Identification in the Ancient Protist *Giardia lamblia* of Two Eukaryotic Translation Initiation Factor 4E Homologues with Distinctive Functions†

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Eukaryotic translation initiation factor 4E (eIF4E) binds to the m7GTP of capped mRNAs and is an essential component of the translational machinery that recruits the 40S small ribosomal subunit. We describe here the identification and characterization of two eIF4E homologues in an ancient protist, *Giardia lamblia*. Using m7GTP-Sepharose affinity column chromatography, a specific binding protein was isolated and identified as *Giardia* eIF4E2. The other homologue, *Giardia* eIF4E1, bound only to the m2,2,7-GpppN structure. Although neither homologue can rescue the function of yeast eIF4E, a knockdown of eIF4E2 mRNA in *Giardia* by a virus-based antisense ribozyme decreased translation, which was shown to use m7GpppN-capped mRNA as a template. Thus, eIF4E2 is likely the cap-binding protein in a translation initiation complex. The same knockdown approach indicated that eIF4E1 is not required for translation in *Giardia*. Immunofluorescence assays showed wide distribution of both homologues in the cytoplasm. But eIF4E1 was also found concentrated and colocalized with the m2,2,7-GpppN cap, 16S-like RNA, and fibrillarin in the nucleolus-like structure in the nucleus. eIF4E1 depletion from *Giardia* did not affect mRNA splicing, but the protein was bound to *Giardia* small nuclear RNAs D and H known to have an m2,2,7-GpppN cap, thus suggesting a novel function not yet observed among other eIF4E homologues in eukaryotes.

Most eukaryotic mRNAs, small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs) are modified posttranscriptionally in the nucleus at their 5’end by addition of a 7-methylguanosine (m7G) cap linked by a 5’-5’-triphosphate bridge to the first transcribed residue (11, 45). The cap of snRNAs and snoRNAs is then further methylated at its N2 position in the cytoplasm or nucleus to yield an N2,N2,7-methylguanosine (m2,2,7-G) cap (41). The m7GpppN-cap structure is required for recruitment of mRNA by the translational machinery, whereas the m2,2,7-GpppN-cap plays crucial roles in gene expression, such as mRNA splicing, methylating, pseudouridylation, and rRNA processing and ribosome assembly (41).

The m7GpppN-cap of mRNA is recognized by eukaryotic initiation factor 4E (eIF4E), which interacts also with scaffold protein eIF4G (13, 30). The latter binds to an ATP-dependent RNA helicase, eIF4A, and ribosome-bound eIF3 to recruit the 40S small ribosomal subunit to the 5’end of an mRNA for translation initiation (14, 16, 20, 24). eIF4E is not only a translation initiation factor but also a protein that modulates the overall rate of translation and the mRNA selectivity of the translation apparatus (11, 13). The functional importance of eIF4E is illustrated by the lethality of eif4e gene disruption in *Saccharomyces cerevisiae* (3). Both the function and structure of eIF4E have been conserved throughout evolution; human and yeast homologues are 31% identical at the amino acid level, and human eIF4E can rescue *eif4e* gene disruption in *S. cerevisiae* (3). In spite of the variable N and C termini among the eIF4Es, a core of about 170 amino acids within the eIF4E molecule has been apparently well conserved among all eukaryotes (28, 32, 49). Deletion analysis of eIF4E in *S. cerevisiae* showed that this core region alone is sufficient for cap recognition (49). The three-dimensional structures of cap analogue-bound eIF4Es from mice (28), humans (48), and *S. cerevisiae* (29) have been resolved. They each contain an eight-stranded antiparallel β-sheet on top of three α-helices in a cupped-hand shape. Two Trp residues, located within a narrow cavity inside the concave surface, hold the guanine residue of the cap analogue through π-π stacking interaction (4, 28, 29, 51), which is further stabilized by the hydrogen bonds among the purine base, the polypeptide backbone, and a conserved Glu residue. A third Trp residue recognizes the N7-methyl group of the cap structure, thus contributing to the specificity of cap binding (43).

Recently, multiple eIF4E homologues have been reported in mammals (43), *Drosophila melanogaster* (25), *Caenorhabditis elegans* (18, 21), plants (44), and *Schizosaccharomyces pombe* (38). This multiplicity could reflect simple redundancy but may also suggest more complex roles for the eIF4E isoforms beyond mere involvement with translation initiation. The issue was partially addressed in *C. elegans*, in which the existence of several eIF4E-like proteins was attributed to the necessity of recruiting mRNAs with different caps (7- and 2,2,7-methylguanosine) to the initiation complex (18, 21). In another case, one of the two eIF4E homologues in zebra fish, eIF4E-1B, cannot bind to the m7GpppN-cap structure and plays no apparent role in translation initiation. Though it shares 66% identity with human prototypical eIF4E and possesses a conserved cap-binding...
domain, its function remains unknown (19). In most other cases, the biological significance in multiple eIF4E-like proteins remains unclear. To distinguish the prototypical eIF4E homologues in the initiation complex from the other eIF4E homologues in humans, the former has been renamed eIF4E1 (20).

*Giardia lamblia,* a parasitic protozoan, is one of the deeply branched and most primitive eukaryotes based on phylogenetic analysis of its small rRNA, as well as many other proteins (1, 17, 42, 46). Within its small 12-Mb genome, most of the genes reported so far do not contain introns (36). Their transcripts have exceedingly short 5′ untranslated regions (5′-UTRs), ranging from 0 to 14 nucleotides, and similarly short 3′-UTRs of 10 to 30 nucleotides (2). In a previous study, we demonstrated that translation of an mRNA in *Giardia* could initiate efficiently from the first initiation codon located only 1 nucleotide downstream from the m7Gpppn-capped structure (26). When the 5′-UTR between the cap and AUG was lengthened beyond 9 nucleotides, translation initiation was decreased drastically. This is in contrast to what were observed in yeast and mammalian systems, in which a minimum of 20 nucleotides are required in the 5′-UTR to allow optimal ribosome scanning and prevention of scanning leakiness (22, 23). This interesting discrepancy between *Giardia* and higher eukaryotes suggests the presence of a unique and perhaps much simpler protein synthetic machinery in *Giardia* that may not depend on ribosome scanning but recognize the simple structure of cap-AUG as a sufficient signal for translation initiation. The precise cap structure in *Giardia* RNAs has, however, not yet been determined, even though m7Gppnpn-capped mRNA introduced into the cells appeared to express well (26). Eight m2,2,7GpppN-capped snRNA species were also identified in *Giardia* (35). Two of them, snRNA D and snRNA H, shared some common features with snoRNAs and were associated with fibrillarin, which suggested their location in the nucleolus and their potential role in rRNA processing and ribosome maturation (12, 34, 35).

To begin elucidating the structure of translation initiation machinery in *Giardia,* we describe in this report the identification and characterization of two eIF4E homologues. eIF4E2 binds to m7Gpppn-capped snRNA and is apparently responsible for translation initiation as a functional equivalent of human eIF4E1, whereas eIF4E1 binds to the m2,2,7GpppN-capped snRNA-like molecules and is not involved with translation initiation. The protein is associated with a nucleolus-like structure in *Giardia* and could perform the function of a snoRNA cap-binding protein for rRNA processing and ribosome biogenesis (15).

**MATERIALS AND METHODS**

**Construction of recombinant expression plasmids.** Two pairs of primers were designed to amplify the two *Giardia* eIF4E homologues by reverse transcription (RT)-PCR. 5′-GGCATATGACCAGCTACTGCGCCG-3′ (Ndel site underlined) and 5′-GGAAAGCTTTACCGAGCTTTTGCCTGCGG-3′ (Hind III site underlined) were used for amplifying the 654-bp eIF4E1 cDNA encoding a 218-amino-acid 25-kDa protein, whereas 5′-GGCATATGGACGACATAT ACCTGAGG-3′ (Ndel site underlined) and 5′-GGAAAGCTTTACCGAGCTTTG ATGGGAGATCGC-3′ (HindIII site underlined) were employed for synthesizing the 504-bp eIF4E2 cDNA coding for a 168-amino-acid 20-kDa protein. The two full-length cDNA fragments were cloned into pGEM-T Easy vector, released with Ndel/HindIII digestion, and inserted into the PET28b expression vector (Novagen). The recombinant plasmids pET41e1 and pET42e2 thus produced were verified by DNA sequencing and transformed into the cells of Escherichia coli BL21(DE3)/pLySs for expression. **Expression and purification of *G. lamblia* eIF4Es from transformed *E. coli.* A colony of the transformed *E. coli* cells was cultured overnight in 50 ml of LB medium with 50 µg/ml kanamycin at 37°C. The culture was transferred into 1 liter of LB medium and incubated until the optical density at 600 nm reached ~0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to 200 µM, and the cells were incubated for another 2 h. The cells were harvested by centrifugation and suspended in lysis buffer (100 mM Na2HPO4, 10 mM Tris-Cl, 8 M urea, pH 8.0) at a ratio of 5 ml of buffer per g of wet cells. The C-terminally His-tagged recombinant protein was purified from the lysate under denatured conditions as suggested by the manufacturer (QIAAGEN). Eluents thus collected were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot assay using mouse anti-His tag monoclonal antibody (1:1,000 dilution). Purified eIF4E1 and eIF4E2 (10 mg of each) were used to raise polyclonal antibodies in rabbits at Animal Pharm Services. The antiserum thus obtained were titrated against the recombinant antigens by enzyme-linked immunosorbent assay and used for subsequent immunoblotting and immunofluorescence assays.

**m7GTP-Sepharose affinity column chromatography.** Trophozoites of *Giardia* strains WB and WBI (WB strain infected with giardiavirus) in 1.4-liter fresh cultures (50) were cooled on ice for 20 min, harvested at 3,000 × g for 30 min, washed in phosphate-buffered saline (PBS) (137 mM NaCl, 8 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4), and suspended in 10 ml of buffer A (20 mM HEPES [pH 7.4], 150 mM NaCl, 2 mM dithiothreitol) with 1 mM EDTA. 2 mM dithiothreitol was added to a 5 ml of buffer A and 20 CV of buffer A plus 0.1 mM m7GTP. Protein still attached to the column was eluted with 1 ml of buffer A plus 0.1 mM m7GTP four consecutive times.

**Protein identification by MALDI-TOF mass spectrometry.** Proteins in the final elute were separated in SDS-PAGE, and the gel was stained with E-Zinc as instructed by the manufacturer (Invitrogen). The specific protein band at about 20 kDa was cut off the gel and destained with the desaturing buffer (Invitrogen). After reduction and alkylation, the protein band was treated with 12.5 µg/ml trypsin (Promega) at 37°C overnight. The digest, cleaned with ZipTip-C18, was analyzed with a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry instrument (Voyager DE-STR mass spectrometer; Applied Biosystems). Molecular weights of individual peptide peaks were compared to the National Center for Biotechnology Information genome database using MS-FIT of the ProteinProspector program, version 4.0.5, from the University of California San Francisco (5).

**Western blot analyses.** Electrotrope of protein was obtained at 50 µg in 10% (vol/vol) methanol–25 mM Tris-Cl (pH 7.4)–192 mM glycerol at 4°C for 1 h. Polyvinylidene difluoride membranes were blocked with 5% milk in TBST buffer (50 mM Tris-Cl pH 8.0, 200 mM NaCl, 0.1% Tween 20) and incubated with mouse monoclonal anti-enc IgG (1:5,000 dilution) and rabbit polyclonal anti-Giardia eIF4E1 or eIF4E2 antibodies. Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (1:10,000 dilution; Amersham) were then added prior to washing and visualization with enhanced chemiluminescence (Amersham).

**Cap-binding specificity assay.** A 200-bp DNA fragment containing the T7 promoter and an AAGAAGCC stretch before the ATG initiation codon was digested from pGlyLu (26) with EcoRI/BstXI and gel purified. The fragment was used as the template for in vitro synthesis of the 178-nucleotide RNA molecule with T7 RNA polymerase in the presence of 12 mM mG5′ppp(5′)G or 12 mM m2,2,G(5′)ppp(5′)G as suggested by the manufacturer (Ambion). The product was purified by phenol-chloroform extraction and ethanol precipitation and examined for integrity by electrophoresis in a 1.5% agarose gel. The concentration of each mRNA sample was estimated at 260 nm in a Beckman DU 7 spectrophotometer.

The RNA sample (3 µg) in 50 µl of TE buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA) was labeled with biotin using the BrightStar Psoralen-Biotin nonisotopic labeling kit in accordance with the instructions of the manufacturer (Ambion). The excess biotin was removed by n-butanol extraction, and the biotinylated
RNA samples were used in the in vitro pulldown experiments described as follows.

Constructs pGBK4E1 and pGBK4E2 with T7 promoters and HA or c-myc located at the 5' ends of the eIF4E1 and eIF4E2 open reading frames, respectively, were each used as a template in the rabbit reticulocyte TnT quick coupled transcription-translation system (Promega) for in vitro synthesis of the corresponding proteins. The reaction mixtures (5 μl of eIF4E1 and 20 μl of eIF4E2) were each incubated with 1 μg of the biotinylated RNA sample described above in 500 μl of the binding buffer (50 mM HEPES-KOH [pH 7.6], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 5%[vol/vol] glycerol) at 4°C for 60 min and centrifuged at 12,000 rpm for 3 min to remove any aggregated material. Pre-blocked streptavidin-Sepharose beads (Novagen) were added to the supernatant, and the mixture was incubated at 4°C for 60 min with gentle shaking. The beads were washed twice with the binding buffer, and the bound proteins were eluted off the beads with 200 mM GTP, m7GTP, or m2,2,7GpppG. Aliquots of the collected fractions were analyzed by SDS-PAGE.

Complementation in S. cerevisiae. The pGADT7 vector (Invitrogen) was converted to pGADT7 by removing the AD domain and the hemagglutinin (HA) tag peptide domain. pGADT7 was used to construct the expression cassette of Giardia eIF4E1 and eIF4E2 in yeast. The two cDNAs were removed from pET4E1 and pET4E2 with NdeI/XhoI digestion and inserted into pGADT7.

Yeast eIF4E in pYCP33-supex2 (a kind gift from John McCarthy of UMIST) was
FIG. 2. Giardia eIF4Es are incapable of complementing the function of S. cerevisiae eIF4E. S. cerevisiae yTHC strain YSC1180-7428777 was transformed with the Leu-selectable vector pGADT7 containing cDNAs encoding yeast eIF4E, Giardia eIF4E1, and Giardia eIF4E2, respectively. Following selection on the –Leu plate under G418 selection, cells from individual colonies were serially diluted and applied to synthetic complete medium plates lacking leucine but containing doxycycline.

removed by NdeI/EcoRI and inserted into pGADT7 as the positive control. The recombinant constructs were verified by restriction enzyme digestion and DNA sequencing.

The S. cerevisiae yTHC strain YSC1180-7428777 (Open Biosystems) was grown in liquid YPD broth containing 200 μg/ml Geneticin to be made competent for transformation. Cells transformed with the construct were plated on –Leu and Geneticin plates and incubated at 30°C for 3 days. Cell colonies were selected, and the cells from individual colonies were used in various dilutions to inoculate plates with 10 μg/ml doxycycline and incubated at 30°C for 2 days.

Ribozyme knockdown of gene expression in G. lamblia. Fragments (~400 bp) of the 5′ ends of the Giardia eIF4E1 and eIF4E2 cDNAs were each inserted into the pC631neo plasmid in which neo was used to substitute for pac in the pC631pac plasmid (40). A hammerhead ribozyme sequence was introduced into the constructs by site-directed mutagenesis using two pairs of primers (7, 8, 33): 4E1RZ1 (CGAGGAAGAGCTCTGTCGTTTCGTCCTCACGGACTCATCAG GTCGATAAACATCCC) plus 4E1RZ2 (GGAGTATTACGACTGTAAGGT GCGGAAGCAAAACGCAGGACCCAGTCTCCTG) and 4E2RZ1 (GTCTATC AGTTTTTGCTCTGCTACTCAATCGGCGCCAACGTATT GAC) plus 4E2RZ2 (GTCATAAGCCTTGGCCCTGATGTCGCTGAG GACGAAACGCAAACACTGATAGC). The two final constructs, pC631neo-eIF1RZ and pC631neo-eIF2RZ, together with pC631neo as a negative control, were each linearized by NdeI and transcribed in vitro with T7 RNA polymerase as instructed by the manufacturer (Ambion). The RNA thus synthesized, containing an antisense sequence of eIF4E1 or eIF4E2 plus an inserted hammerhead ribozyme, was electroporated into Giardia WBI trophozoites by a previously described procedure (26, 52). G418 (200 μg/ml) was added to the cell culture for selection of transfectants. When cells grew to confluency, G418 was increased stepwise to 4 mg/ml and the incubation continued until confluency was reached again each time. The efficiency in knocking down the target mRNA was examined by semiquantitative RT-PCR, and the growth of transfectants was monitored in three independent experiments and presented with calculated standard deviations.

Semiquantitative RT-PCR. Total RNA was extracted from antisense RNA-ribozyme-transfected Giardia trophozoites, and the concentration of each RNA sample was measured spectrophotometrically. An equal quantity of RNA was used as the template in a semiquantitative one-step RT-PCR (Invitrogen) using primers of corresponding genes as instructed by the manufacturer. DNA fragments resulting from RT-PCR and identified in agarose gel were quantitated by laser densitometer tracing.

In vitro transcription, RNA transfection of Giardia, and luciferase assay. The luciferase-containing plasmid pGiaLuc mentioned above (26) was linearized with SacII and used as the template for in vitro synthesis of the luciferase mRNA as suggested by the manufacturer (Ambion) in the presence of 15 mM GTP, 12 mM mG(5′)ppp(5′)G, or 12 mM mG(2′,3′)G(5′)ppp(5′)G. The products were purified by phenol-chloroform extraction and ethanol precipitation and examined for integrity by electrophoresis in 1.5% agarose gel. The concentration of each mRNA sample was estimated by its absorbance at 260 nm in a Beckman DU7 spectrophotometer, and 20 μg of each in vitro transcript was introduced into Giardia WBI trophozoites by electroporation (26). The luciferase activity thus expressed in Giardia was assayed h after electroporation as previously described (26).

Pulldown of Giardia snRNAs by GST-fused eIF4E1. The Giardia eIF4E1 gene was cloned into pGEX4T-3 vector between BamHI and NotI restriction sites, and the recombinant plasmid, pGEX4E1, confirmed by DNA sequencing, was transformed into E. coli BL21(DE3) cells. The eIF4E1 protein fused with glutathione S-transferase (GST) at its N terminus was overexpressed and purified following the instruction of the manufacturer (Amersham). Four micrograms of the fusion protein was incubated with 40 μg of Giardia total RNA for 1 h. Glutathione-Sepharose 4B beads (50 μl), thoroughly washed with PBS and preincubated with 1 mg of yeast tRNA and 1 mg of salmon sperm DNA, were incubated with the protein-RNA mixture for 30 min at room temperature. After a thorough wash, the beads were eluted with glutathione and the eluate was extracted with phenol and precipitated. One-tenth of the RNA sample was used for semiquantitative RT-PCR assay using primers RNA D5 (5′-GTCCTAGAC GCCTCTGTGGGAAATGCT-3′) and RNA D3 (5′-AAGGACTATAGGGGGCG GTGATTACGG-3′) for snRNA D, primers RNA H5 (5′-CTGCTCTCTGATGGCAG-3′) and RNA H3 (5′-GAATTCGATTAGCAGAAAACACTTTCG-3′) for snRNA H, and primers RNA J5 (5′- AATGTCAGCGAACCACGGCG AACG-3′) and RNA J3 (5′-ATTAGTAAAGGAAAAAGCTCCGGACATC-3′) for snRNA J.

Immunofluorescence assays. Giardia trophozoites cultivated in vitro were harvested, washed with PBS three times, and fixed in 4% paraformaldehyde in PBS for 30 min. The cells were then washed three times in PEM buffer (100 mM PIPES, 2 mM EDTA, 1 mM MgSO4, pH 6.9), permeabilized with 0.1% Triton X-100 for 5 min, and washed another three times with PEM buffer. The fixed cells were blocked in blocking buffer (2% bovine serum albumin and 0.1% Triton X-100 in PBS) for 60 min at room temperature and incubated with the following primary antibodies for another 60 min: purified anti-eIF4E1 rabbit polyclonal antibodies (1:1000 final dilution), purified anti-eIF4E2 rabbit polyclonal antibodies (1:1000 final dilution), mouse monoclonal antibody against yeast fibrillarin (1:500 final dilution), and mouse monoclonal antibody against the m2,5-G cap structure (1:1000 final dilution). Alexa Fluor 488- or 555-conjugated secondary donkey antibodies against mouse or rabbit IgG (1:1000 dilution; Invitrogen) were then applied to the cells and incubated for another 60 min at room temperature. Slides were mounted in the presence of 1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) and examined under an Olympus 1X70 microscope equipped with bright-field and epifluorescence optics. The images were acquired with the MetaVue software.

RNA fluorescence in situ hybridization. Giardia trophozoites were harvested, suspended in PBS, placed on coverslips pretreated with 0.5% poly-L-lysine, and incubated at 37°C for 30 min to allow the trophozoites to adhere. The cells were then fixed in 4% paraformaldehyde for 30 min at room temperature and washed with PBS and 2× SSC (300 mM NaCl, 30 mM sodium citrate) each for 5 min. Triton X-100 (0.5%) was added and incubated for 15 min at room temperature, and the cells were dehydrated in 70% and 100% ethanol and denatured in 70% formamide in 2× SSC at 70°C for 2 min and dehydrated again in 70% ethanol
RESULTS

Presence of two eIF4E homologues in G. lamblia. Homology searches using human eIF4E-1, 4E-HP, and S. cerevisiae eIF4E protein sequences revealed in the Giardia genome database two homologous genes encoding two eIF4E isoforms. These two genes were designated Giardia eIF4E1 and eIF4E2.

The sequence of Giardia eIF4E1 encodes a putative protein of 218 amino acids with an estimated molecular mass of 25,129 Da. It shares 13% identity and 29% similarity with human eIF4E-1 (Fig. 1A) and contains four out of the eight conserved Trp residues in human eIF4E-1 and three of the other four residues are two Tyr and one Phe. For the two Trp residues (Trp56 and Trp102) in human eIF4E-1 that are known to hold the guanine moiety of the cap, Trp56 is replaced with Phe whereas Trp102 remains unchanged at the corresponding positions in Giardia eIF4E1. The conserved Glu103 in human eIF4E-1 that stabilizes the interaction with the cap and a third Trp residue (Trp166 in human eIF4E-1) that recognizes the N7-methyl group of the cap structure remain also conserved in Giardia eIF4E1. Thus, in spite of the relatively poor sequence identity with human eIF4E-1, Giardia eIF4E1 could still function as a cap-binding protein. There is also in Giardia eIF4E1 a basic amino acid-rich sequence, RRSSRAPSEERSRTHKR, at the C terminus (Fig. 1A), which was predicted to be a bipartite nuclear localization signal, suggesting that eIF4E1 may locate and even function in the nucleus.

The Giardia eIF4E2 gene (Fig. 1A) encodes a putative protein of 168 amino acids with a molecular mass of 19,874 Da. It is also quite divergent from other eIF4Es, with only 14% identity and 27% similarity with human eIF4E-1. However, Trp56 and Trp102 in human eIF4E-1 are substituted only by Phe, whereas Glu103 and Trp166 are conserved in Giardia eIF4E2. This protein is thus likely capable of binding to the cap as well. There is no apparent nuclear targeting signal in this protein, which could mean that it cannot enter the nucleus and remains and performs its function in the cytoplasm of Giardia (see below).

Alignment of the two Giardia eIF4E amino acid sequences with each other showed that they are not similar to each other at all except for some of the conserved residues in the 170-amino-acid core. Even there, there are only 8% identity and 20% similarity (Fig. 1A), suggesting that though both proteins may be able to bind to the cap structure, they may perform distinctive biological functions in Giardia.

Phylogenetic analysis revealed that the two Giardia eIF4Es are highly divergent from all the other eIF4Es (Fig. 1B). While eIF4E1 is the farthest removed from the others, eIF4E2 shows a closer relationship only to C. elegans eIF4E4 and Arabidopsis thaliana nCBP. It verifies the fact that Giardia is one of the most deeply branched eukaryotes and raises the interest in identifying the precise functions of these two proteins in Giardia.

cDNAs containing the complete open reading frame sequences of eIF4E1 and eIF4E2 were amplified by RT-PCR from Giardia mRNAs. The two recombinant proteins, each tagged with a hexahistidine peptide at the C terminus, were overexpressed in transformed E. coli, purified under denaturing conditions (see Fig. S1 in the supplemental material), and
Neither Giardia homologue can complement an eIF4E knockout in *S. cerevisiae*. Since human eIF4E-1 is capable of complementing the eIF4E function in *S. cerevisiae* (3) in spite of the sequence divergence (31% identity), we tested the two homologues from Giardia for complementing the function of yeast eIF4E (3). In *S. cerevisiae* yTHC strain YSC1180-742877, the promoter of endogenous eIF4E was replaced with a tetracycline-titratable promoter. Thus, the expression of endogenous eIF4E can be switched off by doxycycline, forcing dependence of cell growth on an ectopic expression of another functional eIF4E. cDNAs of the two Giardia eIF4Es, as well as yeast eIF4E, were each cloned into pGADT7/H11032, which has a LEU selection marker and allows insert expression from the constitutive ADH1 promoter. Following transformation and selection for transformants in leucine-deficient medium, cell cultures were applied to doxycycline-containing plates in serial dilutions. The outcome (Fig. 2) indicates that neither eIF4E1 nor eIF4E2 from *Giardia* is capable of rescuing the growth of yeast lacking endogenous eIF4E. The failure could be attributed to the significant sequence divergence between *Giardia* and yeast eIF4Es (18% identify for eIF4E1 and 15% for eIF4E2). It could also raise doubt about whether the two homologues perform a function in *Giardia* similar to that of yeast eIF4E in *S. cerevisiae*.

**Purification and identification of cap-binding proteins from *G. lamblia***. With the identification of two eIF4E homologues in the *Giardia* genome database and expression of their protein products in *E. coli*, it will be important to verify if these two proteins are also expressed in *Giardia* and perform the anticipated cap-binding function.

**Lysate of *Giardia*** trophozoites was subjected to m^7^GTP-Sepharose affinity column chromatography. Column eluates were fractionated by SDS-PAGE and visualized with E-Zinc staining (Fig. 3A). One protein band with an estimated molecular mass of 20 kDa was eluted with m^7^GTP and tentatively designated a cap-binding protein (Fig. 3A, lane 5). The gel containing this protein band was removed, digested with trypsin, and subjected to MALDI-TOF mass spectrometric analysis (see Fig. S2 in the supplemental material). A database search using the peptide masses thus obtained resulted in identification of a single match, eIF4E2, with 46% of its sequence covered by the identified peptides. There is thus little doubt that eIF4E2 is expressed in *Giardia* and that it binds to the m^7^GpppG-cap structure.

From the same m^7^GTP-Sepharose affinity column chromatography, eIF4E1 was, however, not identified in the eluent (Fig. 3A), which was confirmed by subsequent Western blot analysis (Fig. 3B). The results suggest that eIF4E1 may be either not expressed in *Giardia* trophozoites at all or incapable of binding to the m^7^GpppN-cap structure. Unfortunately, both eIF4E1 and eIF4E2 were undetectable in a Western blot from a crude lysate of 10^7^ Giardia trophozoites (data not shown). It was not until the subsequent immunofluorescence assays that the expression of both proteins in the trophozoites was verified (see below). eIF4E1 is thus present in *Giardia* but most likely incapable of binding to m^7^GTP.

**Distinctive cap-binding specificities of eIF4E1 and eIF4E2**. The cap-binding properties of HA-tagged eIF4E1 and c-myc-
tagged eIF4E2, both synthesized from the rabbit TnT quick coupled transcription-translation reticulocyte system, were further examined by m7GTP affinity column chromatography. Effluents from the column were examined by Western blot analysis. The results indicated that eIF4E1 was not retained by the column (Fig. 4A), whereas eIF4E2 was retained and eluted only by m7GTP (Fig. 4B).

To probe into the possibility that eIF4E1 may bind to a different cap structure such as m2,2,7GpppN, known to exist in Giardia (35), c-myc-tagged eIF4E1 was examined in another binding assay with RNA molecules of different caps added as potential substrates. The RNA molecules were biotinylated for pulldown by streptavidin-Sepharose beads. The data in Fig. 4C and D show that while eIF4E1 cannot be pulled down by the m7GpppG-capped RNA, it is coprecipitated with the m2,2,7GpppN-capped RNA. The protein is thus likely an m2,2,7GpppN-binding protein. Testing of eIF4E2 in a similar experiment showed that it was pulled down by m7GpppG-cap but not by m2,2,7GpppN-cap (data not shown).

Only eIF4E2 plays an essential function in protein synthesis in Giardia. To elucidate whether eIF4E1, eIF4E2, or both perform an essential function in Giardia, an antisense-ribozyme strategy with a viral vector, which has been proven efficient for specific gene knockdowns in Giardia (7, 8, 33), was utilized. An in vitro transcript of the antisense sequence from a fragment of eIF4E1 or eIF4E2 cDNA with an inserted hammerhead ribozyme sequence was introduced into Giardia trophozoites, multiplied by the giardiavirus RNA replicating machinery in the cells, and enriched under drug pressure. Semiquantitative RT-PCR and densitometer tracing showed that in the eIF4E1 knockdown experiment, the mRNA of eIF4E1 was reduced to 37% of the original level, while that of eIF4E2 remained relatively unchanged (Fig. 5B, inset). For the eIF4E2 knockdown, the eIF4E2 mRNA was decreased to 19% whereas eIF4E1 mRNA remained unaffected (Fig. 5C inset). Growth of the eIF4E1 knockdown cells reached about 80% of the control (Fig. 5B), whereas eIF4E2 knockdown resulted in only 30% cell growth (Fig. 5C). These outcomes suggest that
each protein may perform certain role in *Giardia* trophozoites, but eIF4E2 appears to play a more essential function in cell growth.

These ribozyme-transfected cell lines were also used to monitor the effect of knocking down eIF4E1 or eIF4E2 on m7GpppN-cap-dependent translation initiation in *Giardia*. The m7GpppG-capped in vitro transcript of firefly luciferase was electroporated into the antisense ribozyme-transfected cells, and the luciferase activity thus expressed in the cells was assayed (33). The results indicated that the translation initiation in eIF4E1 knockdown cells reached 66.4% of the control, whereas luciferase expression in eIF4E2-depleted cells was reduced to 39.7% of the wild-type control (Fig. 5D). These data suggest that eIF4E2, but not eIF4E1, could be the cap-binding protein in the translation initiation complex of *Giardia*.

To verify if translation initiation in *Giardia* is mediated by m7GpppN-cap, m2,2,7GpppN-cap or no cap at all, a firefly luciferase transcript without a cap or with the m7GpppG-cap or m2,2,7GpppG-cap structure was electroporated into *Giardia* trophozoites. No luciferase activity could be detected from the cells transfected with the transcript without a cap or with the m2,2,7GpppG cap structure (see Fig. 7A), but cells transfected with the m7GpppG-capped luciferase transcript expressed a significant level of luciferase activity (Fig. 6A), indicating that m7GpppN-cap is most likely the cap of *Giardia* mRNAs and functions in translation initiation. The observation also agrees with the previous findings that eIF4E2 is most likely the cap-binding protein involved in translation initiation (Fig. 5D) and that it binds to the m7GpppG-cap structure (Fig. 3 and 4B).

This identification of the function of eIF4E2 left the function of eIF4E1 still unclear, except that it binds to the m2,2,7GpppG-cap (Fig. 4C) and is thus most likely associated...
with the snRNAs or snoRNAs in *Giardia* (35). snRNAs are known to play important roles in RNA splicing (6, 40, 41). An intron-containing gene, encoding a 2Fe-2S ferredoxin, was recently identified in *Giardia* (36), which prompted us to look into the potential involvement of eIF4E1 in converting the pre-mRNA into the mature mRNA of this particular gene. Using RT-PCR, the ratios of pre-mRNA to mature mRNA of 2Fe-2S ferredoxin in the wild-type, eIF4E1, and eIF4E2 knockdown cells of *Giardia* (Fig. 5) were compared. The results (Fig. 6B) showed no apparent change in the ratios of pre-mRNA to mature mRNA from the three cell lines, suggesting that eIF4E1 does not function in mRNA splicing in *Giardia*. The snRNAs with the m2,2,7GpppN cap structure identified so far in *Giardia*, snRNA D and snRNA H, are associated with fibrillarin and share some common features with snoRNAs rather than snRNAs (35). To verify if eIF4E1 could be associated with snRNA D and snRNA H, N-terminally GST-tagged eIF4E1 was overexpressed and purified from transformed *E. coli*, incubated with *Giardia* total RNA, and pulled down by glutathione Sepharose 4B beads. Results from semiquantitative RT-PCR (Fig. 6C) indicated that m2,2,7GpppN-capped RNA D and RNA H were indeed pulled down with eIF4E1, but not snRNA J, which is without an m2,2,7GpppN cap structure (Fig. 6C). eIF4E1 is thus most likely bound to the m2,2,7GpppN-capped snRNAs in *Giardia*.

**Localization of the two eIF4E proteins in *Giardia* trophozoites.** As the two eIF4Es bind to different cap structures and may perform distinctive functions in *Giardia*, we asked whether this might be reflected in their intracellular distribution. Immunofluorescence assays using purified rabbit polyclonal antibodies against eIF4E1 and eIF4E2 revealed very different patterns of distribution for the two proteins (Fig. 7). Although both proteins were identified in the cytoplasm of *Giardia*, eIF4E1 also showed a concentrated presence in a nucleolus-like structure in the nucleus (Fig. 7, top panel). To verify this observation, two more monoclonal antibodies against the m2,2,7GpppN-cap structure and the nucleolus marker fibrillarin (37) were used in the immunofluorescence assay and showed that eIF4E1 was indeed co-localized with the cap and fibrillarin in the nucleolus-like structures in both nuclei of *Giardia* (Fig. 8A). The nucleolus-like structure was mostly located at the anterior end of the nucleus. Its identity was further verified in an RNA fluorescence in situ hybridization study using FITC-conjugated oligonucleotide complementary to the 3’ end of *Giardia* 16S-like rRNA molecule. The results indicated the presence of the 16S-like rRNA in the nucleolus-like organelles at the anterior ends of the nuclei (Fig. 8B) similar to those structures containing eIF4E1, m2,2,7GpppN-cap, and fibrillarin (Fig. 7, top panel, and 8A).

The cytoplasmic distribution of eIF4E2 was punctate in ap-
pearance, with four concentrated spots located at the conjunc-
tions where flagella emerged across the cell membrane of Gi-ardia (Fig. 7, bottom panel). The potential significance in this
punctate appearance of eIF4E2 remains unclear.

FIG. 8. Colocalization of Giardia eIF4E1 with m2,2,7GpppN cap, fibrillarin, and 16S-like rRNA. (A) Giardia WB cells stained with rabbit polyclonal antibody against eIF4E1, mouse monoclonal antibody against m2,2,7GpppN cap (top), and mouse monoclonal antibody against yeast fibrillarin (bottom). (B) Giardia WB trophozoites were fixed and stained with an FITC-conjugated 30-mer oligonucleotide complementary to the 3’ end of the Giardia 16S-like rRNA. Concentrated stains were visible in the nucleolus-like structures at the anterior ends of both nuclei.

DISCUSSION

In this report we showed that there are two eIF4E homologues in Giardia. eIF4E1 carries a putative bipartite nuclear localization signal and was found concentrated in a nucleolus-like structure in the nucleus of Giardia. It does not bind to m7GpppN-cap and plays no apparent role in translation initiation in Giardia. But it binds to the m2,2,7GpppN-cap, is colocalized with it in the nucleolus-like structure, and interacts with the snRNA molecules with an m2,2,7GpppN-cap structure. These snRNAs represent an abundant, evolutionarily ancient group of noncoding RNAs among the eukaryotes (3, 44). They perform highly diverse functions ranging from methylation and pseudouridylation of RNA and nucleolytic processing of
rRNAs to synthesis of telomeric DNA. eIF4E1 could be involved in some or all of these activities in Giardia. Its apparent lack of involvement in mRNA splicing (Fig. 6B) agrees with the fact that it is colocalized with the cap only in the nucleolus-like structure and is most likely a snRNA binding protein. Giardia eIF4E1 may thus play important roles in RNA and ribosome maturation in the nucleolus (10). To the best of our knowledge, this is the first case in which an eIF4E homologue has been found to bind to m^2,2,7GpppN-capped snRNA-like molecules in a nucleolus-like structure instead of m^7GpppN-cap mRNAs for translation initiation. The only protein identified so far that interacts specifically with m^2,2,7GpppN-cap but not m^7GpppN-cap, Snurportin1, functions as an snRNP-specific nuclear import receptor (15). However, Giardia eIF4E1 shares little sequence homology with that protein.

The structural basis for the specific binding between eIF4E1 and m^2,2,7GpppN-cap is a little more difficult to envision. The five eIF4E variants found in C. elegans have eIF4E-3 and eIF4E-4 binding only to the m^7GpppN-cap, whereas eIF4E-1, eIF4E-2, and eIF4E-5 bind to both m^7GpppN- and m^2,2,7GpppN-cap (21). Molecular dynamic simulation suggested that the width and depth of the cap-binding cavity were smaller in eIF4E-3 than in eIF4E-5 (31). Site-directed mutations of eIF4E-5 aimed at reducing the size of its cap-binding cavity resulted in a mutant binding only to m^7GpppN-cap. This well-derived structural basis for selective cap binding cannot, however, explain the specific binding to only the trimethyl-cap by Giardia eIF4E1. In view of the vastly divergent amino acid sequence of eIF4E1 compared with those of the other eIF4Es, it is possible that a different structural basis may underlie the binding specificity of this Giardia protein, which will have to wait for the resolution of its three-dimensional structure.

Giardia eIF4E2 binds only to m^7GpppN-cap and is most likely involved in translation initiation in Giardia. The fact that only transcripts with the m^7GpppN-cap are translated in Giardia further confirmed this conclusion. This is the first translation initiation protein identified in Giardia thus far.

By the knowledge from other eukaryotes, His37, Pro38, Val69, Trp73, Leu131, Glu132, and Leu135 in eIF4E (by human eIF4E numbering) are involved in interacting with the scaffold protein eIF4G and 4E-BPs (27). Val69 and Trp73 are located in a conserved sequence (S/T)V(E/D)(E/D)FW in which substitution of the third residue E in yeast eIF4E with a glutamic acid residue does not completely explain the specific binding to only the trimethyl-cap by Giardia eIF4E1. In view of the vastly divergent amino acid sequence of eIF4E1 compared with those of the other eIF4Es, it is possible that a different structural basis may underlie the binding specificity of this Giardia protein, which will have to wait for the resolution of its three-dimensional structure.

Giardia eIF4E2 binds only to m^7GpppN-cap and is most likely involved in translation initiation in Giardia. The fact that only transcripts with the m^7GpppN-cap are translated in Giardia has further confirmed this conclusion. This is the first translation initiation protein identified in Giardia thus far.

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