 Aas   KHPLNTKWTL WYTKP     6 Aas   SWSDLLRPVT SFQTVEEFWA IIQNIPEPHE
AteIF4E    1        MAVEDTPKSV   48 Aas   SHPLEHSWTF WFDNP     7 Aas   SWGSSLRPVT TFSTVEEFWS LYNNMKHPSK
PfeIF4E    1        MKYLTFNKNN   19 Aas   PLLLQYNWVI WEQVS     8 Aas   NYKDYTRPLA KFNSVQKFWQ LWNRLPQPSD
GleIF4E    1        ME....... ..........  DIILDDEWTL FFSNQ    10 Aas   DFMDNIHPIM TTDSLKAFFN CYQFLRRPNV
TbEIF4E1   1        MMAESSAKEM   27 Aas   RHLLNRPWTL WYDSV     6 Aas   QWELSLIEVM TVRTVEDFFA MLHYCKPPHV
LmEIF4E1   1        MSAPSSVPPH    4 Aas   LHKLQRAWTL WYDSP     6 Aas   NWEMSLVPIM TVHSVEEFFV MLRYMKPLHA
TbEIF4E2   1        MQTLLRPRPG   11 Aas   CTQLWGVWEM WCVLP    30 Aas   TWLDQVRSIG LFDSAEGFWG IITCTLNPSQ
LmEIF4E2   1        MDPNTCAPAS    7 Aas   LTLLWGTWEM WCDMP    11 Aas   NWLEQVKSIG LFDSAEGFWG IFNCTILPSQ
TbEIF4E3 221        MLPSLFQVEV    8 Aas   PVRFNTVWAL YADEH     8 Aas   AYH..PVLVH LVGDVECFWR LWRHLPPPST
LmEIF4E3 122        KLPKLFGCHN    8 Aas   AIPLHASWDL YADDH    16 Aas   SFE..PIFVS NVGDVESFWR LWRYLPAPSA
TbEIF4E4 227        KPSPFTLTPD    2 Aas   DMRFGDPWCL FYLPV     9 Aas   TYD..PTLVF RMDCISSFWK VFNNIPEPTR
LmEIF4E4 244        QPSPLALAPS    8 Aas   EMMLDDLWCL FYLPT     9 Aas   DYN..PTLVF RVDSILTFWR VVNNIAAPSE


                                  *⇓#        ⇓  *                         *↓      ↓
HseIF4E-1  85       LMPGC..... ..........   DYSLEKDGIE PMWEDEKNKR GGR.WLITLN ..........   .KQQRRSDLD RFWLETLLCL
SceIF4E    87       LPLKS..... ..........   DYHVFRNDVR PEWEDEANAK GGK.WSFQLR ..........   .GKGAD..ID ELWLRTLLAV
AteIF4E   111       LAHGA..... ..........   DFYCEKHIIE PKWEDPICAN GGK.WTMTFP ..........   .KEKS....D KSWLYTLLAL
PfeIF4E    83       LLAQRSMTRF SEDGIFRIVD   ALMIFRDNIQ PMWEDPANSG GGH.FEYKIL ..........   PKDYPYSQID EFWNNLVLAI
GleIF4E    58       IDCNQ..... ..........   GYHFEKKGIR PAFEDPAHVK CIRIKIMIAS ..........   ......GYAS MVWEYLLMMI
TbEIF4E1   89       LRVSA..... ..........   QYHFEREGVK PMWEDPNNKA GGKLWVSLDD    20 Aas   ADADKKPELD TVWENVLIAL
LmEIF4E1   66       LRTSS..... ..........   QYHFFQEGVK PMWEDPANKK GGKLWVNLDI    20 Aas   SAAEAKTDLD KAWENVLMAT
TbEIF4E2   97       LPPGF..... ..........   NYYLFRRNIA PMWEHEANRR GGR.WVMRFR    17 Aas   AAAEGQLPVD RAWEALCVAM
LmEIF4E2   74       LPPNG..... ..........   SMYLFRKHIA PMWEHEANRR GGK.WVIPF.     5 Aas   RSEGDLQPVD EAWQTLCLSA
TbEIF4E3  290       LLPAF..... ..........   TYHWFRRDIR PNWEHTRNKN GGTITFVIFD     3 Aas   PGLNNKQTMD DAFMAMLMAC
LmEIF4E3  199       LPTVY..... ..........   TMSWFRKDIK PEWHPRNKK GGTISIVVFD     3 Aas   SGLSDKQVLD DVFMAMLVGA
TbEIF4E4  291       MCAG..... ..........    TLYLFRDGIN PKWEDLRNRD GGIVRAKVR. ..........   .....PQVVD DAWLHLLCRT
LmEIF4E4  314       LQLS...... ..........   TLYLFRDGID PKWEDPANRD GGIVKVKAT. ..........   .....AAQVD EAWELLLCRT


                    ↓  ↓                        ⇓                        ⇓   *
HseIF4E-1 138       I.GESFDDYS .......DDV   CCAVVNVRAK GD........ .....KIAIW TTECENREAV THIGRVYKER LGLPPK....
SceIF4E   138       I.GETIDEDD .......SQI   NCVVLSIRKG GN........ .....KFALW TK.SEDKEPL LRIGGKFKQV LKLTDD....
AteIF4E   160       I.GEQFDHG. .......DEI   CCAVVNIRGK QE........ .....RISIW TKNASNEAAQ VSIGKQWKEF LDYN......
PfeIF4E   152       I.GCSLKHYD .......LI    TCIRLVDKLS TTRYG..... ...YIRIEIW YTTITDESVK NYLRKDLEEH MCNRIDGS..
GleIF4E   107       FRGL.LDECP .......KV    TCIEFRRGKV VAN....... ....EFIAIW MRDKTDEDQK NLLAILKREF NLQD......
TbEIF4E1  164       V.GEYLDYG. ....VEGEHI   MCVVLTKRKY CN........ ....RIALW LKDASDSDAV AAIEKQLVKE AGLLPAT...
LmEIF4E1  141       V.GEYLDCVD KKDTPTEPFV   TCIVMSKRKY HN........ ....RLAVW VSDASATDKI EALKKALTKE ASLAPIA...
TbEIF4E2  168       I.GEQLPGDE .......TEI   CCAVVRRAER R......... ..RDWKLSLW TRTAADRCTQ ERIGFFVKDL LHLE......
LmEIF4E2  132       I.GELFPGDE .......EEI   CCVTVSRGRQ RTLPSGHATS ALSEWKLCLW TRSADNRGSQ IRIAEYIRKQ LHLQPPSKEA
TbEIF4E3  348       S.GESLAEST .......TNL   NCVMLKVRQN KPT....... .....TLQIW TASSDELKLR SLARSLRTLL EKVIGPK...
LmEIF4E3  257       I.GESFHECS .......TTL   NCIMLKVRSN KPV....... .....TLQLW TAHSEVGKLK AFANSVRDTL TKIMGAK...
TbEIF4E4  339       V.GESWSRSV R......NSV   NCIALKVR.A AAF....... .....MLEVV VTEQT....S ELMSDISELL HKFLGDA...
LmEIF4E4  362       I.GDSWSPSV R......ETV   NCVVLKVR.E RAY....... .....WLELW VTKNS....S ALQKDLAELW HPILGAS...


HseIF4E-1 192       .......... ....IVIGYQ   SHADTATKSG     9 Aas   217
SceIF4E   191       .......... ....GHLEFF   PHSS...ANG     9 Aas   213
AteIF4E   211       .......... ....NSIGFI   IHED.AKKLD     9 Aas   235
PfeIF4E   212       .......... TIFPPRVKSL   SHIHR                227
GleIF4E   160       .......... .......TSM   FHIQP                168
TbEIF4E1  221       .......... .....KPIFT   AHGASKA              233
LmEIF4E1  203       .......... .....SMVFT   KHGEAS               214
TbEIF4E2  222       .......... ...DGSLQYF   SHRELMQASE    12 Aas   251
LmEIF4E2  204       [   30 Aas   ]  SGIPSAMTYV AHRDLMEAKQ    28 Aas   281
TbEIF4E3  404       .......... ..PLQKLEYF   SHQRTQV...    23 Aas   442
LmEIF4E3  313       .......... ..TLQKLEYY   SHHQKQA...    21 Aas   349
TbEIF4E4  391       .......... ....FQVPYI   PHSVAQERAA    20 Aas   427
LmEIF4E4  414       .......... ....FATTYL   THAMMQERS.    18 Aas   447
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Fig. 1.

Sequence analysis of the four *T. brucei* eIF4E sequences. Clustal W alignment of the *T. brucei* and *L. major* eIF4E homologues with sequences from representative eukaryotic lineages. Amino acids identical in more than 60% of the sequences are highlighted in dark gray, while amino acids defined as similar, based on the BLOSUM 62 Matrix, on more than 60% of the sequences, are shown in pale gray. When necessary, spaces were inserted within the various sequences (dots) to allow better alignment. The first two boxes highlight regions which share conserved elements in the N-terminus of the EIF4E3 and 4 homologues from

Leishmania and *Trypanosoma* species, with phosphorylated residues identified in *Tb*EIF4E3 [49] indicated by the symbol ▾. The third box highlight the unique insertion observed in *Lm*EIF4E2. * indicates the conserved tryptophan residues. Large arrows indicate non-tryptophan residues required for the interaction with the cap structure (based on [20]). Thin arrows indicate conserved non-tryptophan residues shown to be involved in eIF4G binding [41]. The symbol # marks the conserved D residue which is replaced by H solely in the trypanosomatid EIF4E2 and EIF4E3 sequences. GenBank Accessions for the non-trypanosomatid sequences: *Hs* (human) eIF4E-1 – **NP_001959**; *Sc* (*Saccharomyces cerevisaei*) eIF4E – **NP_014502**; *At* (*Arabidopsis thaliana*) eIF4E – **NP_193538**; *Pf* (*Plasmodium falciparum*) eIF4E1 – **XP_001351220**; *Gl* (*Giardia lamblia*) – **XP_001710318**.
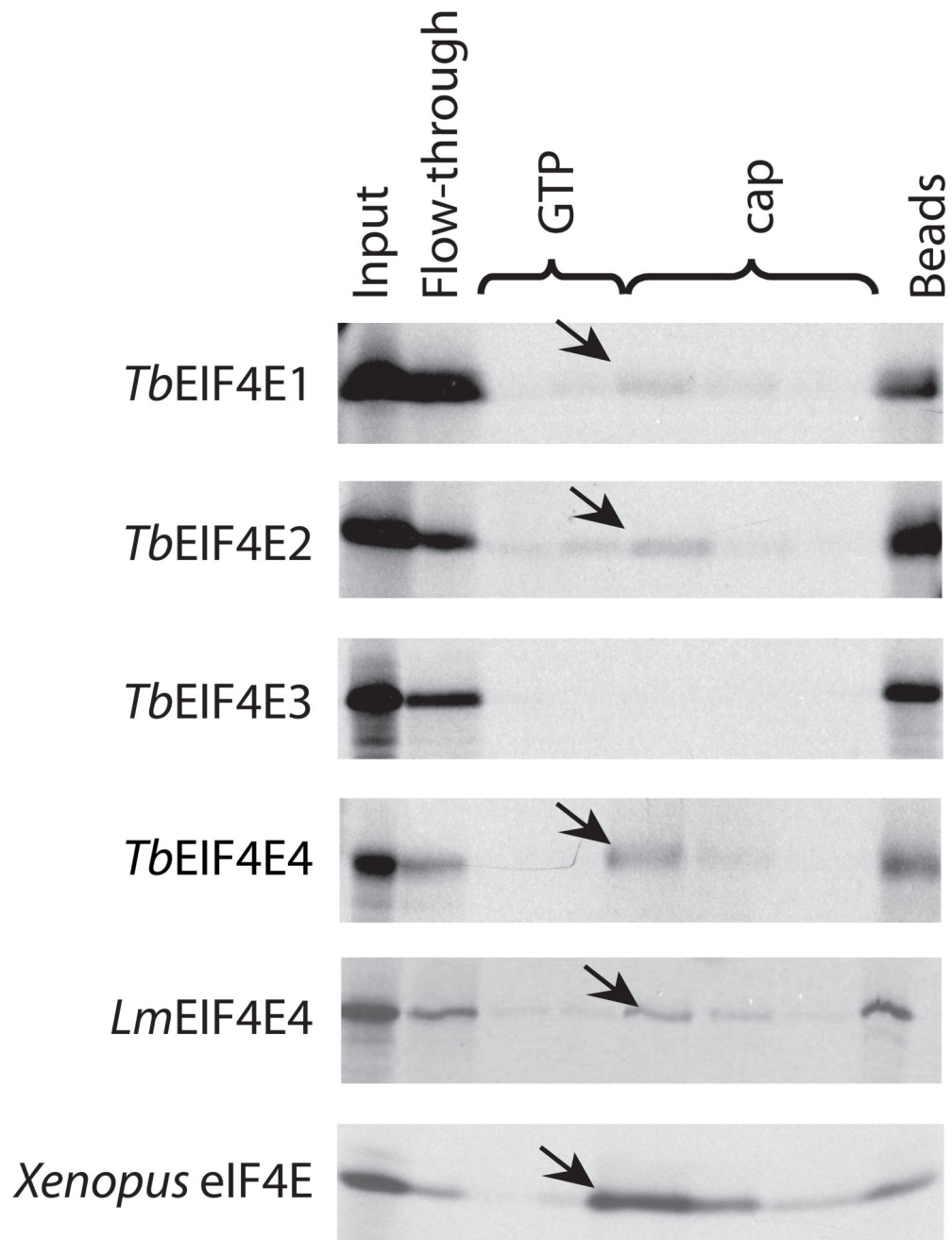
Fig. 2.

Cap binding assays. Autoradiography of the cap binding chromatography performed with the four ^{35}S-labelled *T. brucei* eIF4E homologues, the *Leishmania Lm*EIF4E4 and the *Xenopus leavis* eIF4E homologue as positive control. Aliquots of the various washes were ran on SDS-PAGE and compared with samples from the original translation reaction (Input) as well as the non-bound fraction (Flow-through) and any protein remaining bound to the beads (Beads). The arrows indicate the proteins eluted by the cap analogue. The assay shows

the affinity of *Tb*EIF4E1, 2 and 4, as well as *Lm*EIF4E4, for the 7-methyl-GTP Sepharose resin.
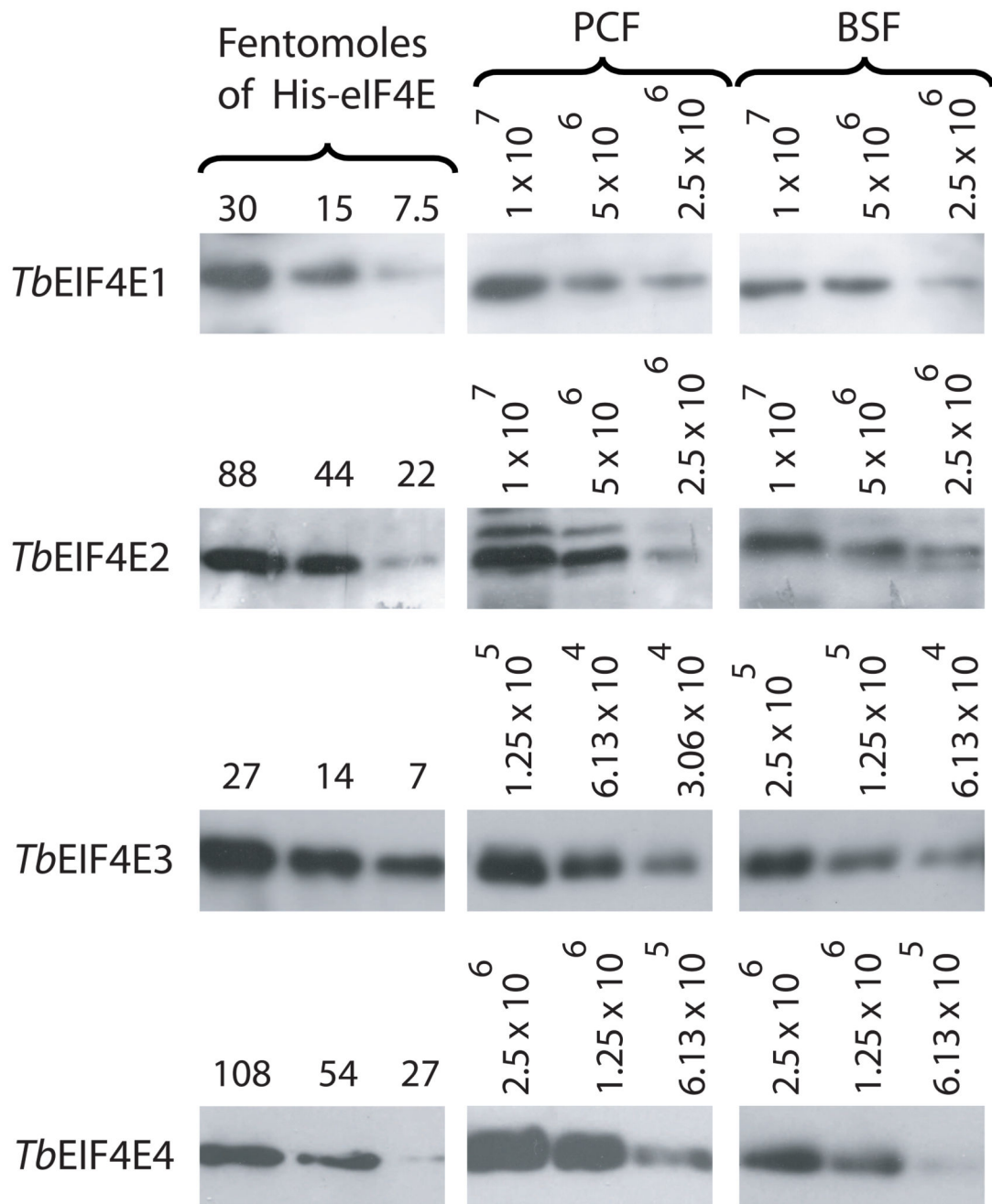
Fig. 3.
Expression analysis of *Tb*EIF4E1 through 4 in procyclic and bloodstream procyclic cells. Quantitation and expression analysis of *Tb*EIF4E1, 3 and 4 proteins in procyclic/ bloodstreams forms. Recombinant His tagged *Tb*EIF4E1, 2, 3 and 4 were quantitated, diluted to defined concentrations (in fentomoles) and run on SDS-PAGE gels with whole parasite extract obtained from known number of cells from both procyclic and bloodstream forms.
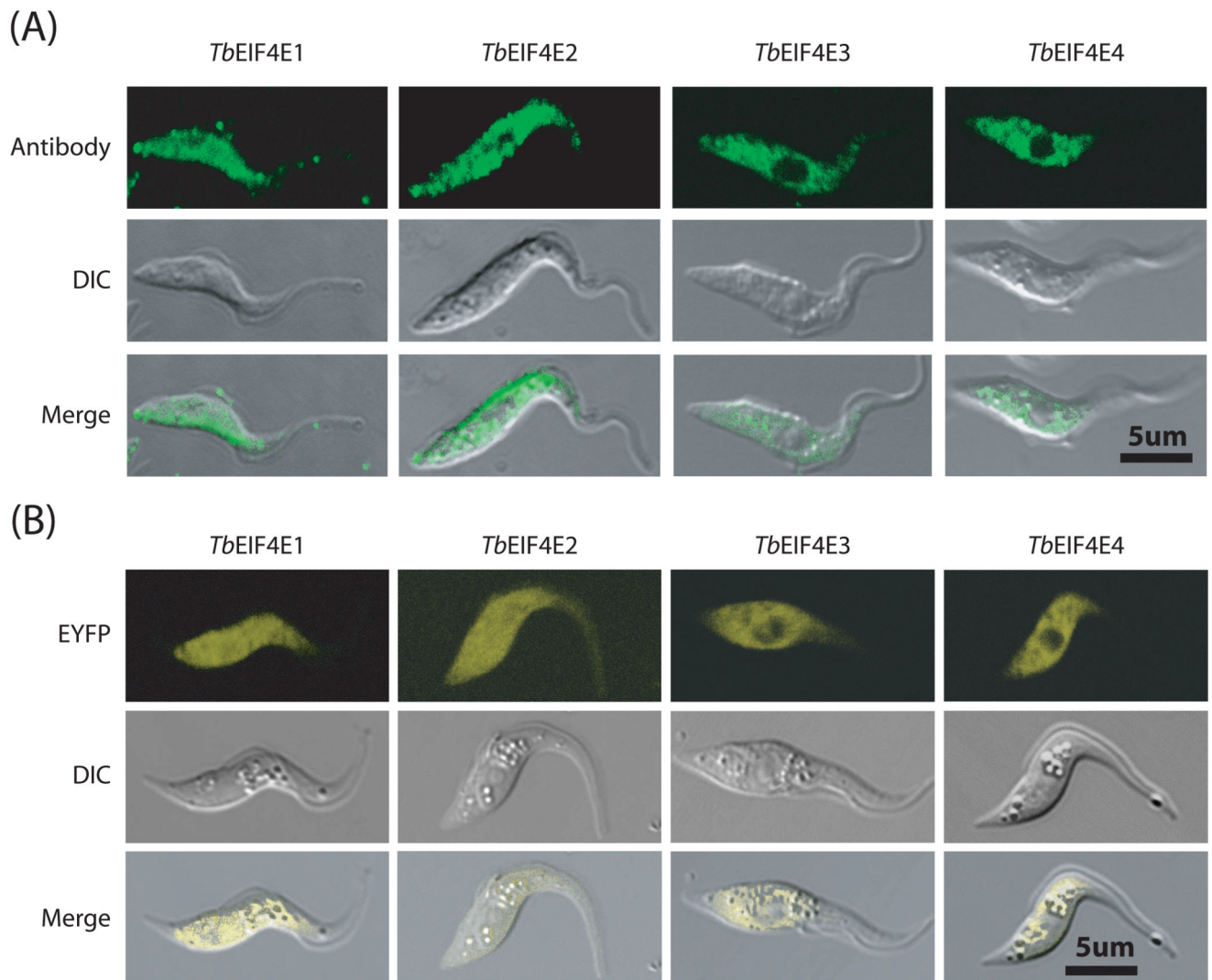
Fig. 4.
Subcellular localisation of *Tb*EIF4E1 through 4 in *T. brucei* procyclic forms. (A) Subcellular localisation of the four eIF4E homologues in wild type *T. brucei* procyclic cells (WT 427) by indirect immunofluorescence. This was performed using the antibodies directed to the various homologues followed by incubation with the Alexa Fluor 488 conjugated secondary antibody. (B) The localisation of *Tb*EIF4E1 through 4 was also confirmed through the expression of eYFP fusion proteins in transfected *T. brucei* procyclic cells examined under the confocal microscope.
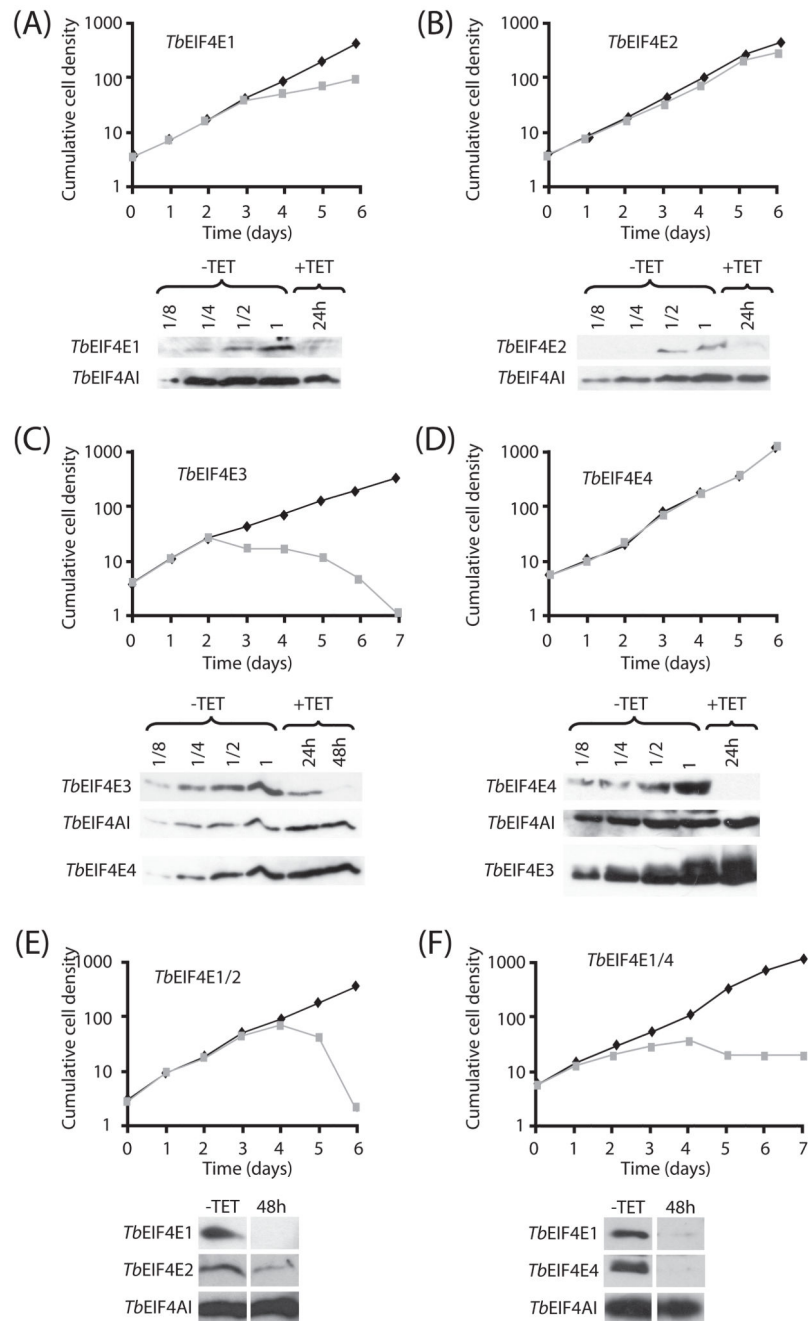
Fig. 5.

RNA interference of *Tb*EIF4E1 through 4 in procyclic cells. Procyclic *T. brucei* cells were transfected with the p2T7-177 derived plasmid containing the individual eIF4E genes (*Tb*EIF4E1 – (A), *Tb*EIF4E2 – (B), *Tb*EIF4E3 – (C), *Tb*EIF4E4 – (D)) or the double constructs *Tb*EIF4E1/2 (E) and *Tb*EIF4E1/4 (F). Transfected cells were selected after growth in the presence of phleomycin and RNA interference induced after tetracycline addition. At regular intervals, cellular growth was monitored by counting the number of viable cells of cultures with and without tetracycline and the resulting values used to plot the

curves shown (plus tetracycline – gray; minus tetracylcline – black). The values shown are indicative of the cumulative cell density in 10^5 cells per ml. Below each graph are Western-blot analysis of the proteins being targeted, using the affinity-purified antisera specific for each protein. These were performed with a single sample derived from the curves minus tetracycline (-TET) as well as selected samples from the curves produced after the RNAi induction (in hours after tetracycline addition). For the single protein knock-downs, note the various dilutions of the -TET sample (1 to 1/8 cell dilution – 1 equals to the amount of cells used in the +TET lane) which allows for a better estimate of the RNAi efficiency. In general the same blot was reprobed with affinity purified antibodies against the endogenous control TbEIF4AI. The blot from the TbEIF4E3 RNAi was further incubated with the anti-TbEIF4E4 antibodies and likewise the blot from the TbEIF4E4 experiment was assayed with the anti-TbEIF4E3 antibodies. All RNAi results shown are representative of at least three different experiments performed with at least two distinct transfection events. In F only the first 7 days of the curve are shown but +TET RNAi curves, from different transfection events, were kept for 15 days or more without any increase in cell numbers.
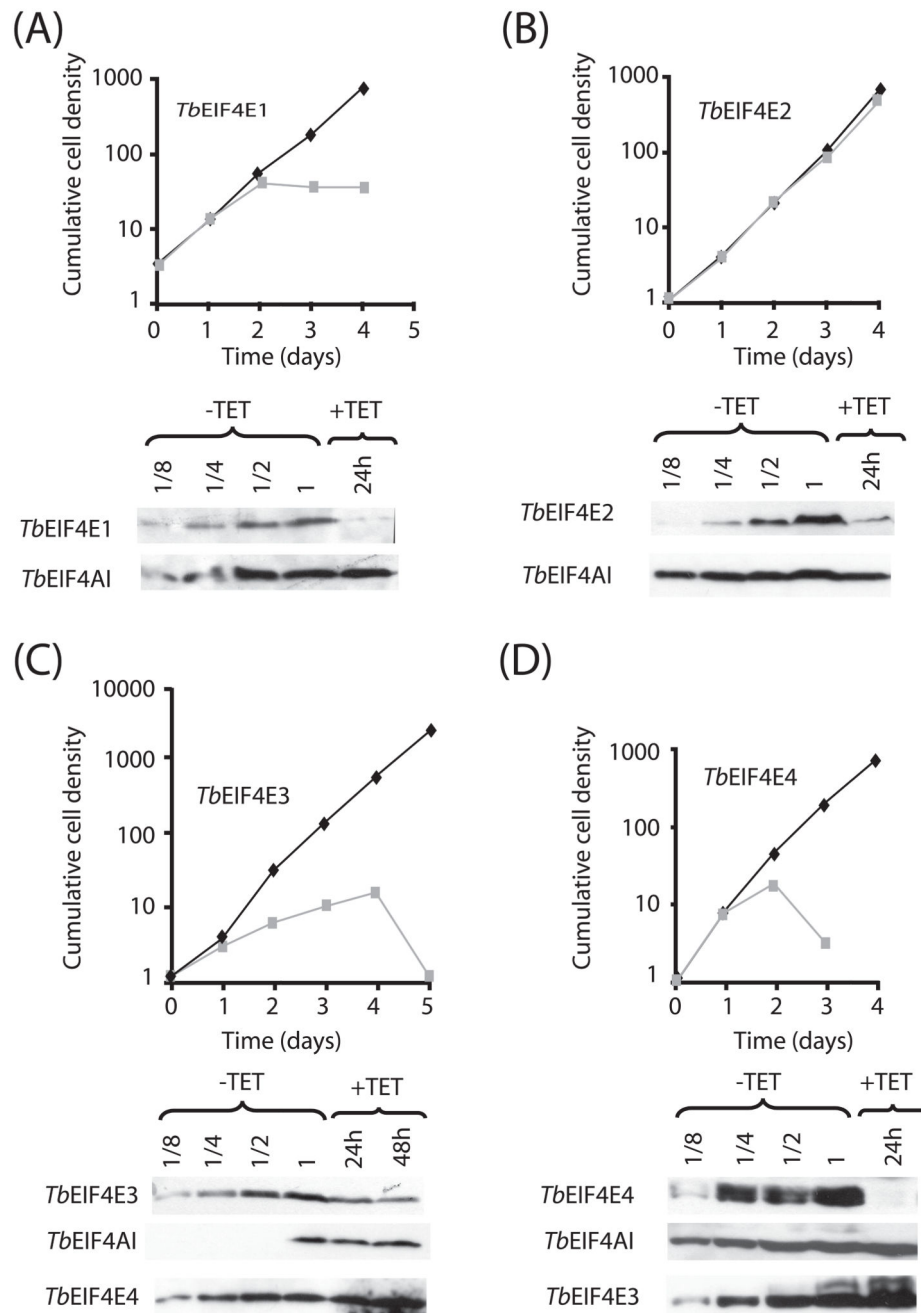
Fig. 6.

RNA interference of *Tb*EIF4E1-4 in bloodstream cells. Bloodstream *T. brucei* cells were transfected with the p2T7-177 derived plasmids containing the eIF4E genes and monitored for cellular growth in the presence (gray curve) or absence (black curve) of tetracycline (*Tb*EIF4E1 – (A), *Tb*EIF4E2 – (B), *Tb*EIF4E3 – (C), *Tb*EIF4E4 – (D)). For all four RNAi procedures, expression of the targeted genes as well as the endogenous *Tb*EIF4AI control was assayed in a sample from the control curve (-TET, diluted from 1 to 1/8 – 1 equals to the amount of cells used in the +TET lane) as well as in selected samples from the curve

supplemented with tetracycline (+TET). The *Tb*EIF4E3 and 4 RNAi blots were further assayed with the anti-*Tb*EIF4E4 and anti-*Tb*EIF4E3 antibodies, respectively. All procedures were done as described for Fig. 5.

(A)

Relative [^{35}S]-methionine incorporation +TET/-TET			
Days after TET addition	3	4	8
E3	58%	36%	X
E1/E2	101%	85%	X
E1/E4	43%	28%	0%

(B)
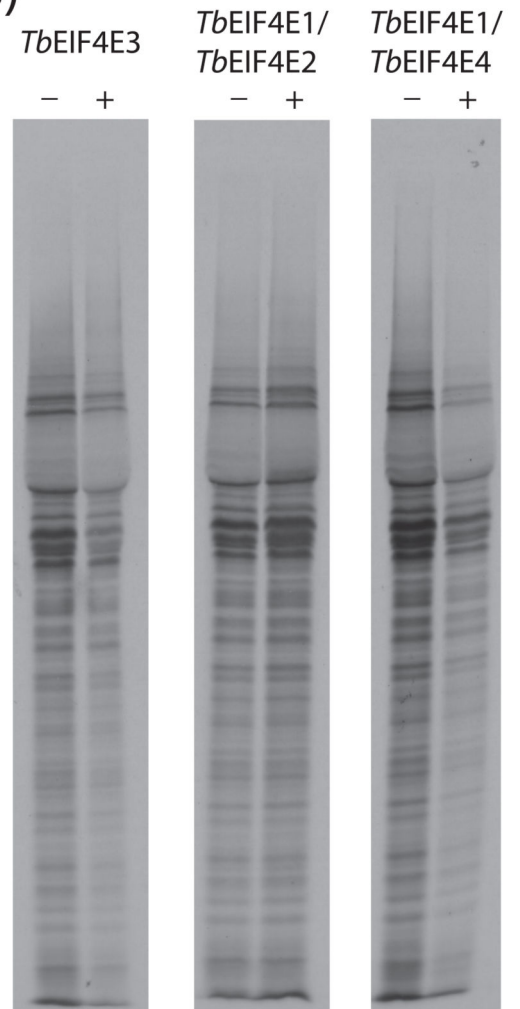
Fig. 7.

Metabolic labelling of procyclic cells from select RNAi curves. (A) Total protein synthesis was estimated after RNAi for *Tb*EIF4E3 (E3) and the double constructs *Tb*EIF4E1/2 (E1/E2) and *Tb*EIF4E1/4 (E1/E4). At selected time points (3 and 4 days for the first two samples and 3, 4 and 8 days for *Tb*EIF4E1/4), aliquots of the cells were incubated in the presence of ^{35}S methionine followed by TCA precipitation and quantitation of the incorporated radioactivity. To estimate the efficiency of translation in the RNAi treated cells in comparison with the minus tetracycline controls, the normalized incorporation values after two hours of incubation were used to calculate the ratios shown in the figure. (B) For a qualitative analysis of the proteins being synthesized, aliquots of $2x10^6$ cells derived from an independent labeling experiment (4 days after tetracycline addition) were ran on an SDS-PAGE gel followed by autoradiography.
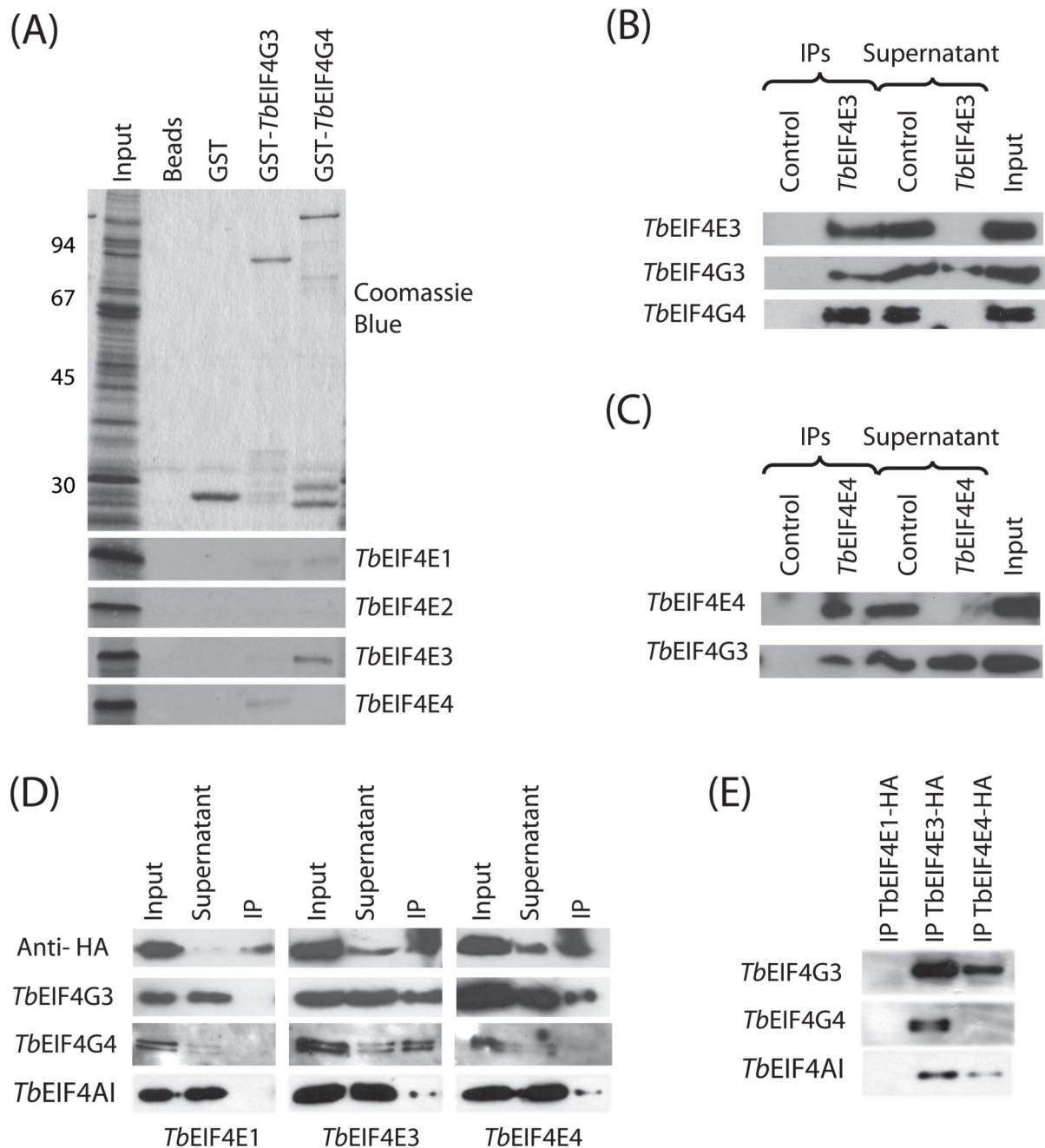
Fig. 8.

Analysis of the interactions between *Tb*EIF4E3/*Tb*EIF4E4 and *Tb*EIF4G3/*Tb*EIF4G4. (A) Pull-down assay using GST-tagged *Tb*EIF4G3 and 4 and ^{35}S-labelled *Tb*EIF4E1 through *Tb*EIF4E4. As controls GST on its own or only the glutathione sepharose beads were tested for their ability to bind the labeled protein. Upper panel: Coomassie Blue stained gel showing total translation extract (Input) as well as recombinant GST or GST- *Tb*EIF4G3 and 4. Bottom panels: autoradiographies showing strong and specific binding between GST- *Tb*EIF4G3 and labeled *Tb*EIF4E4 and between GST-*Tb*EIF4G4 and labeled *Tb*EIF4E3. (B)

Immunoprecipitation (IP) assay confirming interactions between *Tb*EIF4E3 and *Tb*EIF4G4 and between *Tb*EIF4E3 and *Tb*EIF4G3 *in vivo*. *T. brucei* cytoplasmic extracts were immunoprecipitated with the anti-*Tb*EIF4E3 affinity purified antibodies (2 μg) or 5 μl of the pre-immune total serum as control (Control). Precipitated immunocomplexes were then used in Western blot assays with the *Tb*EIF4E3, *Tb*EIF4G3 or *Tb*EIF4G4 antisera. In the same blots, an aliquot of the non-bound supernatant (Supernatant) was compared with an equivalent aliquot of the cytoplasmic extract used in the IP reaction and blot (Input). Equivalent aliquot means that if 20% of an IP was blotted (the real % values varied according to the protein assayed), then 20% of the same volume of the original cytoplasmic extract used for the IP, or its supernatant, was blotted as well. (C) Assay confirming the interaction between *Tb*EIF4E4 and *Tb*EIF4G3 *in vivo*. Immunoprecipitation was carried out as described in B with anti-*Tb*EIF4E4 antibodies followed by Western-blots with the *Tb*EIF4E4 and *Tb*EIF4G3 antisera. (D) IP of HA-tagged eIF4E homologues. Cytoplasmic extracts from transfected *T. brucei* cells expressing HA-tagged *Tb*EIF4E1, 3 and 4 (left, middle and right panels, respectively) were incubated with anti-HA beads and the precipitated samples, as well as equivalent aliquots of the non-bound supernatant and the input extract, were assayed for the presence of the HA-tagged proteins, *Tb*EIF4G3 and 4 and *Tb*EIF4AI. (E) To highlight the results from the IP lanes shown in D, these were ran on another gel and blotted again with the *Tb*EIF4G3 and 4 and *Tb*EIF4AI antisera, confirming the potential for both *Tb*EIF4E3 and 4 to reconstitute functional eIF4F complexes.

Table 1

Estimate of the intracellular levels of the various *T. brucei* and *L. major* eIF4E homologues. ND – Not determined.

Homologue	Protein levels (molecules/cell)		
	T. brucei procyclics	*T. brucei* bloodstream	[1] *L. major* promastigotes
EIF4E1	$3\text{-}8 \times 10^3$	$1.5\text{-}5 \times 10^3$	$2\text{-}4 \times 10^3$
EIF4E2	$1\text{-}5 \times 10^3$	$1\text{-}5 \times 10^3$	$\sim1 \times 10^3$
EIF4E3	$5\text{-}10 \times 10^4$	$2\text{-}4 \times 10^4$	$4\text{-}10 \times 10^4$
EIF4E4	$2\text{-}4 \times 10^4$	$1\text{-}2 \times 10^4$	ND

[1]Based on reference [33].