Short communication

The eIF4E subunits of two distinct trypanosomatid eIF4F complexes are subjected to differential post-translational modifications associated to distinct growth phases in culture

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**ABSTRACT**

The eukaryotic eIF4F complex, the cap binding complex, functions during translation initiation through interactions mediated by its three subunits (eIF4E, eIF4G and eIF4A), other initiation factors and the ribosome. In trypanosomatids, various eIF4E and eIF4G homologues were identified, with two eIF4F-like complexes confirmed (EIF4E4/EIF4G3/EIF4AI and EIF4E3/EIF4G4/EIF4AI). Here, the expression pattern of these complexes was investigated during *Leishmania amazonensis* and *Trypanosoma brucei* growth. The two sets of eIF4E and eIF4G homologues were found represented by phosphorylated isoforms with multiple phosphorylation events targeting the two eIF4E homologues. Expression of these multiple isoforms was differentially affected by inhibitors of mRNA synthesis/processing and translation. Phosphorylated EIF4E4 was consistently associated with early/active growth phases in both organism studied. In *T. brucei* phosphorylation of both EIF4E3 and 4, overexpressed as HA-tagged fusions, was partially mapped to their N-terminuses. Our results indicate that phosphorylation is associated with a further layer of complexity in translation initiation in trypanosomatids.

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The 5’ end of the majority of eukaryotic mRNAs is defined by the presence of the modified cap nucleotide (7-methyl-GTP), a structure which is required for efficient mRNA recognition by the translation machinery and which is specifically recognized by the translation initiation factor eIF4E (the cap binding protein). eIF4E is part of the heterotrimeric translation initiation complex eIF4F, which also includes the RNA helicase eIF4A and the large scaffolding protein eIF4G. Interactions between eIF4F, through its eIF4G subunit, with other initiation factors facilitate the recruitment of the 40S ribosomal subunit to the mRNAs and the initiation of translation [1]. The whole translation initiation process can be regulated by events which target both eIF4E and eIF4G subunits of eIF4F [2].

Trypanosomatids are strictly parasitic organisms which have diverged very early on from the main line of eukaryotic evolution. These pathogens are known to possess a number of unique features in their basic biologic processes, such as the constitutive transcription of their protein coding genes into polycistrionic pre-mRNAs [3]. The resulting transcripts are subsequently processed through trans-splicing to generate mature monocistrionic mRNAs, all containing an identical 39 nucleotide spliced-leader (SL) with a modified cap nucleotide, called cap4, at their 5’ end [4]. Regulation of gene expression is then mediated almost entirely by post-transcriptional processes acting at the level of mRNA processing, stability and possibly translation [5].

Multiple homologues for the eIF4A (two), eIF4E (four) and eIF4G (five) subunits of eIF4F have been described in both *Leishmania* and *Trypanosoma* species [6]. EIF4AI has been found to be the sole eIF4A homologue involved in translation [7], whilst preliminary characterization of EIF4E1 through 4, from both *T. brucei* and *Leishmania* species, have shown them to vary in cap binding affinity, subcellular localization and requirement for cell viability [8]. The eIF4G homologues (EIF4G1 through 5) have been the focus of less attention, but two of those (EIF4G3 and 4) have been shown to associate with EIF4E3 and 4, the two largest eIF4E homologues and which are characterized by unique features in common, such as their long N-terminal extensions and strictly cytoplasmic localization. A minimum of two distinct eIF4F complexes have then been found centered on the interactions between EIF4E4/EIF4G3/EIF4AI and EIF4E3/EIF4G4/EIF4AI [10]. So far only the first complex has been clearly implicated as performing a critical role in translation initiation, since both eIF4E and eIF4G subunits migrate with polysomes ...
in sucrose gradients [11]. EIF4G3 also binds specifically to PABP1, the first of the three PABP homologues identified in Leishmania species, an interaction which further supports a role in translation [12]. Nevertheless, depletion by RNAi of EIF4E3 substantially inhibits protein synthesis in T. brucei, implying a role in this process for the complex based on EIF4E3/EIF4G4 [10].

Here, to investigate the possibility that the two known trypanosomatid elf4F complexes might be associated with protein synthesis under different growth stages, and having the availability of serum directed against their subunits from both Leishmania and Trypanosoma species, we set out to investigate the expression pattern of these subunits during growth in culture of two representative species. First, their expression was evaluated in L. amazonensis, a model species which can be readily recovered from infected mice to start fresh cultures of cells not affected by long periods of cultivation. In order to enhance reproducibility, cultures were set up in which the cells were allowed to reach stationary phase prior to passing followed by dilution to the same pre-established cell density. At selected time points after passing, samples were harvested, resuspended directly into SDS-PAGE sample buffer and then submitted to denaturing SDS-PAGE and blotting with either whole rabbit polyclonal serum or affinity purified antibodies directed against the various polypeptides under study. Sera against Leishmania EIF4E3, EIF4G3 and EIF4AI have been previously described [6], whilst sera directed against EIF4E4 and EIF4G4 were generated during the course of this work (see Supplementary Data S1).

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Both sets of elf4F (EIF4E3 and 4) and elf4G (EIF4G3 and 4) homologues, as well as EIF4AI, were seen to be expressed throughout the L. amazonensis curves evaluated, although only EIF4AI and EIF4G3 were seen to be represented by single bands of roughly equivalent intensities in all samples analyzed (Fig. 1A). For EIF4E3, EIF4E4 and EIF4G4 a pattern of various isoforms emerged for each protein, with overall abundance for the elf4F homologues being greater during exponential growth. Whilst for EIF4G4 the different isoforms were equally represented throughout the curve, EIF4E4 was represented by isoforms of lower molecular weight immediately after passing which increased in size in subsequent time points (lag and early exponential growth phases) and later returned to their original size when the cells reached stationary phase. In contrast, EIF4E3 was first found mainly as an isoform of higher molecular weight which rapidly decreased in size during lag and exponential phases and later returned to the large size, single band, which was typical for stationary phase cells. Overall the results shown are consistent with simultaneous expression of both elf4F complexes throughout the growth curves of the insect stage but with post-translational modifications differentially acting on the elf4F homologues and presumably interfering with their function in translation.

To better define the mechanisms behind the changes in expression profile observed for EIF4E3 and 4, growth curves were set up as described above but in media supplemented with inhibitors of translation (either Cycloheximide or Puromycin), transcription (Actinomycin D) or mRNA processing (Sinefungin). The expression of EIF4E3 and 4 and EIF4AI (as internal control) was then investigated at selected time points (Fig. 1B). At 24 h, cells from all conditions tested displayed in general a phenotype for both proteins which is typical of stationary cultures and may represent a common pattern for stressed cells. At earlier time points (up to 6 h), however, incubation with protein synthesis inhibitors did not interfere with the changes in molecular weight previously seen for EIF4E4 at the start of a new culture. Treatment with Cycloheximide (stabilizes the polysomes) seemed even to increase the presence of the high molecular weight EIF4E4 isoforms observed soon after passing (2 and 6 h time points), whilst treatment with Puromycin (induces polysome dissociation) led to a less pronounced effect. In contrast, treatment with inhibitors of mRNA transcription and processing induced a substantial inhibition on the increase in size seen for the EIF4E4 isoforms. For EIF4E3, the shift for the low molecular weight isoform seen upon passing to new media was substantially blocked in the presence of inhibitors of both protein synthesis and mRNA processing but only mildly affected by the transcription inhibitor Actinomycin D. These results are consistent with no new translation being required for the rapid increase in size observed for EIF4E4 during passing to fresh media, whilst the decrease in size observed under equivalent conditions for EIF4E3 was directly dependent on it. Both proteins were, however, affected by treatment with the mRNA processing inhibitor Sinefungin, indicating the need for new mRNA to arrive in the cytoplasm for the observed changes in post-translational modifications to take effect. The quick response induced upon treatment with Sinefungin [13] might be responsible for its stronger effect as compared to that seen with Actinomycin D. Treatment with the phosphatase inhibitor Okadaic Acid and the proteasome inhibitor Lactacystin did not impact on the changes observed for either of the proteins investigated (data not shown).

In metazoa, both elf4F and elf4G subunits of the elf4F complex have been shown to be actively regulated by modifications such as phosphorylation [2,14]. The results described so far are then indicative of different Leishmania elf4F subunits being modified through phosphorylation and with this type of modification being associated with some or all of the multiple isoforms observed for EIF4E3, EIF4E4 and EIF4G4. To investigate this possibility, total protein extracts from exponentially grown L. amazonensis promastigotes were subjected to purification of their phosphoproteome fraction followed by blotting to assay for the presence of the two sets of elf4F subunits. Both sets of elf4F and elf4G homologues were detected not only in the load and flowthrough fractions, but also in the elution sample which includes the phosphorylated proteins (Fig. 1C), and for both EIF4E3 and 4 the higher molecular weight isoforms were only found in the elution and control samples. In contrast, only a minor detection in the elution lane was observed for EIF4AI. Two-dimensional (2D) gel electrophoresis was then carried out in order to better define the differences in expression profile observed for the two L. amazonensis elf4F homologues during exponential growth. EIF4E4 was represented by a large number of different spots (eight or more) (Fig. 1D) which generally increased in size simultaneously with small reductions in pl. In contrast, EIF4E3 displayed a markedly distinct profile, with two spots (1 and 2) representing the higher molecular weight band seen in Fig. 1A and two spots (3 and 4), representing the smaller band. For EIF4AI only one isoform predominates but a second isoform of lower intensity is also observed, indicating minor, if any, phosphorylation. The differences in isoelectric point (pl) observed between the various isoforms of the two elf4F homologues are overall consistent with pl prediction analysis carried out for both proteins using the ScanSite 2.0 program (available at http://scansite.mit.edu/calc_mw_pl.html), capable of estimating the effect of multiple phosphorylation events on the proteins' pl [15]. Whilst one or two phosphorylation events in EIF4E3 substantially change the protein's pl, minor changes are predicted even after two phosphorylations on EIF4E4.

Multiple phosphorylation events to the extent observed here have not been reported from other elf4F homologues and the residue targeted for phosphorylation in the C-terminus of metazoan elf4E (S209 in the mammalian sequence) is not conserved in the trypanosomatid sequences. To evaluate if the results seen for the phosphorylation of L. amazonensis elf4F subunits are representative of different trypanosomatid species, the expression pattern
of EIF4E3 and 4, as well as their direct partners, was also investigated in a second trypanosomatid model species, T. brucei. Growth curves of procyclic cells were set up following harvest at selected time points for the production of total protein extracts, as described for L. amazonensis. Previously described sera produced against T. brucei EIF4AI, EIF4E3 and 4 and EIF4G3 and 4 [7,10] were then used to assay for expression of these proteins during growth of the procyclic life stage. As shown in Fig. 2A, all selected proteins were simultaneously expressed, with EIF4E3, EIF4E4 and EIF4G4 being represented by multiple isoforms whilst EIF4G3 and EIF4AI were represented by single bands. Only EIF4E4 displayed changes in isoform expression profile, showing a marked decrease in size in stationary cells, compatible with what was observed for its L. amazonensis orthologue, and the two sets of EIF4E and EIF4G homologues were confirmed to be phosphorylated although that was not seen to be the case for EIF4AI (Supplementary Fig. S1A). The pattern derived from 2D analysis of T. brucei EIF4E3 and 4 was very similar to that observed for L. amazonensis EIF4E4, and contrasting to that seen for its EIF4E3 counterpart, with eight or more spots being identified which differ by slight increases in sizes and are accompanied by equivalent decreases in pl (Supplementary Fig. S1B). As before, these are in agreement with prediction data for both proteins when targeted by multiple phosphorylation events (not shown).

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Using high throughput phosphoproteomic analysis, phosphorylated serine or threonine residues have been previously identified within the N-terminal regions of L. infantum EIF4E4 [16] and T. brucei EIF4E3 [17]. These early assays uncovered single or double phosphorylation events which support our observation but gave no clue to the real extent of the phosphorylation seen here for these proteins. Here to confirm the localization of the multiple phosphorylation events, full-length, HA-tagged, EIF4E3 and 4 were expressed in T. brucei and their expression pattern compared with constructs generated where the unique N-terminus of both proteins was removed. At single-dimension SDS-PAGE, the full-length proteins were represented by fatter bands, indicative of multiple isoforms, whilst their truncated equivalents were represented by single slim bands, suggesting a reduction in the number of isoforms (Fig. 2B). Indeed, through 2D gels, both full-length proteins showed a multiplicity of isoforms compatible with those observed for the native proteins (Fig. 2C) and consistent with the prediction data for multiple phosphorylation events. In contrast, the number of isoforms for the truncated proteins (EIF4E3ΔN-HA and EIF4E4ΔN-HA) was seen to be significantly reduced, although four isoforms were still visible for each. These results are indicative of the majority of phosphorylation events targeting the proteins’ N-terminal segments, with nevertheless, some (most likely three) still targeting the core EIF4E segment of both proteins. They are also mostly compatible with data from a very extensive phosphoproteomic analysis of T. brucei procyclic and bloodstream forms published whilst this paper was under review [18]. Up to ten phosphorylation sites in EIF4E3 and nine in EIF4E4 were mapped, all of them localized to the two proteins’ N-terminal regions. The same analysis also identified six sites for T. brucei EIF4G4, again in agreement with what is reported here. Contrasting with the current results, no phosphorylation was seen for EIF4G3 or within the core eIF4E segment of EIF4E3 and

Fig. 1. Expression analysis of homologues of eIF4F subunits during growth in culture of L. amazonensis. (A) Western-blots showing the expression of the proteins chosen for this study during different stages of L. amazonensis (MHOM/77/LT80016) promastigotes’ growth. Cultures were started in Schneider’s medium (supplemented with fetal calf serum, glutamine and antibiotics), pH 7.2, at 26°C, with stationary cells recently isolated from mouse foot pad lesions and diluted to a density of 10⁶ cells/ml. Aliquots were taken immediately after passing (0 h), at the 2 and 6 hours’ time points and daily after that. Equal loads were run in each lane under denaturing conditions and blotted with polyclonal serum directed against each individual protein. Representative molecular weight markers are shown on the left. (B) Expression analysis of selected homologues upon inhibition of mRNA translation, transcription or processing. Representative aliquots from a standard promastigote growth curve (Control) or curves produced in the presence of Cycloheximide (50 μg/ml), Puromycin (50 μg/ml), Actinomycin D (10 μg/ml) or Sinefungin (1 μg/ml) were analyzed. (C) Phosphoprotein purification assays of protein extracts made from L. amazonensis promastigotes. The procedure was carried out using the Phosphoprotein Purification Kit (Qagen) following the manufacturer’s recommendations. Roughly ~5 × 10⁸ cells were used for extract preparation and ~2.5 mg of the resulting protein solution was loaded per phosphoprotein column provided by the kit. Non-bound (Flowthrough) and bound (Elution) samples were analyzed through Western blots, using as positive control aliquots of the extracts loaded in the columns. (D) Two-dimensional analysis of eIF4E and eIF4A homologues. These were carried out starting with ~40–100 μg of the, urea soluble, cellular extract precipitated with TCA/acetone and loaded onto immobilized dry-strips, pH 6–11 (EIF4E3 and EIF4E4) or pH 4–7 (EIF4AI), for isoelectric focusing in an Ettan IPGphor 3 apparatus (GE-HealthCare). For the second dimension the strips were equilibrated in a modified SDS-PAGE loading buffer, followed by the second dimension run on SDS-PAGE gels and blotting. All four spots seen on the EIF4E3 analysis are numbered. The TryTripDB accession numbers for the L. major homologues of the different proteins studied here are: EIF4AI – LmjF01.0780/LmjF01.0770; EIF4E3 – LmjF28.2500; EIF4E4 – LmjF30.0450; EIF4G3 – LmjF16.1600; EIF4G4 – LmjF16.6060.
4, whilst EIF4A1 was found to be targeted by a single phosphorylation event. Overall this independent approach confirms the high level of phosphorylation detected here for EIF4E3 and 4 and EIF4G4 whilst further studies will be required in order to understand the discrepancies observed for EIF4G3 and EIF4A1 and regarding the core segment from EIF4E3 and 4.

Despite a number of efforts designed to unravel the role of EIF4E phosphorylation in the proteins’ function in mammals, this has not been clarified so far [2], although phosphorylated mammalian EIF4G has been linked to enhanced translation in response to specific stimulus, such as increased levels of branched-chain amino acids [19]. The evidence presented here indicates that phosphorylation may play a significant role in regulating the activity of individual translation initiation factors and protein synthesis in trypanosomatids and phosphorylated EIF4E4 is strongly correlated with active growth and presumably enhanced protein synthesis. In T. brucei, the multiple phosphorylation events on both EIF4E3 and 4 targeting residues in both their N-terminius highlight the uniqueness of the phosphorylation events of the trypanosomatid proteins. In a recent paper EIF4E3 has been shown to be subject to changes in isoform expression profile during nutritional stress, also in L. amazonensis [20], but the differences reported here regarding the phosphorylation profiles observed for this protein in the two trypanosomatid species investigated indicate that this mechanism might not be conserved. Indeed, the number of phosphorylation events targeting EIF4E3 in L. amazonensis seems to be more limited, when compared to EIF4E4 or its T. brucei orthologue, and the differences in size between the two sets of spots identified do not seem to be accompanied by changes in pl compatible solely with phosphorylation changes (compare spots 2 and 3 in Fig. 1D, which differ in size but not in pl), so a second type of post-translational modification might be causing the observed shift in size. The functional significance of these modifications on EIF4E3 function, and protein synthesis, remains to be identified although it may reflect functional differences for this EIF4E homologue between major trypanosomatid groups. Overall, genomic analysis have previously identified a considerably large number of protein kinases in trypanosomatids [21], leading to speculation that novel events of protein phosphorylation, among the post-translational modifications, may have a prominent role in regulating different processes in these organisms. The results presented here indicate that this clearly seems to be case for the initiation stage of protein synthesis.

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References


