Binding Specificities and Potential Roles of Isoforms of Eukaryotic Initiation Factor 4E in Leishmania

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The 5′ cap structure of trypanosomatid mRNAs, denoted cap 4, is a complex structure that contains unusual modifications on the first four nucleotides. We examined the four eukaryotic initiation factor 4E (eIF4E) homologues found in the Leishmania genome database. These proteins, denoted LeishIF4E-1 to LeishIF4E-4, are located in the cytoplasm. They show only a limited degree of sequence homology with known eIF4E isoforms and among themselves. However, computerized structure prediction suggests that the cap-binding pocket is conserved in each of the homologues, as confirmed by binding assays to m7GTP, cap 4, and its intermediates. LeishIF4E-1 and LeishIF4E-4 each bind m7GTP and cap 4 comparably well, and only these two proteins could interact with the mammalian eIF4E binding protein 4EBP1, though with different efficiencies. 4EBP1 is a translation repressor that competes with eIF4G for the same residues on eIF4E; thus, LeishIF4E-1 and LeishIF4E-4 are reasonable candidates for serving as translation factors. LeishIF4E-1 is more abundant in amastigotes and also contains a typical 3′ untranslated region element that is found in amastigote-specific genes. LeishIF4E-2 bound mainly to cap 4 and comigrated with polysomal fractions on sucrose gradients. Since the consensus eIF4E is usually found in 48S complexes, LeishIF4E-2 could possibly be associated with the stabilization of trypanosomatid polysomes. LeishIF4E-3 bound mainly m7GTP, excluding its involvement in the translation of cap 4-protected mRNAs. It comigrates with 80S complexes which are resistant to micrococcal nuclease, but its function is yet unknown. None of the isoforms can functionally complement the Saccharomyces cerevisiae eIF4E, indicating that despite their structural conservation, they are considerably diverged.

Trypanosomatids are ancient eukaryotes that cycle between invertebrate vectors and mammalian hosts, causing a wide range of diseases. Leishmania parasites exist as extracellular flagellated promastigotes in the alimentary canal of female invertebrate vectors and cells of the immune system, transforming into amastigotes. Leishmania parasites are exposed to a broad range of environmental conditions, and stage differentiation is triggered by changes in temperature and pH. Trypanosomatids are characterized by a variety of unique molecular features, including polycistronic transcription, which involves the propagation of protein-coding genes (48, 60) and trans splicing (37), whereby a small leader RNA of 39 nucleotides, denoted spliced leader RNA (SL RNA), is spliced onto the 5′ ends of all mRNAs, providing the cap structure. The trypanosomatid cap is a highly modified structure that, in addition to m7GTP, contains 2′-O-methylations on the ribose moieties of the first four transcribed nucleotides and unusual base methylations on the first adenine and last uridine of the SL RNA. We recently reported on the chemical synthesis of the cap 4 analogue m7Gpppm6AmpAmpCmpm3Um (35). Cap 4 formation in trypanosomatids is essential for trans splicing, and drugs that inhibit methylation stop RNA processing (38). Mutagenesis of the capped nucleotides in the SL RNA also has a negative effect on trans splicing, showing that cap 4 formation is essential for RNA processing (39). Similar to other eukaryotes, the trypanosomatid cap structure is expected to play a key role in translation initiation, although the details of how cap 4 functions in this process have not yet been elucidated.

Cap-dependent translation initiation in eukaryotes is a highly regulated rate-limiting step, which involves assembly of eukaryotic initiation factor 4F (eIF4F), a multiprotein complex on the 5′ cap of the mRNA. eIF4F consists of at least three proteins: the cap-binding protein eIF4E, the ATP-dependent RNA helicase eIF4A, and the scaffold protein eIF4G. The last protein interacts with the other eIF4F subunits as well as with the poly(A) binding protein to create a close mRNA circle during translation initiation (24, 58). The three-dimensional structure of eIF4E from mouse (40), human (59), and Saccharomyces cerevisiae (42) shows conservation of its overall tertiary structure, specifically in the amino acids comprising the cap-binding pocket.

While all eukaryotes express eIF4E, which is essential for translation, several eIF4E homologues that can serve as tissue-specific translation factors or as gene-specific repressors have been identified. All three mammalian eIF4E isoforms bind m7GTP but vary in their ability to bind eIF4G. Only one isoform, eIF4E-1, can rescue the growth of a yeast mutant that fails to express its own eIF4E gene (31). Differences in the ability to complement the yeast eIF4E were also reported for the two isoforms from Arabidopsis thaliana. At-eIF4E-1 can...
fully replace the yeast eIF4E gene, whereas At.eIF4E-2, which encodes eIF(iso)4E, provides only partial complementation and the recovered yeast cells grow very slowly (54). The genome of Drosophila melanogaster also encodes multiple isoforms of eIF4E that vary in their function (28). d4EHP can bind to the cap structure; however, it fails to interact with eIF4G and prevents assembly of the eIF4F complex. A recent study showed that d4EHP functions as a translation repressor that is involved in axis formation during embryonic development. It interacts with Bicoid, a regulatory protein that is expressed in the anterior part of the embryo, and binds a 3’-untranslated region (UTR) element in caudal mRNA. Thus, d4EHP blocks the formation of the translation initiation complex eIF4F by impeding the binding of eIF4E to the cap structure (13). Five eIF4E isoforms were identified in Caenorhabditis elegans. This organism uses both cis and trans splicing and generates transcripts that are capped by m’GTP and trimethyl guanosine (TMG), respectively. IF4E-1, IF4E-2, and IF4E-5 can bind both m’GTP and TMG, whereas IF4E-3 and IF4E-4 can exclusively bind m’GTP (32, 45). Knockout of IF4E-4, the 4EHP homologue in C. elegans, using RNA interference or a null mutation produced a pleiotropic phenotype that included egg-laying defects (17).

Cap-binding proteins in trypanosomatids are expected to have gone through structural adaptations that enable them to interact with the unusual cap 4 structure. Additionally, the nuclear cap-binding protein CBP20 of Trypanosoma brucei was identified and shown to specifically bind cap 4 (36). We formerly described the biochemical and cellular features of a cytoplasmic eIF4E homologue, LeishIF4E-1, and determined its binding affinities to different cap structures (62). Basic features of the other homologues in Leishmania were also reported (16), yet their roles and their binding specificities remain vague. Here, we provide new biochemical information that can shed light on the potential roles of the different LeishIF4E homologues. We describe their binding affinities for a collection of cap analogues, including m’GTP, cap 4, and its intermediates. We also follow their association with high-molecular-weight complexes and assay their interaction with the mammalian translation repressor 4E-BP1. Finally, we show that none of the four isoforms can complement the missing function of eIF4E in yeast, suggesting that they have diverged considerably throughout evolution.

Materials and Methods

Organisms. Leishmania major (Friedlin) and Leishmania amazonensis were cultured in Schneider’s medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, and 25 µg/ml gentamicin.

Cloning and expression of the Leishmania eIF4E isoforms in bacteria for protein purification. The open reading frames of eIF4E isoforms from Leishmania were amplified by PCR, using L. major genomic DNA as a template. The primers were derived from the amino and carboxy termini and included anchor sequences that added restriction enzyme sites. The amplified fragments were cloned into the pHIs-parallel expression vector, yielding plasmids pHISLeishIF4E-1 through pHISLeishIF4E-4. These plasmids were transformed into Escherichia coli BL21 cells, and expression was induced at 20°C in low-phase cultures by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Cells were harvested, resuspended in sonication buffer 1 (SB1) (20 mM Tris-HCl, pH 8, 0.5 mM NaCl, 20 mM imidazole) or sonication buffer 2 (SB2) (20 mM HEPES, pH 7.6, 1 mM dithiothreitol [DTT], 2 mM EDTA, 5% glycerol), and disrupted by sonication. Following sonication, the cell extracts were clarified by centrifugation for 30 min at 20,000 × g. For purification over Ni-nitrilotriacetic acid (Ni-NTA) columns, the cell pellets were disrupted in SB1 and the supernatant was loaded on a HiTrap column using AKTA fast protein liquid chromatography (Amersham Bioscience). Initial washes were done in SB1 containing 20 mM and 100 mM imidazole. The proteins were then eluted with SB1 containing 250 mM imidazole and dialyzed against the fluorescence buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 50 mM NaCl). For purification over m’GTP-Sepharose, the cell pellets were sonicated in SB2 and the supernatant was loaded on m’GTP-Sepharose (Amersham). The protein was eluted by SB2 containing high salt (600 mM NaCl) or the free ligand (m’GTP). The purified proteins were dialyzed against fluorescence buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Antibodies. Recombinant LeishIF4E-1 through LeishIF4E-4 were affinity purified over m’GTP-Sepharose (LeishIF4E-1, LeishIF4E-3, and LeishIF4E-4) or Ni-NTA (LeishIF4E-2), emulsified with complete Freund’s adjuvant, and injected subcutaneously into New Zealand White rabbits (500 µg per rabbit). Two boosts of the protein emulsified in incomplete Freund’s adjuvant were given in 2-week intervals. Serum samples from immunized animals were obtained 10 to 20 days after the boost. Where necessary, Western blot analyses were carried with antibodies that were affinity purified over the recombinant protein that was immobilized on nitrocellulose blots. Mouse monoclonal antibodies against Hsp70 were obtained from R. Morimoto, Northwestern University, and antibodies directed against the murine eIF4E were a gift from N. Sonenberg, McGill University.

Sequence alignment and homology modeling. Four open reading frames encoding homologues of the murine eIF4E were identified in the L. major genome database using a BLAST search. The annotated genes LmjF27.1620, LmjF19.1500, LmjF28.2500, and LmjF30.0450 were denoted LeishIF4E-1 through LeishIF4E-4. The amino acid sequences of all four isoforms were aligned using the MUSCLE multiple sequence alignment algorithm (20) and viewed with Jalview editor (14).

Homology modeling for each of the predicted structures was performed using SwissPDBViewer, based on the published structure of the mouse eIF4E (Protein Data Bank no. 1EJ1a). The models with the lowest Z score of the overall structure were visualized.

Measurements of binding affinity for the different LeishIF4E isoforms. Cap 4 and its intermediates were prepared as previously described (35). The equilibrium association constants (Kd) for complexes of Leishmania eIF4E isoforms with different cap analogues were determined by intrinsic protein fluorescence quenching, as described previously (49, 62). Freshly prepared protein samples were filtered through 0.2-µm polycarbonate filters (Roth) prior to spectroscopic measurements. Protein concentrations were determined by absorption, assuming the following: εm7GTP = 47,500 cm⁻¹ M⁻¹ for LeishIF4E-1, εm7GTP = 63,660 cm⁻¹ M⁻¹ for LeishIF4E-2, εm7GTP = 54,600 cm⁻¹ M⁻¹ for LeishIF4E-3, and εm7GTP = 67,970 cm⁻¹ M⁻¹ for LeishIF4E-4 (calculated from amino acid composition) (23). Fluorescence titration measurements were carried out on an LS-50B spectrophotofluorometer (PerkinElmer Co.) in 50 mM HEPES, pH 7.2, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT at 20°C using protein concentrations that ranged between 0.2 µM and 1 µM. The fluorescence intensity was monitored at 295 nm and observed between 320 and 345 nm recording time and accounting for the sample dilution, the inner filter effect, and the instability of protein fluorescence. The analysis was repeated with proteins that were obtained from multiple preparations and analyzed over SDS-PAGE for their integrity. In all cases, the results were highly reproducible. All measurements were done with freshly prepared proteins that were not exposed to freezing and thawing.

GST–4E-BP pull-down assay. Glutathione S-transferase (GST) was expressed from the pGEX vector, and 4E-BP fused to GST was expressed from pGEX-6p1-h4EBP1 (kindly provided by N. Sonenberg). GST and GST–4E-BP were expressed in E. coli BL21 cells, and the cells were harvested and disrupted by sonication in phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Sigma). GST or GST–4E-BP was immobilized on glutathione-agarose beads and washed with binding buffer (40 mM MOPS [morpholinopropanesulfonic acid], pH 7.2, 50 mM KCl, 250 mM NaCl, 7 mM MgCl₂, 20 mM N-mercaptoethanol, 2 mM MgCl₂ containing 0.5% Triton X-100, and the beads were incubated with supernatants of lysed bacteria that expressed either the mouse eIF4E or the four different LeishIF4E isoforms in binding buffer. The beads were washed extensively five times with 100 volumes of binding buffer containing 0.5% Triton X-100, and the proteins were eluted with Laemmli’s SDS-PAGE sample buffer, separated on 15% SDS-PAGE, and subjected to Western analysis using specific antibodies directed against the murine eIF4E or the four different LeishIF4E isoforms. The GST–4E-BP pull-down assay was a gift from N. Sonenberg, McGill University.

GSTM–4E-BP pull-down assay. Glutathione S-transferase (GST) was expressed from the pGEX vector, and 4E-BP fused to GST was expressed from pGEX-6p1-h4EBP1 (kindly provided by N. Sonenberg). GST and GST–4E-BP were expressed in E. coli BL21 cells, and the cells were harvested and disrupted by sonication in phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Sigma). GST or GST–4E-BP was immobilized on glutathione-agarose beads and washed with binding buffer (40 mM MOPS [morpholinopropanesulfonic acid], pH 7.2, 50 mM KCl, 250 mM NaCl, 7 mM MgCl₂, 20 mM N-mercaptoethanol, 2 mM MgCl₂ containing 0.5% Triton X-100, and the beads were incubated with supernatants of lysed bacteria that expressed either the mouse eIF4E or the four different LeishIF4E isoforms in binding buffer. The beads were washed extensively five times with 100 volumes of binding buffer containing 0.5% Triton X-100, and the proteins were eluted with Laemmli’s SDS-PAGE sample buffer, separated on 15% SDS-PAGE, and subjected to Western analysis using specific antibodies directed against the murine eIF4E or the four different LeishIF4E isoforms. The GST–4E-BP pull-down assay was a gift from N. Sonenberg, McGill University.

Functional complementation in yeast. p301-eIF4E constructs were transformed into S. cerevisiae derivative strain CWO4Δ36 (genotype, MATA ade2 trp1 ura3 leu2 his3 tfα4LEU2 [pVTURA3-4E]). This strain does not grow on media containing 5-fluorouracil acid (5-FOA), as its unique eIF4E gene copy is localized on a plasmid with the gene URA3 as a selectable marker (pVTURA3-
Differential expression of LeishIF4E isoforms under different environmental conditions. L. amazonensis cells were propagated in Schneider’s medium at 26°C, and log-phase cultures were shifted to 33°C for 18 h. The cells were washed twice in PBS, resuspended in lysis buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol and protease inhibitor cocktail), and subjected to immunoblotting using antibodies against LeishIF4E, α-tubulin, and Hsp83. Subcellular fractionation. For total protein extracts, Leishmania parasites (10⁶ cells) were harvested, washed twice in PBS, and extracted in SDS-PAGE sample buffer (33). Protein concentrations were determined by the bicinchoninic acid protein assay kit (Pierce). All fractionation was performed following published protocols (11). The cells were washed twice, and the pellet was frozen in liquid nitrogen and then resuspended in Dignam buffer (10 mM HEPES, pH 7.6, 1.5 mM MgCl₂, 120 mM NaCl, 0.5 mM DTT, 100 µg/ml cycloheximide, and 1 mg/ml heparin). The cell pellet was resuspended in Dignam buffer, and the protein concentrations of the cytoplasmic fraction and the resuspended pellet were determined. Samples of whole-cell extracts and of the subcellular fractions containing equal protein loads were analyzed over 15% SDS-PAGE. The protein blots were reacted with antibodies raised against LeishIF4E, α-tubulin, and Hsp83.

Sucrose gradients and polysome analysis. L. major promastigote cells (5 × 10⁷ to 10 × 10⁷ per gradient) were incubated for 5 min in 5 ml of medium containing 100 µg/ml cycloheximide. After centrifugation, the cells were washed twice in PBS containing 100 µg/ml cycloheximide and once with lysis buffer (15 mM Tris-HCl, pH 7.4, 0.3 M KCl, 5 mM MgCl₂, 0.5 mM DTT, 100 µg/ml cycloheximide, and 1 mg/ml heparin). The cell pellet was resuspended in lysis buffer and incubated on ice for 10 min. The cells were lysed by the addition of Triton X-100 to a final concentration of 1%. The cell lysates were centrifuged at 12,000 × g for 15 min at 4°C. The cell pellets were resuspended in Dignam buffer, and the protein concentrations of the cytoplasmic fraction and the resuspended pellet were determined. Samples of whole-cell extracts and of the subcellular fractions containing equal protein loads were analyzed over 15% SDS-PAGE. The protein blots were reacted with antibodies raised against LeishIF4E-1, LeishIF4E-3, and LeishIF4E-4.

RESULTS

Structural conservation of the cap-binding pocket in the different Leishmania eIF4E isoforms. We identified in the genome database of Leishmania (LeishDB) four different isoforms of eIF4E, denoted LeishIF4E-1 to LeishIF4E-4, based on their homology with the mouse and yeast translation factor eIF4E. We previously reported on the characterization of LeishIF4E-1, and now we describe in detail all four isoforms.

Sequences of eIF4E isoforms from Leishmania and a variety of other eukaryotes were aligned, showing that all four LeishIF4E isoforms had a relatively low degree of homology to the mouse and yeast proteins (30 to 40%); however, the core region that contains residues encompassing the cap-binding pocket is mostly conserved (see Fig. S1 in the supplemental material). The LeishIF4E proteins extend to various sizes of 214, 281, 349, and 308 amino acids for LeishIF4E-1 through LeishIF4E-4, respectively. LeishIF4E-1 lacks the region that corresponds to the carboxy terminus of the mouse protein (amino acids 205 to 217), which is known to serve as a phosphorylation site (56, 66). LeishIF4E-1 and LeishIF4E-2 each possess insertions that are adjacent to the conserved cap-binding pocket. LeishIF4E-3 and LeishIF4E-4 have a long extension at their amino terminus that has no corresponding sequences in the mouse homologous proteins. Amino acids at positions that are expected to promote binding to eIF4G are partially conserved, including the mouse Trp73 and Val69 (26, 41). These partial conservations, combined with the variability in the amino terminus, are not sufficient to predict the ability of each isoform to bind eIF4G (61).

A computerized prediction of the different eIF4E isoforms in Leishmania was carried out, based on the known tertiary structure of the mouse eIF4E (40). Homology modeling suggests that the structure of the cap-binding pocket is basically conserved in each of the four proteins (Fig. 1), including the typical stacking interactions between two Trp residues (corresponding to Trp56 and Trp102 in the mouse protein) and van der Waals contacts between Trp166 and the m⁷G base. These three Trp residues were conserved in LeishIF4E-1 and LeishIF4E-2. In LeishIF4E-4, the equivalent of Trp56 is replaced by Tyr, but this replacement maintains an aromatic residue in this position and therefore is not expected to affect cap binding. In LeishIF4E-3, however, the position that corresponds to the mouse Trp56 was occupied by a Met residue, and despite this, the protein readily interacts with m⁷GTP-Sepharose. Only a few substitutions of Trp56 can be tolerated without affecting binding to m⁷GTP. The replacement of this amino acid with an aromatic residue has little effect on cap binding (1); however, replacement with a Leu residue eliminated completely the ability of the mutated protein to bind m⁷GTP (46). Interestingly, introducing an amino acid that contains a sulfur atom does not eliminate cap-binding activity (31). The basic residues of the cap-binding pocket, which are expected to interact with the phosphate backbone of the cap nucleotides, are conserved in LeishIF4E-1 and LeishIF4E-2. In LeishIF4E-3 and LeishIF4E-4, the only position that contains a basic residue corresponds to Arg157 (according to mouse eIF4E numbering), whereas the other two basic amino acids are replaced with Thr in LeishIF4E-3 and with Ile and Trp in LeishIF4E-4. We assume that the methylations in cap 4 mask the negative charges of the phosphate backbone, allowing exchange of the basic amino acid residues, which are conserved in most eIF4E homologues in metazoa. However, it should be noted that the Lys and Arg amino acids in the cap-binding pocket interact with the phosphate groups of m⁷GpppN and that Thr may also form hydrogen bonds as an electron donor.

Binding affinities between the LeishIF4E isoforms and different cap structures. To examine whether the LeishIF4E proteins could indeed bind m⁷GTP, as suggested by the computerized structure prediction, the recombinant proteins were subjected to affinity purification on m⁷GTP-Sepharose. Three of the isoforms, LeishIF4E-1, LeishIF4E-3, and LeishIF4E-4,
bound to the affinity resin and could be eluted with m7GTP or with high salt buffer (Fig. 2). However, LeishIF4E-2 could not be purified over m7GTP-Sepharose. This result correlated with the Trp fluorescence quenching analysis that showed preferential binding of this protein to cap 4.

Conservation of Trp residues in the cap-binding pocket of the LeishIF4E proteins enabled us to use the Trp fluorescence time-synchronized titration and to determine the $K_\text{in}$ for complexes of eIF4E isoforms and different cap analogues. This method is based on measuring the fluorescence quenching of Trp residues in response to binding of a given ligand (49). The fluorescence spectra of the LeishIF4E isoforms are typical for eIF4E proteins; however, the emission spectra of LeishIF4E-1, LeishIF4E-2, and LeishIF4E-4 are slightly shifted towards the red wavelength compared to that of the mouse eIF4E (data not shown). The fluorescence quenching data are shown in Fig. 3. For LeishIF4E-1 and LeishIF4E-2, in which the sandwich stacking of m7G with two of the Trp residues as well as the Van der Waals contacts with the third Trp in the cap-binding pocket are conserved, the total protein fluorescence was reduced up to approximately 60% and 40% of the original level, respectively (Fig. 3, insert). This range was also typical for the mouse protein (data not shown). The slightly lower level of quenching that was observed for LeishIF4E-2 is possibly due to a masking effect caused by the presence of two additional Trp residues in the sequence outside the cap-binding pocket. For LeishIF4E-3 and LeishIF4E-4, total fluorescence quenching was two- and threefold lower than that observed for LeishIF4E-1. This effect could originate mostly from the replacement of the Trp residue at position 56 (mouse numbering) by either Met or Tyr in LeishIF4E-3 or LeishIF4E-4, respectively.

Binding affinities for the different cap analogues were tested with each of the LeishIF4E isoforms and the mouse eIF4E. The ligands used were m7GTP, an analogue of the consensus eukaryote cap structure, m32,2,7GTP, the hypermethylated cap structure which is found on U snRNAs, an analogue of cap 4 (m7Gppp6AmpAmCmpm3Um) which was recently synthesized (35) along with the nonmethylated tetranucleotide m7GpppAACU (cap 0), and several cap 4 intermediates. The intermediates analyzed were m7Gppp6AmpAm (cap 1), m7Gppp6AmpAmCmp (cap 2), and m7Gppp6AmpAmCmpm3Um (cap 3). The results shown in Table 1 reveal that the association constants for complexes of LeishIF4E homologues with different cap structures were two orders of magnitude lower than those of the mouse protein. We assume that their binding may be further stabilized by association with additional components of the cap-binding complex. However, evaluation of the binding of each protein to various cap analogues indicates that there are basic differences between the LeishIF4E isoforms. LeishIF4E-1 and LeishIF4E-4 each bound cap 4 and m7GTP with similar efficiencies. However, their binding to cap 0 was higher than to cap 4, suggesting that hypermethylations...
on cap 4 have a destabilizing effect on cap binding. LeishIF4E-2 showed clear preferential binding to the methylated cap 4 structure, and the absence of methyl bases (m7GTP or cap 0) decreased its ability to bind this cap analogue. An opposite effect was observed for LeishIF4E-3, which bound specifically to m7GTP ($K_{as} = 0.32 \pm 0.07 \, \mu M^{-1}$) and showed very weak binding to cap 4 ($K_{as} = 0.046 \pm 0.002 \, \mu M^{-1}$) (mean ± standard deviation). A similar range of reduction in the binding affinity caused by hypermethylations was observed for the mouse protein. Among LeishIF4E isoforms, the highest binding affinity for m7GTP and cap 4 was observed for LeishIF4E-4 ($K_{as} = \sim 0.8 \, \mu M^{-1}$ for both ligands). Although the basic amino
identified with certainty in eIF4G, which in mammals is a large protein, has not yet been the missing eIF4E activity in a heterologous organism (28, 53). The criteria for evaluating whether a given protein serves as a translation factor are often based on its ability to interact with the scaffold protein eIF4G and to complement repression (52). The highest binding was observed for the murine eIF4E, which was pulled down by GST 4EBP1, though the interaction with LeishIF4E-1 was weaker.

None of the LeishIF4E proteins complement for yeast eIF4E. The in vivo activity of eIF4E has been evaluated in complementation experiments of a conditionally lethal yeast mutant strain deficient in eIF4E. Each of the four LeishIF4E isoforms varied in their binding specificities, suggesting that they are responsible for different functions.

### Interaction of LeishIF4E isoforms with the mammalian 4EBP1

Different roles can be assigned to homologues of eIF4E that can serve as general translation factors or as repressors (52). The criteria for evaluating whether a given protein serves as a translation factor are often based on its ability to interact with the scaffold protein eIF4G and to complement the missing eIF4E activity in a heterologous organism (28, 53). eIF4G, which in mammals is a large protein, has not yet been identified with certainty in Leishmania, although there are several candidates in the L. major genome database (16; Y. Yoffe et al., unpublished data). As eIF4G interacts with conserved residues in eIF4E and these are also targets for binding of translation regulators that compete with eIF4G (25), we examined the abilities of the different LeishIF4E proteins to interact with mammalian 4EBP1 in pull-down assays.

Bacterial extracts that express GST or the 4EBP1-GST fusion protein were immobilized on glutathione beads and further incubated with the soluble fraction of bacterial extracts expressing each of the recombinant LeishIF4E isoforms or the mouse protein. The eluted complexes were subjected to Western analysis with antibodies raised specifically against each LeishIF4E. As can be seen in Fig. 4, LeishIF4E-1 and LeishIF4E-4, as well as the murine eIF4E, were pulled down by GST 4EBP1, though the interaction with LeishIF4E-1 was weaker.

None of the LeishIF4E proteins complement for yeast eIF4E. The in vivo activity of eIF4E has been evaluated in complementation experiments of a conditionally lethal yeast mutant strain deficient in eIF4E. Each of the four LeishIF4E isoforms varied in their binding specificities, suggesting that they are responsible for different functions.

### Table 1. Equilibrium association constants for complexes of Leishmania eIF4E isoforms and mouse eIF4E

| Cap analogue | Mouse eIF4E (28-217) | LeishIF4E-1 | LeishIF4E-2 | LeishIF4E-3 | LeishIF4E-4 |
|--------------|----------------------|-------------|-------------|-------------|-------------|
| m7GTP        | 121.4 ± 3.0          | 0.16 ± 0.02 | 0.037 ± 0.006 | 0.32 ± 0.07 | 0.8 ± 0.08 |
| m7GpppA      | 4.4 ± 0.1           | 0.072 ± 0.002b | 0.016 ± 0.010 | 0.020 ± 0.005 | 0.21 ± 0.03 |
| Cap 1 m7Gppp3,6,6,2, A | 1.45 ± 0.03         | 0.065 ± 0.004b | 0.020 ± 0.004 | 0.029 ± 0.007 | 0.075 ± 0.031 |
| Cap 2 m7Gppp3,6,6,2, Apm2, A | 10.7 ± 1.4         | 0.067 ± 0.005 | 0.092 ± 0.008 | 0.063 ± 0.010 | 0.23 ± 0.06 |
| Cap 3 m7Gppp3,6,6,2, Apm2, Apm2, C | 22.2 ± 1.0         | 0.19 ± 0.01 | 0.24 ± 0.02 | ND | ND |
| Cap 4 m7Gppp3,6,6,2, Apm2, Apm2, Cpm2,3,2, U | 26.1 ± 0.7         | 0.253 ± 0.003b | 0.28 ± 0.04 | 0.046 ± 0.02 | 0.77 ± 0.08 |
| Cap 0 m7GppApApCpU | 73.3 ± 1.8         | 0.467 ± 0.005c | 0.089 ± 0.013 | 0.12 ± 0.03 | 2.0 ± 0.4 |
| TMG m7G2,2,2,2,GTP | 0.64 ± 0.06 | <0.01 | <0.003 | <0.04 | <0.006 |
| GTP          | 0.032 ± 0.004         | <0.01 | <0.003 | <0.01 | <0.01 |

* Data from reference 22.
* Data from reference 6.
* Data for LeishIF4E-1 (amino acids 12 to 214) (22).
* Data are presented as means ± standard deviations. ND, not determined.
isoforms was cloned into the yeast expression vector p301, and the constructs were transformed into S. cerevisiae strain CWO4#368 (See Materials and Methods). Upon transformation, yeast cells were replicated on minimal medium containing SGal or SGal plus 0.5% 5'-FOA. After 3 days of growth, none of the four Leishmania eIF4E gene constructs was able to grow on plates containing 5'-FOA, and only p301-DmeIF4E-1 (Drosophila eIF4E-1) and p301-eIF4E (expressing yeast eIF4E [yeIF4E], clones 1 and 2) could complement for the loss of endogenous yeast eIF4E. Expression in yeast of the foreign genes tested is shown at the bottom, using extracts of cells freshly transformed and grown in SGal (in the absence of 5'-FOA). The top row shows expression of yeast eIF4E from the pVTURA3-4E plasmid, and the bottom row shows expression of the exogenous genes LeishIF4E-1 to LeishIF4E-4 and deIF4E (the yeast gene expressed from pVTURA3 cannot be distinguished from that expressed from p301).

eIF4E was verified by Western blot analysis of total yeast cell extracts using antibodies against the different eIF4E isoforms analyzed (Fig. 5). It therefore appears that the parasite cap-binding translation factor has diverged throughout evolution, presumably to adapt for binding to cap 4. Thus, the conservation of its sequence and structure is not sufficient to rescue a yeast strain lacking endogenous eIF4E.

Differential expression of LeishIF4E under different environmental conditions. Stage-specific gene expression in Leishmania is controlled by the 3' UTR (3, 30, 44, 50, 65), and an

FIG. 5. None of the LeishIF4E isoforms complements for yeast eIF4E. p301-TRP1-eIF4E constructs were transformed into S. cerevisiae strain CWO4#368. Upon transformation, yeast cells were replicated on minimal medium containing SGal or SGal plus 0.5% 5'-FOA. After 3 days of growth, none of the four Leishmania eIF4E gene constructs was able to grow on plates containing 5'-FOA, and only p301-DmeIF4E-1 (Drosophila eIF4E-1) and p301-eIF4E (expressing yeast eIF4E [yeIF4E], clones 1 and 2) could complement for the loss of endogenous yeast eIF4E. Expression in yeast of the foreign genes tested is shown at the bottom, using extracts of cells freshly transformed and grown in SGal (in the absence of 5'-FOA). The top row shows expression of yeast eIF4E from the pVTURA3-4E plasmid, and the bottom row shows expression of the exogenous genes LeishIF4E-1 to LeishIF4E-4 and deIF4E (the yeast gene expressed from pVTURA3 cannot be distinguished from that expressed from p301).

FIG. 6. Differential expression of the LeishIF4E isoforms in promastigotes and axenic amastigotes. (A) Sequence alignment of the 450-nucleotide amastin element of Leishmania infantum (GenBank accession no. AF195531) with the 3' UTR of LeishIF4E-1 from L. major (AAZ09911) performed with ClustalX. (B) L. amazonensis promastigotes were grown at 26°C and transferred overnight to 33°C. Cells were harvested, extracted, and analyzed on Western blots with antibodies against the different eIF4E isoforms, with anti-Hsp70 and antitubulin antibodies as controls.
amastigote-specific element has been identified (44). Such an element (bearing 68% identity) was found in the 3’UTR of LeishIF4E-1, suggesting that expression of this protein is higher in amastigotes (Fig. 6A). Stage transformation of several Leishmania species can be obtained under axenic conditions (8, 9); thus, a promastigote culture of L. amazonensis that was exposed to 33°C resulted in morphologically altered cells, which changed from flagellated promastigotes to rounded amastigote-like cells. Western analysis of LeishIF4E isoforms isolated from cells exposed to different conditions revealed that the level of LeishIF4E-1 was clearly higher in axenic amastigotes, as expected from the finding of an amastigote-specific element in its 3’UTR. The other isoforms showed milder and less-significant changes at the different temperatures. Protein loads were adjusted by comparing signal intensities to those obtained with antibodies against α-tubulin and antibodies against Hsp70. Both proteins are expressed at all life stages, with a slight increase in the steady-state level of Hsp70 at elevated temperatures (Fig. 6B) (12).

Subcellular distribution of LeishIF4E isoforms. The basal translation factor eIF4E and related homologues that function as translation regulators are located mainly in the cytoplasm, although they can be detected at low levels in the nucleus (34). The eIF4E homologues are distinct from the more distant nuclear cap-binding protein CBC20, which was recently identified in trypanosomes (36). Subcellular fractionation experiments, followed by Western analysis, that were performed on Leishmania promastigotes indicate that all four LeishIF4E isoforms were expressed in the cytoplasm (Fig. 7). Polysome analysis indicated that only LeishIF4E-2 and LeishIF4E-3 comigrated with high-molecular-weight fractions on sucrose gradients (Fig. 8). While the migration profile suggests that LeishIF4E-2 cosediments with polysomes (and with 80S particles), LeishIF4E-3 is found only in the slower-migrating fractions that cosediment with 80S complexes. The addition of EDTA, which dissociates polysomes and other high-molecular-weight complexes, to cell extracts eliminated LeishIF4E-2 and LeishIF4E-3 from the Western blots, most probably due to their degradation. LeishIF4E-1 and LeishIF4E-4 migrated at the top of the sucrose gradient with and without EDTA, indicating that they are not associated with polysomes. Since eliminating Mg²⁺ can affect many complexes in the cell, polysome disintegration was also performed by digestion with micrococcal nuclease. Under limiting conditions, this nuclease cleaves the mRNA between the ribosomes, thus generating 80S particles (18). As shown in Fig. 8, nuclease treatment eliminated the polysomes and shifted the migration of LeishIF4E-2 towards the top of the gradient, supporting the association between LeishIF4E-2 and polysomes. However, migration of LeishIF4E-3 remained unaltered, suggesting that it associates with nonpolysomal complexes.

**DISCUSSION**

The Leishmania genome encodes four isoforms of eIF4E, all of which are located in the cytoplasm. The different isoforms...
show a relatively low degree of conservation with eIF4E of other eukaryotes or among themselves, and the genome of *Trypanosoma brucei* contains four homologues which are highly similar to the *Leishmania* proteins (see Fig. S1 in the supplemental material). Computerized modeling suggests that all of the LeishIF4E isoforms contain a typical cap-binding pocket. Using fluorescence titration measurements, we show that these isoforms demonstrate differential binding capacities for the various cap analogues. The results were supported in part by affinity chromatography, although slight quantitative differences between the two approaches were apparent, for reasons not fully clear to us. LeishIF4E-1 and LeishIF4E-4 each bind to m7GTP and cap 4 with comparable affinities, unlike the mouse eIF4E, which binds to m7GTP fivefold better than to cap 4. This pattern was less apparent when we compared binding between cap 4 and cap 0, where only a slight preference for cap 4 was monitored with LeishIF4E-1, indicating that the length of the analogue should be taken into account. Despite this, the length of the cap analogue does not change the preference of LeishIF4E-2 to cap 4. Overall, it appears that the LeishIF4E isoforms have diverged from their eukaryotic isoforms, possibly in conformity with the appearance of a hypermodified cap structure. The outcome of these changes is reflected in the reduced binding affinity of the recombinant proteins to both m7GTP and cap 4 compared to that of the mammalian protein. The difference between the binding parameters of the mouse and parasite proteins could also be attributed to variations in the purification protocols, as noted elsewhere (49). While the totally insoluble recombinant mouse protein was subjected to a denaturation-renaturation process, the different LeishIF4E isoforms were purified from the soluble fraction of the corresponding bacterial extracts over m7GTP (or Ni-NTA) columns.

It is also possible that the presence of accompanying factors that bind eIF4E is critical for stabilizing the interaction between this protein and its cap ligand. It was shown that the purified trypanosome nuclear complex could discriminate between cap 4 and m7GTP-protected SL RNAs, with preference for cap 4, and this could be attributed to the use of purified multiprotein complexes in the binding assays (36). Cap-binding activity of the *T. brucei* CBC20 complex was ~30-fold higher than that measured for the recombinant LeishIF4E-4. We therefore expect that an eIF4G-like protein is required for stabilizing this interaction, as previously shown for heterologous organisms (61).

The binding efficiency to the dinucleotide analogues consistently drops for all of the eIF4E proteins that were tested, as also noted for the mouse protein (49). In the mononucleotide, there is an additional OH group on the last phosphate that is absent from the dinucleotide. This group is partially ionized and could therefore strengthen the interaction with the positively charged amino acids at the entrance of the cap-binding pocket.

LeishIF4E-3 is a cytoplasmic protein that binds mainly to m7GTP and hardly interacts with cap 4. This most probably excludes it from serving as a basal translation factor in *Leishmania*, since cap 4 is found at the 5′ end of all trypanosomatid mRNAs. The potential role of LeishIF4E-3 remains elusive. However, it is possible that it is involved in the biogenesis of other small nuclear U RNAs, for example, those carrying a TMG cap structure (4, 63). The hypermethylation of m7GTP that generates TMG-protected snRNAs occurs in the cytoplasm of mammalian cells (43) or in the nucleolus of yeast cells, where snRNPs are not obliged to transit through the cytoplasm during their biogenesis (47). It is not clear at this stage which pathway prevails in kinetoplastids. The possibility that LeishIF4E-3 is involved in the modification process of SL RNA is less feasible. If this were the case, we would expect to find this protein where cap 4 methylations take place. It was reported that the enzymes responsible for the 2′-O-methylations of the ribose residues of the SL RNA are nuclear (5, 6, 64) and that SL RNA modification and Sm assembly take place in the nucleus (10, 38). LeishIF4E-3 is associated with a slow-migrating complex in sucrose gradients but not with polysomes. However, additional characterization of this complex is required.

The observation that LeishIF4E-2 binds mainly to cap 4 is rather intriguing since this cap ligand contains m7GTP, and the computerized modeling of this protein suggests that the cap-binding pocket is conserved. The binding of LeishIF4E-2 to cap 4 intermediates increases with their length; however, the highest $K_{\text{on}}$ values were observed for the hypermethylated cap 3 and cap 4 analogues but not for the nonmethylated cap 0 tetranucleotide. We therefore assume that the originally weak binding of the m7GTP moiety by LeishIF4E-2 may be stabilized by the hypermethylations in cap 4. A recent study of the nuclear CBC20 in *T. brucei* also shows a stronger binding to RNAs protected by cap 4 than to RNAs protected by cap 2 (5, 6).

LeishIF4E-2 is a cytoplasmic protein that comigrates with polysomal fractions on sucrose gradients. However, this does not indicate that LeishIF4E-2 is a basal translation factor, since in higher eukaryotes, the majority of the eIF4E is found in the postribosomal supernatant and in 48S complexes. A recent report describing the structure of the 80S ribosome from *Trypanosoma cruzi* revealed that the 40S ribosomal subunit has a unique structure that forms a large helix located in the vicinity of the mRNA exit channel. The authors of that report suggest that this region could serve as a target for direct interaction with the RNA and could contain a distinct factor for stabilizing the interaction between the ribosome and cap 4-protected mRNAs (21). LeishIF4E-2 could therefore function as a mediator between the ribosome and the parasite mRNAs.

A bioinformatics approach did not reveal unequivocal homologues of eIF4G or 4EBP in *Leishmania*. Five candidates of eIF4G were recently identified in LeishDB, all of which contain the middle HEAT domain of eIF4G, but only LmEIF4F3 could bind eIF4A (15, 16). In the absence of a more detailed analysis of the eIF4G candidates, we tested the capacity of each LeishIF4E to interact with mammalian 4EBP1, a translation repressor that competes with eIF4G on binding to eIF4E (24, 27). A strong binding to h4EBP1 was observed with LeishIF4E-4 and, to a lesser extent, with LeishIF4E-1; thus, if 4EBP1 and eIF4G interact with the same residues in eIF4E, LeishIF4E-4, and LeishIF4E-1 could be part of the eIF4F complex. The eIF4E-eIF4G interaction is promoted by the cap-distal structure of eIF4E. In addition to conservation of specific residues such as Trp73 and Val 69 (26, 41), other regions of the molecule are involved, including the N terminus (61). Thus, conservation of these residues cannot account for...
the ability to bind 4EBP1, as was also noted for 4EHP, a cap-binding protein that does not interact with 4EBP1, despite sequence conservation of specific amino acids which are dedicated to this interaction (13, 55).

Neither LeishIF4E-1 nor LeishIF4E-4 is found in the polyribosomal fraction. This is in accordance with the analysis of eIF4E in rabbit reticulocyte lysates, where eIF4E is found in the postsorbal supernatant and in the 48S fraction (29, 51). Indeed, analysis of the sucrose gradient profile shows that LeishIF4E-1 and LeishIF4E-4 are found at the top of the gradient and that LeishIF4E-4 comigrates with 48S particles (Fig. 8).

LeishIF4E-1 contains an RNA sequence element in its 3′ UTR, which bears 68% homology to the amastigote-specific element found in the amastin gene family and Hsp100, which show a stage-specific pattern of expression (11, 44). Indeed, expression of LeishIF4E-1 in axenic amastigotes of L. amazonsis is much higher. Expression of the other three isoforms was subject to much milder changes, (~30%).

Functional analysis of eIF4E homologues can be deduced from complementation studies. S. cerevisiae contains a single eIF4E isoform which is essential for its survival. eIF4E isoforms from heterologous organisms can replace yeast eIF4E, indicating that these proteins are evolutionarily conserved (28, 53, 54). None of the LeishIF4E isoforms could complement yeast eIF4E, possibly due to their high structural divergence. Unfortunately, there is yet no in vitro translation system for any trypanosomatid that can initiate translation. The only cell-free system that was developed for T. brucei can elongate but not initiate protein synthesis (19). Thus, we base the functional evaluation of different eIF4E isoforms in Leishmania mainly on biochemical analysis.

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