Deoxyhypusine Modification of Eukaryotic Translation Initiation Factor 5A (eIF5A) Is Essential for Trypanosoma brucei Growth and for Expression of Polyprolyl-containing Proteins*

Suong Nguyen1, Christopher Lejia1, Lisa Kinch1, Sandesh Regmi1, Qiong Li1, Nick V. Grishin2, and Margaret A. Phillips1

From the Departments of1Pharmacology and 2Biophysics and Biochemistry and 3Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9041

The eukaryotic protozoan parasite Trypanosoma brucei is the causative agent of human African trypanosomiasis. Polyamine biosynthesis is essential in T. brucei, and the polyamine spermidine is required for synthesis of a novel cofactor called trypanothione and for deoxyhypusine modification of eukaryotic translation initiation factor 5A (eIF5A). eIF5A promotes translation of proteins containing polyprolyl tracts in mammals and yeast. To evaluate the function of eIF5A in T. brucei, we used RNA interference (RNAi) to knock down eIF5A levels and found that it is essential for T. brucei growth. The RNAi-induced growth defect was complemented by expression of wild-type human eIF5A but not by a Lys-50 mutant that blocks modification by deoxyhypusine. Bioinformatics analysis showed that 15% of the T. brucei proteome contains 3 or more consecutive prolines and that actin-related proteins and cysteine proteases were highly enriched in the group. Steady-state protein levels of representative proteins containing 9 consecutive prolines that are involved in actin assembly (formin and CAP/Srv2p) were significantly reduced by knockdown of eIF5A. Several T. brucei polyprolyl proteins are involved in flagellar assembly. Knockdown of TbeIF5A led to abnormal cell morphologies and detached flagella, suggesting that eIF5A is important for translation of proteins needed for these processes. Potential specialized functions for eIF5A in T. brucei in translation of variable surface glycoproteins were also uncovered. Inhibitors of deoxyhypusine modification would be expected to cause a pleomorphic effect on multiple cell processes, suggesting that deoxyhypusine/hypusine biosynthesis could be a promising drug target in not just T. brucei but in other eukaryotic pathogens.

Human African trypanosomiasis, also known as sleeping sickness, is a fatal vector-borne disease caused by the single celled parasitic protozoan Trypanosoma brucei (1–4). Although the disease reached epidemic levels in the 1990s, the World Health Organization now reports fewer than 10,000 cases, although millions in sub-Saharan Africa remain at risk (5). Gains made against the disease have been attributed to renewed control efforts and the effectiveness of the new combination therapy nifurtimox/eflornithine. Eflornithine (α-difluoromethylornithine) is a suicide inhibitor of the polyamine biosynthetic enzyme ornithine decarboxylase (6). The polyamines putrescine and spermidine are cationic amines that are essential for growth in all eukaryotic cells (7–9). They play roles in transcription, translation, and ion channel regulation, although the exact nature of their function in many of these processes remains poorly understood. In trypanosomatids, polyamines play a specialized role in the formation of trypanothione, a unique glutathione-spermidine conjugate that mediates redox balance in these species (10–12).

The one demonstrated universal role for polyamines in eukaryotic cells is the requirement for spermidine to serve as a substrate in the enzyme-catalyzed hypusine (N4-(4-amino-2-hydroxybutyl)lysine) modification of eIF5A (13–15). eIF5A was originally thought to function in translation initiation but was subsequently found to be one of only three universally conserved translation elongation factors (14, 15). It has been shown to be essential in a number of eukaryotes including mammalian cells and yeast, suggesting that it will be required for growth in all eukaryotes (16–19). Bacteria express a functional counterpart of eIF5A, elongation factor P, that is modified by lysine rather than spermidine, but in contrast, it is not an essential protein. Both eIF5A (mammalian and yeast) and elongation

* This work was supported, in whole or in part, by National Institutes of Health Grants 2R01 AI034432 (to M. A. P.), GM007062 (to C. L.), and GM094575 (to N. V. G.). This work was also supported by Welch Foundation Grants I-1257 (to M. A. P.) and I-1505 (to N. V. G.). The authors declare that they have no conflicts of interest with the content of this article.

† Holds the Beatrice and Miguel Elias Distinguished Chair in Biomedical Science and the Carolyn R. Bacon Professorship in Medical Science and Education. To whom correspondence should be addressed: Dept. of Pharmacology, UT Southwestern Medical Center, 6001 Forest Park Rd., Dallas, TX 75390. Tel.: 214-645-6164; E-mail: margaret.phillips@UTSouthwestern.edu.

** This article contains supplemental Tables 1 and 2.
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factor P have recently been demonstrated to promote peptide bond formation during the translation of polyprolyl (poly-Pro) tracts consisting of consecutive prolines (3 or greater) and of triplets of PPX (where X = Gly, Trp, Asp, or Asn) (20–22). The covalent modifications of eIF5A with hypusine and of elongation factor P by lysinylation were both necessary to the function of these proteins in alleviating the ribosome stalling that occurs at these motifs. Although eIF5A/elongation factor P function in the translation of only a subset of proteins, the percentage of proteins containing proline-stalling motifs is high, and it is greater in eukaryotes than in bacteria (33% (human) versus 5.8% (Escherichia coli)) (14, 23). Although it is still yet to be determined whether poly-Pro proteins are involved in the control of specific cellular processes, in yeast it was found that a high fraction of proteins involved in cytoskeleton organization and active polarization contains poly-Pro tracts (24).

eIF5A is the only protein that is modified by hypusination (13–15). Modification of eIF5A occurs on a conserved lysine residue (Lys-50 in human eIF5A) and is catalyzed by two enzymatic reactions: transfer of the 4-aminobutyl moiety from spermidine catalyzed by deoxyhypusine synthase (DHS) and hydroxylation of the second carbon by deoxyhypusine hydroxylase (DOHH) forming hypusine (Fig. 1A). DHS has been shown to be essential in all species where it has been studied including mammals and yeast (25) and more recently in the kinetoplastids T. brucei (26) and Leishmania (27). Furthermore, the kinetoplastids and Entamoeba encode two DHS genes, and it was shown for T. brucei that the two gene products (one catalytically active but impaired and the other catalytically inactive) associate to form a heterotetrameric enzyme, which is the functionally active form of the enzyme (26). This novel mechanism of enzyme activation by a catalytically inactive paralog was also observed for T. brucei S-adenosylmethionine decarboxylase, which is required for formation of the DHS substrate spermidine (28, 29). The hydroxylase reaction is not found in all eukaryotes; however, the kinetoplastids do contain a homolog of DOHH, and it has recently been shown to be a functional enzyme in Leishmania (30). The finding that DHS is essential in T. brucei and other eukaryotes has sparked interest (31). The finding that DHS is required for optimal expression of T. brucei DHSp (Tb927.10.2750), and protein synthesis during bloodstream form (BSF) and insect stage procyclic form (PF) parasite development (13–15). Modification of eIF5A occurs on a conserved lysine residue (Lys-50 in human eIF5A) and is catalyzed by two enzymatic reactions: transfer of the 4-aminobutyl moiety from spermidine catalyzed by deoxyhypusine synthase (DHS)2 and hydroxylation of the second carbon by deoxyhypusine hydroxylase (DOHH) forming hypusine (Fig. 1A). DHS has been shown to be essential in all species where it has been studied including mammals and yeast (25) and more recently in the kinetoplastids T. brucei (26) and Leishmania (27). Furthermore, the kinetoplastids and Entamoeba encode two DHS genes, and it was shown for T. brucei that the two gene products (one catalytically active but impaired and the other catalytically inactive) associate to form a heterotetrameric enzyme, which is the functionally active form of the enzyme (26). This novel mechanism of enzyme activation by a catalytically inactive paralog was also observed for T. brucei S-adenosylmethionine decarboxylase, which is required for formation of the DHS substrate spermidine (28, 29). The hydroxylase reaction is not found in all eukaryotes; however, the kinetoplastids do contain a homolog of DOHH, and it has recently been shown to be a functional enzyme in Leishmania (30). The finding that DHS is essential in T. brucei and other eukaryotes has sparked interest in identifying inhibitors of DHS as potential drugs for the treatment of proliferative diseases (14).

Herein we studied the function of eIF5A in T. brucei by characterizing the effects of RNA interference (RNAi)-induced gene knockdown. We found that eIF5A was essential in both bloodstream form (BSF) and insect stage procyclic form (PF) parasites where knockdown of eIF5A led to morphological abnormalities including cell rounding and detached flagella. We also demonstrate that modification of the conserved lysine with deoxyhypusine is required for eIF5A function in T. brucei. Bioinformatics analysis was performed to identify T. brucei proteins containing poly-Pro tracts, and we found that actin-related proteins (formins), cysteine proteases, and flagellar- associated proteins were highly represented in this set. Steady-state protein levels of two representative poly-Pro proteins involved in the actin cytoskeleton were analyzed after eIF5A knockdown, demonstrating that they were reduced after depletion of eIF5A. Thus eIF5A and deoxyhypusine modification are essential in T. brucei and are required for optimal expression of proteins containing poly-Pro tracts.

Experimental Procedures

Gene Accession Numbers—The accession numbers for genes discussed in this study are as follows: TriTrypDB TbEIF5A (Tb927.11.740), TbdHsc (Tb927.10.2750), and TbdHsp (Tb927.1.870); UniProtKB/Swiss-Prot HsEIF5A (P63241.2), T. brucei telomerase reverse transcriptase (TERT) (Tb927.11.10190); formin, putative (Tb927.5.2300); G-actin-binding protein, CAP/Srv2p, putative (Tb927.10.9250; also known as Tb10.6k15.1160); and flagellum adhesion glycoprotein 2 (FLA2) (Tb927.8.4060) as described (31).

Oligonucleotide Primers—Primers used for cloning, mutagenesis and real time (quantitative) polymerase chain reaction (qPCR) can be found in Table 1.

Anti-TbEIF5A Antibody Production—Antibodies were raised in rabbits against purified recombinant Tb EIF5A (Covance Inc.) that was purified as previously described (26). T. brucei Cell Culture—T. brucei brucei strain Lister 427 and the derived strains that constitutively expresses T7 polymerase and tetracycline (Tet) repressor protein (bloodstream form “single marker” and procyclic form 29-13) were used for cell-based experiments (10). BSFs were cultured in HMI-11 medium supplemented with 10% fetal bovine serum (FBS) (Tet-free, heat-inactivated; Atlanta Biologicals) at 37 °C with 5% CO2 (33). PFs were cultured in SDM-79 medium (Sigma) supplemented with 15% FBS at 28 °C. Antibiotics were added for maintenance of recombinant cell lines based on the nature of the integrated plasmids at the following concentrations: G418 (BSF, 2.5 µg ml−1; PF, 15 µg ml−1; Sigma), hygromycin B (BSF, 2.5 µg ml−1; PF, 25 µg ml−1; Sigma), phelemycin (BSF and PF, 2.5 µg ml−1; Invivogen), blasticidin S (BSF, 2 µg ml−1; PF, 10 µg ml−1; Invivogen), and Tet (BSF, 1 µg ml−1; PF, 10 µg ml−1).

Cloning of the RNAi Construct Targeting TbEIF5A—A 451-base pair region (2–452) of the Tb EIF5A coding sequence was identified using RNAiIt. This segment was PCR-amplified from BSF single marker genomic DNA (primers in Table 1) and TA-cloned into the Gateway entry vector pCR®8/GW/TOPO® (Life Technologies). The entry clone (100 ng) was combined with the destination vector pTrypRNAiGate (100 ng) using the Gateway® LR Clonase® reaction to generate a Tet-inducible stem-loop (Life Technologies) as described (34). Upon transfection, the pTrypRNAiGate-TbEIF5A construct was integrated into the rRNA gene array and conferred resistance to phelemycin. Addition of Tet induced expression of the hairpin RNA, leading to knockdown of TbEIF5A mRNA.

Cloning of the Human eIF5A and eIF5A-K50A T. brucei Expression Constructs—The previously described E. coli expression vector for codon-optimized expression of Hs eIF5A (26) was used a PCR template, and the Hs eIF5A gene was

2The abbreviations used are; DHS, deoxyhypusine synthase; DOHH, deoxyhypusine hydroxylase; CAP/Srv2p, G-actin-binding protein; BSF, bloodstream form; T. brucei; PF, procyclic form; T. brucei Tet, tetracycline; Tb, T. brucei; Hs, Homo sapiens; TERT, telomerase reverse transcriptase; qPCR, real time (quantitative) polymerase chain reaction; DHODH, dihydroorotate dehydrogenase; N, nucleus; K, kinetoplastid; VSG, variant surface glycoprotein; FLA, flagellum adhesion glycoprotein.
amplified with flanking 5’ HindIII and 3’ BamHI restriction sites (see primers in Table 1). The construct was cloned into pLew100v5-BSD (Addgene plasmid 27658 from Cross and coworkers (10)) to generate pLew100v5-BSD-HsEIF5A, which when integrated into the rRNA locus confers resistance to blasticidin and drives Tet-inducible expression of HsEIF5A. To generate the K50A mutant, the AAA codon was mutated to GCA by Phusion PCR mutagenesis. The PCR mixtures (50 μl) contained 1× Phusion HF buffer, 200 μM dNTPs, 0.5 μM 5’-phosphorylated mutagenesis primers, 30 pg of pCR™-Blunt II-elf5A plasmid template, and 1 unit of Phusion polymerase (New England Biolabs). PCR cycling conditions were denaturation at 98 °C for 30 s and annealing and extension at 72 °C for 3 min for 25 cycles followed by a final extension at 72 °C for 10 min. Plasmid template was digested in a reaction (100 μl) containing 1 μl of DpnI (New England Biolabs), 1× Cutsmart buffer, and 25 μl of PCR mixture. Digested PCR product was purified using the High Pure PCR Product Purification kit (Roche Applied Science). The product was recircularized using the Quick Ligation™ kit (New England Biolabs) in a reaction (21 μl) containing 25 ng of PCR product, 1× ligation buffer, and 1 μl of T4 DNA ligase. Chemically competent E. coli Top10 cells (75 μl) were transformed with 2 μl of the ligation reaction, and colonies were selected with kanamycin (50 μg ml⁻¹). pCR-Blunt II-HsEIF5A-K50A plasmids were identified by sequencing using M13 primers. The HsEIF5A-K50A mutant was cloned into the pLew100v5 expression plasmid as detailed above for wild type to generate pLew100v5-HsEIF5A-K50A. K50A eIF5A mutant plasmids were identified by sequencing using M13 primers.

Transfection of Trypanosomes—For BSF, 10⁷ parasites were combined with NotI-linearized plasmid (2–5 μg) with Amaxa Nucleofector II as described (35) in Human T Cell Nucleofector Buffer (100 μl; Lonza) using protocol X-001 on an Amaxis Nucleofector (Lonza). Cells were transferred to a flask containing 50 ml of prewarmed medium and allowed to recover at 37 °C in 5% CO₂ for 8 h before selection antibiotics were added. The culture was plated into a 48-well plate at 1 ml/well. Residual culture was brought up to 48 ml in selection medium (1:24 dilution) and plated into a second 48-well plate. After the death of parallel negative transfection controls and density of transfected wells reached >10⁷ ml⁻¹, 4 wells were selected for limited dilution to generate clonal cells lines. For PF, 3 × 10⁷ parasites were combined with linearized plasmid (5 μg) in Human T Cell Nucleofector Buffer (100 μl) and transfected using protocol X-014. The cells were transferred to SDM-79 medium supplemented with 20% FBS (10 ml) and plated into a 24-well plate at 1 ml/well. Cells were allowed to recover at 28 °C for 16 h before addition of appropriate selection antibiotics and additional medium. Independent transfecants were diluted again, and cultures arising from the lowest dilution were used for growth experiments.

Quantitation of mRNA by qPCR—For qPCR analysis of mRNA levels, 5 × 10⁷ cells were collected at the indicated times from Tet-treated cells and untreated controls. Total RNA was purified using the Illustra RNAspin Mini RNA Isolation kit (GE Healthcare) or TRIzol reagent (Life Technologies). For quantitation of TbEIF5A mRNA levels in Figs. 2–4, RNA abundance
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was quantified using iTaq SYBR Green Supermix with ROX™ (Bio-Rad), and relative gene abundance was calculated by the ΔΔCt method with TERT as the reference gene as described previously (26, 36, 37). For mRNA quantification shown in Fig. 7, relative RNA abundance was calculated using the method of Pfaffl (38) with TERT as the reference gene. A standard curve was utilized for each experiment as recommended by the manufacturer (Bio-Rad, CFX qPCR software).

Western Blot Analysis—T. brucei cells (10⁷–10⁸ per assay condition) were harvested by centrifugation (2000 × g, 10 min). For soluble proteins, pellets were washed twice with PBS (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4), resuspended in Tryp Lysis Buffer I (50 mM Hapes, pH 8, 100 mM NaCl, 5 mM β-mercaptoethanol, 2 mM PMSF, 1 μg ml⁻¹ leupeptin, 2 μg ml⁻¹ antipain, 10 μg ml⁻¹ benzamidine, 1 μg ml⁻¹ pepstatin, 1 μg ml⁻¹ chymostatin), and cells were lysed with three freeze/thaw cycles. The lysate was clarified by centrifugation (13 000 × g, 10 min, 4°C), and the supernatant was collected. Protein concentration was determined using the Bio-Rad Protein Assay reagent with bovine serum albumin (BSA) used as the standard curve. Protein (30 μg) was separated by SDS-PAGE for Western analysis. For membrane-associated proteins (e.g. formimin), cells were instead resuspended in Tryp Lysis Buffer II (250 mM Tris, pH 8.0, 0.5 mM PMSF, 100 mM DTT, 4% SDS, 40% glycerol), whereas all other samples were resuspended in Laemmli buffer. All samples were boiled for 5 min and then separated by SDS-PAGE. Proteins from gels were transferred to a PVDF membrane (iBlot®, Life Technologies), which was then blocked with 5% milk in Tris-buffered saline (TBS) (20 mM Tris–HCl, pH 7.6, 137 mM NaCl) and incubated with primary antibody for 1 h at room temperature. Blots were washed with TBS + Tween 20 (0.1%) three times before incubation with secondary antibody for 1 h at room temperature. Blots were exposed to SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and imaged using an ImageQuant LAS4000 (GE Healthcare). Bands were quantified using NIH ImageJ software. Membranes were sequentially blotted with experimental and control antibodies with stripping and reblocking between each. Blots were stripped by incubating in Restore™ Western Blot Stripping Buffer (Life Technologies) for 30 min at room temperature and then washed with TBS for 5 min. The following antibodies were used at 1:10,000, anti-TbIF5A (rabbit polyclonal), anti-binding protein (rabbit polyclonal; kindly provided by James Bangs, University of Wisconsin-Madison), anti-TbHDHODH (39), anti-human eIF5A mouse polyclonal antibody (Sigma), anti-AU1 (Covance), anti-rabbit conjugated with alkaline phosphatase (Sigma), and horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma). The anti-human eIF5A mouse polyclonal antibody showed no cross-reactivity with TbIF5A, nor did the anti-TbIF5A antibody show any cross-reactivity with human eIF5A.

Microscopy—For morphometric analysis (Figs. 3D and 4, D and E) and immune staining (Fig. 2B), cells (~10⁷ log phase parasites) were washed with phosphate-buffered saline (PBS), pH 7.4 and fixed for 30 min in 4% paraformaldehyde. Cells were then washed once in PBS, resuspended in 100 μl of PBS, and stored at 4°C. Fixed cells were settled on Sigma polylysine-coated slides in a moist chamber for 20 min, permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 1 min, and washed three times with PBS. To examine nucleus and kinetoplast configuration, permeabilized cells were mounted with Vectashield Hardset with 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and directly observed under a Zeiss epifluorescence microscope or a Leica TCS SP5 confocal microscope. For immunostaining, cells were blocked for an hour in 4% goat serum (Gibco) in PBS. Rabbit anti-TbIF5A immune serum (1:5000) and preimmune serum (1:5000) were prepared in 0.2% BSA made in PBS, overlaid on the slide, and incubated for 1 h at room temperature. Slides were washed three times (5 min each), incubated for 1 h in goat anti-rabbit Alexa Fluor 488 secondary antibody (Invitrogen) (1:750) in 0.2% BSA, and again washed three times for 5 min each. Slides were counterstained with DAPI with mounting medium (Vectashield) and imaged as above.

Images in Figs. 3C and 4C were obtained as follows. Cells (final density, 1 × 10⁶ cells/ml) were collected by centrifugation, washed in PBS, and resuspended in 1 ml of PBS. The cell suspension (0.2–0.3 ml) was placed on a coverslip for 20 min, the coverslip was then placed cell side up in a well of a 6-well plate, 1 ml of 4% paraformaldehyde (16% paraformaldehyde stock from Electric Microscopy Sciences) was added, and cells were incubated for 30 min at room temperature. Paraformaldehyde was removed by vacuum, and the coverslip was washed twice with 1 ml of cold PBS, one time with PBS plus 0.1% Triton X-100, and finally three times with PBS. Coverslips were removed from the well, and mounting medium (anti-fade; Invitrogen; containing DAPI) (10 μl) was dropped onto the slide followed by mounting of the coverslip with cells. Cells were visualized using a DeltaVision Deconvolution or an AppliedPrecision microscope. The differential interference contrast and fluorescence field images were obtained at the same time. Images were visualized using NIH ImageJ software.

Putrescine Labeling of eIF5A in T. brucei Cultures—For polyamine supplementation experiments, cell culture media were supplemented with chicken serum (Gemini Bio-Products) instead of PBS to avoid amine oxidase-mediated toxicity. Log phase cells were cultured in media supplemented with 50 μM [1,4-¹⁴C]putrescine dihydrochloride (114 mCi/mmol; Amer sham Biosciences) for 6–12 h before cells (BSF, 10⁶; PF, 10⁷) were pelleted and washed with PBS (1 ml) three times. Cells were resuspended in 100 μl of Tryp Lysis Buffer (defined above) and lysed by three cycles of successive free/thaw. Cell lysates were clarified by centrifugation (16,000 × g, 10 min, 4°C), and 30 μg of soluble lysate was incubated with anti-TbIF5A antibody (1:200) for 12 h at 4°C. Dynabeads® Protein A (50 μl; Life Technologies) was added, and incubation was continued for 2 h at room temperature. Antibody-antigen complex was captured with a magnetic stand. Beads were washed three times with TBS, and the antibody-antigen complex was eluted with 40 μl of citrate buffer, pH 3. Eluent was neutralized with 5 μl of 0.1 M NaOH, boiled in SDS dye, and separated by SDS-PAGE. The gel was dried and imaged with x-ray film (Eastman Kodak Co.).

Bioinformatics Analysis to Identify Poly(Pro) Repeat Proteins—Protein sequences from the T. brucei strain 927/4 GU1at10.1 genome were retrieved from NCBI (March 11, 2015). Genes that contain poly(Pro) stretches were identified.
using a pattern search for any range of 3–9+ adjacent proline residues contained within the sequence (supplemental Table 1). The number of proline residues present in each sequence was counted and reported as a percentage of the sequence length (number of Pro/length). Protein sequences of those genes with poly(Pro) stretches of length greater than 7 residues were queried against the NCBI RefSeq database using PSI-BLAST (40) (three iterations; E-value cutoff, 0.005) to identify homologs and determine whether the poly(Pro) stretches extend to human or *Saccharomyces cerevisiae* orthologs. Gene ontology (GO) clusters of gene families enriched in poly(Pro) tracts (supplemental Table 2) were identified using the DAVID server (41).

**Cloning of Select Polyprolyl Repeat Proteins in *T. brucei***

**Expression Constructs**—The putative formin (Tb427.05.2300) and CAP/Srv2p (Tb927.10.9250) genes were PCR-amplified from single marker *B. subtilis* genomic DNA using primers that inserted a C-terminal AU1 tag before the stop codon (Table 1). PCR products were cloned into the HindIII/BamHI-digested pLew90 vector in an In-Fusion cloning (Clontech) reaction (10 μl) containing 1× In-Fusion HD Enzyme mixture, 120 ng of vector, and 100 ng of purified PCR product. The reaction was incubated at 50 °C for 15 min. Chemically competent *E. coli* Top10 cells (100 μl) were transformed with 2.5 μl of the ligation reaction, and colonies were selected with ampicillin (100 μg ml⁻¹). The pLew90-formin and pLew90-CAP/Srv2p expression constructs were confirmed by direct sequencing and transfected into the *T. brucei* BSF *TbEIF5A* RNAi cell line as described above.

**Results**

**Putrescine-dependent Modification of *TbEIF5A* and Subcellular Localization**—Sequence alignment of *T. brucei* elf5A with other eukaryotic elf5As shows that it contains the hypusine amino acid consensus sequence including the key residue Lys-50 (human sequence numbering) (14) (Fig. 1B). To confirm that *TbEIF5A* is modified by deoxyhypusine/hypusine *in vivo*, PF *T. brucei* were incubated with the spermidine precursor [1,4-¹⁴C]putrescine for 48 h to determine whether radioactivity would be incorporated into *TbEIF5A*. [1,4-¹⁴C]Putrescine is converted to [¹⁴C]spermidine by spermidine synthase, providing the radiolabeled form of spermidine to serve as a substrate for deoxyhypusination of *eIF5A*. Labeled [¹⁴C]spermidine was converted to [¹⁴C]spermidine by spermidine synthase, providing the radiolabeled form of spermidine to serve as a substrate for deoxyhypusination of *eIF5A*. Labeled [¹⁴C]spermidine was not supplied directly because spermidine is poorly taken up by *T. brucei* cells (29). After incubation of cells with [1,4-¹⁴C]putrescine, *TbEIF5A* was immunoprecipitated using polyclonal *TbEIF5A* antibody, and protein was separated by SDS-PAGE, transferred to PVDF membrane, and visualized using a phosphor storage screen. A single band (17 kDa) was detected in the labeled cell lysates both before and after immunoprecipitation with anti-*TbEIF5A* antibody; whereas the control without anti-*TbEIF5A* antibody did not yield a radiolabeled band (Fig. 2A). These data show that the [1,4-¹⁴C]putrescine is incorporated into *TbEIF5A*, providing direct biochemical evidence that *TbEIF5A* is covalently modified by deoxyhypusine. Given the presence of a DOHH gene in *T. brucei*, it is likely that hypusine-*TbEIF5A* is the final product.

Immunofluorescence analysis of PF *T. brucei* parasites was performed to determine the subcellular localization of *TbEIF5A*. Cells were stained with anti-*TbEIF5A* antibody followed by an Alexa Fluor 488-conjugated secondary antibody (FITC channel) and DAPI to stain the DNA. A diffuse staining throughout the cell was observed for anti-*TbEIF5A*, but staining was absent in the nucleus, showing that *TbEIF5A* is localized to the cytoplasm as expected for a protein involved in translation (Fig. 2B).

**RNAi Knockdown of *TbEIF5A* in BSF *T. brucei***—To assess the essentiality of *TbEIF5A* in *T. brucei*, RNAi was used to deplete *TbEIF5A* in both BSF and PF *T. brucei* cells using a Tet-controlled inducible system that leads to the expression of a dou-
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**FIGURE 3. Knockdown of *Tb*elf5A in BSF cells.** A, growth of BSF. *Tb*elf5A RNAi cells in the absence (squares) and presence (circles) of Tet over time; the mean is plotted for nine biologic replicates, and the S.D. is represented by a dotted gray line. Cell number = Cell density × Volume × Dilution factor. Inset, representative Western blot analysis of soluble protein (20 μg) in uninduced (−Tet) versus cells induced with Tet for 1 (D1) or 2 (D2) days. Blots were probed with anti-elf5A antibody and with anti-DHODH antibody as a loading control. B, quantification of elf5A mRNA by quantitative RT-PCR after 24 h of Tet induction for growth curves shown in A. Error bars represent the S.D. for n = 9 biological replicates. TERT was used as reference gene, and values are normalized to expression in uninduced cells. C, cell morphological analysis. Images of representative cells ± Tet for 2 days (D2) where the left panels are an overlay of DAPI and differential interference contrast images, middle panels show the differential interference contrast images, and right panels are DAPI staining. The white scale bars represent 5 μm. D, genome analysis by microscopy. Cells were grown ± Tet for 1 or 2 days, stained with DAPI, and visualized by fluorescence microscopy. Minimally, 100 cells were counted per condition, and cells were scored for the number of nuclei (N) and kinetoplasts (K).

The human elf5A gene was chosen as a control with human elf5A. The human homolog was chosen because although many cells were morphologically altered the population is not homogeneous, and it is likely that some cells would be more sensitive to growth arrest caused by loss of endogenous elf5A.

**Validation of the RNAi Phenotype**—To validate the RNAi phenotype, the BSF *elf5A* RNAi cell lines were complemented with human elf5A. The human homolog was chosen because the different nucleotide sequence would allow it to escape RNAi knockdown. Indeed, nucleotide sequence alignment of *Tb*elf5A and *Hs*elf5A sequences revealed no stretches of homology greater than 12 nucleotides, predictive that the human sequence would not be degraded by the *Tb*elf5A-directed RNAi stem-loop constructs. The *Hs*elf5A gene was introduced into the rDNA spacer region under control of the rDNA promoter and Tet repressor elements in both the BSF and PF *Tb*elf5A RNAi cell lines. Expression of human elf5A was able to rescue the growth arrest caused by loss of endogenous elf5A in both BSF and PF parasites (Fig. 5, A and C). Western blot analysis confirmed that upon addition of Tet human elf5A expression was induced and *Tb*elf5A protein levels declined (Fig. 5, B and D). These data confirm that elf5A is essential for growth of both BSF and PF *T. brucei*. Moreover, the ability of...
human eIF5A to complement confirms that the two proteins are functional homologs.

Essentiality of eIF5A Hypusination—To provide additional evidence that hypusination of eIF5A is essential in *T. brucei*, the BSF and PF EIF5A RNAi cell lines were also transfected with the K50A mutant of human eIF5A that cannot be hypusinated. Using the same strategy as above, the HsEIF5A-K50A mutant was expressed in the context of knockdown of endogenous *Tb*EIF5A (Fig. 5). Unlike the wild-type human enzyme, HsEIF5A-K50A was unable to rescue the growth defect caused by loss of eIF5A. To determine whether the growth defect is due to a dominant negative effect of the HsEIF5A-K50A mutant, the K50A mutant was expressed in the context of knockdown of endogenous *Tb*EIF5A. Cell growth of these wild-type cells (data not shown). Thus, the mutant was also overexpressed in wild-type single marker cells. Overexpression of the HsEIF5A-K50A did not cause a change in growth rate of these wild-type cells (data not shown). Thus, the inability of this mutant to rescue the RNAi phenotype shows that hypusine modification of eIF5A is essential for *T. brucei* BSF and PF cell growth.

Identification of Poly(Pro) Proteins in *T. brucei*—To determine which proteins encoded in the *T. brucei* genome would be most sensitive to a block in translation in the absence of eIF5A, we analyzed the *T. brucei* proteome for poly(Pro) motifs and classified the proteins based on the number of consecutive prolines in the protein. Of the 8,712 predicted encoded proteins, only 55 were completely devoid of prolines, and 1306 *T. brucei* proteins (15%) were found to contain consecutive proline stretches of 3 or greater (Fig. 6, Table 2, and supplemental Table 1). Of these, 942 (72%) are annotated as hypothetical proteins due to poor sequence similarity with known proteins. Interestingly, 19 predicted proteins contain poly(Pro) tracts of more than 8 consecutive prolines, and most of these are putative cysteine peptidases (Fig. 6B, Table 2, and supplemental Table 2). Other protein families with a high number of poly(Pro)-containing members include actin-related proteins such as formin and zinc finger proteins (Fig. 6B and Table 2). Further exploration of the poly(Pro) *T. brucei* proteins revealed interesting trypanosome-specific proteins. Atypical variant surface glycoprotein (VSG; Tb927.9.17050) and retrotransposon hot spot protein 3 (RHSP3; Tb927.9.15810) both encode 5 consecutive prolines, whereas flagellum adhesion glycoproteins (FLA) (Tb927.8.4060) contain a 9-proline repeat. One such VSG and putative VSG also contain 3 consecutive prolines. The procyclin (EP) proteins are also proline-rich, but rather than encoding consecutive prolines, they encode glutamine-proline repeats. EP repeats have not been shown to induce ribosome stalling, although the role of eIF5A in *T. brucei* procyclin expression has not been studied.

*Tb*EIF5A Is Essential for Translation of poly(Pro) Proteins in *T. brucei*—To determine whether eIF5A is essential for translation of poly(Pro)-containing proteins in *T. brucei*, we assessed the effects of eIF5A knockdown in BSF parasites on expression of representative proteins containing 9 consecutive prolines.
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including formin (Tb927.5.2300) and CAP/Srv2p (Tb927.10.9250) (Fig. 7). The target proteins were expressed as AU1-tagged proteins in BSF TbEIF5A RNAi cell lines under the control of a constitutive promoter. Protein levels were monitored by Western analysis, and mRNA was quantitated by qPCR before and after (24–48 h) Tet-mediated induction of TbEIF5A knockdown. As in prior studies, TbElF5A protein levels were reduced by 85%, whereas mRNA levels were reduced by >90% after the addition of Tet relative to the TERT (qPCR) and DHODH (protein) controls (Fig. 7). In parallel, formin protein levels decreased by 64%, whereas formin mRNA levels increased 1.7-fold (Fig. 7). The ratio of formin protein to mRNA was decreased by 81% relative to the uninduced control. Formin was shown previously to be an essential protein in T. brucei by a genome-wide RNAi screen (44). Similarly to formin, protein levels of CAP/Srv2p were reduced to 42% of the no-Tet control 48 h after Tet induction, whereas mRNA levels were unchanged to slightly reduced. Overall, the ratio of CAP/Srv2p protein to RNA was reduced to 58% of no-Tet levels after 48 h of Tet induction (Fig. 7). Thus these data show that loss of eIF5A led to a substantial decrease in both formin and CAP/Srv2p steady-state protein levels even after only 24–48 h of Tet induction. These observed changes were not the result of decreases in mRNA levels; indeed, formin mRNA levels actually increased after eIF5A levels were reduced. Thus these data support the conclusion that formin and CAP/Srv2p expression levels decreased after knockdown of eIF5A. However, these studies do not rule out potential changes in protein turnover, although that seems an unlikely consequence of eIF5A knockdown.

Discussion

The translation factor eIF5A is required for efficient translation of mRNAs encoding proteins with poly(Pro) tracts in
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TABLE 2
Annotated T. brucei proteins containing seven or more consecutive prolines

| No. prolines | Gene ID       | Annotated function              | Protein length |
|--------------|---------------|---------------------------------|----------------|
| 7            | Tb927.1.3340  | Hypothetical protein, unlikely  | 62 aa          |
| 7            | Tb927.2.5200  | Hypothetical protein            | 675            |
| 7            | Tb927.3.2770  | Hypothetical protein            | 1136           |
| 7            | Tb927.5.3250  | Hypothetical protein            | 1557           |
| 7            | Tb927.5.3260  | Hypothetical protein            | 1399           |
| 7            | Tb909.211.3370| RNA-binding protein, putative   | 560            |
| 7            | Tb11.03.0760  | Repressor activator protein 1   | 855            |
| 8            | Tb927.4.1700  | Protein kinase                  | 692            |
| 8            | Tb927.4.4520  | Hypothetical protein            | 504            |
| 8            | Tb927.6.990   | Cysteine peptidase, clan CA, family C1, cathepsin L-like | 449 |
| 8            | Tb927.6.1000  | Cysteine peptidase, clan CA, family C1, cathepsin L-like | 449 |
| 8            | Tb927.6.1030  | Cysteine peptidase, clan CA, family C1, cathepsin L-like | 449 |
| 8            | Tb927.6.1040  | Cysteine peptidase, clan CA, family C1, cathepsin L-like | 449 |
| 8            | Tb927.6.1050  | Cysteine peptidase, clan CA, family C1, cathepsin L-like | 449 |
| 8            | Tb927.6.1060  | Cysteine peptidase, clan CA, family C1, cathepsin L-like | 449 |
| 8            | Tb10.6k15.1500| Flagellum-targeting protein kharon 1 (KH1) | 411 |
| 8            | Tb11.01.2330  | Eukaryotic translation initiation factor 4γ | 697 |
| 8            | Tb11.01.3630  | Eukaryotic translation initiation factor 4e | 442 |
| 9+           | Tb927.5.2300  | Formin                          | 943            |
| 9+           | Tb927.5.2390  | Hypothetical protein            | 342            |
| 9+           | Tb927.6.960   | Cysteine peptidase, clan CA, family C1, cathepsin L-like | 450 |
| 9+           | Tb927.6.970   | Cysteine peptidase, clan CA, family C1, cathepsin L-like | 450 |
| 9+           | Tb927.6.980   | Cysteine peptidase, clan CA, family C1, cathepsin L-like | 450 |
| 9+           | Tb927.6.1010  | Cysteine peptidase, clan CA, family C1, cathepsin L-like | 450 |
| 9+           | Tb927.6.1020  | Clan CA, family C1, cathepsin L-like (rhodesain) | 450 |
| 9+           | Tb927.6.1350  | Hypothetical protein            | 345            |
| 9+           | Tb927.6.1370  | Hypothetical protein            | 347            |
| 9+           | Tb927.6.1390  | Hypothetical protein            | 347            |
| 9+           | Tb927.8.4060  | Flagellum adhesion glycoprotein (FLA2) | 590 |
| 9+           | Tb927.8.4110  | Flagellum adhesion glycoprotein | 590 |
| 9+           | Tb927.8.8330  | Calpain, putative, cysteine peptidase | 888 |
| 9+           | Tb909.160.2650| Hypothetical protein            | 1314           |
| 9+           | Tb909.211.0100| Hypothetical protein            | 576            |
| 9+           | Tb10.70.0060  | Hypothetical protein            | 508            |
| 9+           | Tb927.10.9250 | G-actin-binding protein, putative, CAP/Srv2p | 269 |
| 9+           | Tb10.61.1170  | Hypothetical protein            | 451            |
| 9+           | Tb11.02.3470  | Formin, putative, formin-like protein | 1004 |

eukaryotic cells where its role has been best studied in yeast and mammals (14, 15). We have shown that eIF5A is also essential for growth of the parasitic protozoa T. brucei, and we have demonstrated that as in other eukaryotes deoxyhypusination/hypusination is required for eIF5A function. We confirmed that the observed growth phenotype was due to specific knockdown of eIF5A by complementing the RNAi cell lines with RNAi-resistant human eIF5A. The ability of Hs-eIF5A to rescue the growth defect validates the essential function of eIF5A and furthermore demonstrates that human eIF5A and trypanosome eIF5A are functionally homologous. Our data extend studies of eIF5A to a eukaryotic cell that is only distantly related to yeast and mammals; thus these data provide strong evidence that eIF5A is a universally required elongation factor that is essential in all eukaryotic cells.

To more directly address the essentiality of hypusination in T. brucei, we complemented the RNAi cell lines with a Lys-50 to Ala mutant of Hs-eIF5A that cannot be hypusinated (Hs-eIF5A-K50A). This mutant was unable to fully rescue the growth defect caused by knockdown of T. brucei eIF5A, demonstrating that deoxyhypusination/hypusination is essential for the function of Tbel5A. These results are similar to those observed in yeast (45). Interestingly, in BSF parasites, Hs-eIF5A-K50A-complemented cells survived a day longer than those that did not contain a rescue plasmid, suggesting that the non-hypusinated form of Hs-eIF5A is either not entirely without function or that it stabilized Tbel5A levels, leading to less effective knockdown. In contrast, Hs-eIF5A-K50A was unable to rescue the growth deficit in PF cells at any level.

In T. brucei, we have not yet established whether formation of hypusine is required or whether deoxyhypusine modification of Lys-50 is sufficient. Both T. brucei and Leishmania encode a DOHH gene, and the Leishmania DOHH gene has been shown to encode a functional enzyme, but its essentiality has not been demonstrated (30). In S. cerevisiae and Schizosaccharomyces pombe, formation of deoxyhypusine is required, but subsequent hydroxylation to hypusine is not essential, whereas it is required in multicellular eukaryotes (13).

To obtain additional insight into the role of eIF5A in poly(Pro) translation in T. brucei, we used bioinformatics to identify poly(Pro)-containing proteins. Fifteen percent of the trypanosome proteome encoded proteins containing 3 or more consecutive prolines by comparison with 33% of human proteins (14). Because so many T. brucei proteins in the proteome contain three or more consecutive proline stretches, this analysis supports the current popular hypothesis that eIF5A functions to allow translation of poly(Pro) proteins in all eukaryotic cells. However, it remains unknown whether eIF5A will be required for the translation of all poly(Pro)-containing proteins in T. brucei or just a subset. We identified cysteine peptidases, actin-related proteins such as formin, and zinc finger protein families as being particularly highly enriched in proteins that contained long poly(Pro) tracts in T. brucei. We showed that the steady-state protein levels (normalized to mRNA levels) of both formin.
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A. Formin (Tb927.5.2300)

- Tet 6 12 24 h
- α-eIF5A
- α-AU1
- α-DHODH

B. CAP/Srv2p (Tb927.10.9250)

- Tet 48 h
- α-eIF5A
- α-AU1
- α-BIP

FIGURE 7. Effect of eIF5A knockdown on expression of representative poly(Pro) proteins containing 9 consecutive prolines (A and B). A, formin (Tb927.5.2300). Panel i, Western blot analysis of eIF5A and formin protein expression ± Tet for the indicated time (h). Formin was expressed with an AU1 tag to allow visualization, and DHODH was probed as loading control. Panel ii, qPCR analysis of eIF5A and formin mRNA expression relative to the TERT control ± Tet for 24 h. Panel iii, ratio of protein/mRNA expression levels ± Tet for 24 h. B, CAP/Srv2p (Tb927.10.9250). Panel i, Western blot analysis of eIF5A and AU1-tagged CAP/Srv2p protein expression ± Tet at time points 0 and 48 h with BIP as the loading control. Panel ii, qPCR analysis of eIF5A and CAP/Srv2p mRNA expression relative to the TERT reference before and 48 h after induction. Panel iii, ratio of protein/mRNA expression levels ± Tet 48 h after induction. qPCR data were collected in triplicate, and Western analysis was performed either over a time course (formin) or in triplicate (CAP/Srv2p). Error bars represent S.D. for three biological replicates.

and CAP/Srv2p were reduced in cells that had been depleted of TbIF5A by RNAi. These data support the hypothesis that in T. brucei proteins with poly(Pro) tracts also require eIF5A for effective translation. A number of the proteins with long poly(Pro) tracts including for example formin, flagellum adhesion glycoprotein, and calpain were shown in a genome-wide RNAi screen to be required for fitness in T. brucei (44); thus the loss of eIF5A is expected to lead to the depletion of many essential proteins in the parasite and to have a pleomorphic effect on multiple cell processes.

Interestingly many of the proteins in T. brucei with long poly(Pro) tracts are involved in regulation of the actin cytoskeleton, the remodeling of which is essential for diverse cellular processes including cytokinesis, endosome trafficking, cell polarization, and motility (47). The role of eIF5A in promoting translation of actin regulatory proteins correlates with our observation that knockdown of TbIF5A led to abnormal cell morphologies in both BSF and PF parasites. Formin and CAP/Srv2p, which we have shown are depleted after eIF5A knockdown, are both involved in the regulation of actin filament assembly (47, 48). Depletion of eIF5A in S. cerevisiae also resulted in misshapen cells that were more sensitive to ethanol (49). Yeast formin also contains a long poly(Pro) tract, and its translation was also adversely affected by loss of eIF5A, leading to a loss of shmoo formation (24). Additionally, proline-rich regions are known to bind SRC homology 3 domains, which in turn have been shown to be involved in cytoskeleton organization in yeast (50). Thus, regulation of eIF5A expression could provide a mechanism to regulate cytoskeleton changes in eukaryotic cells.

Potential specialized functions for eIF5A in T. brucei are also suggested by our analysis including a role in flagellar attachment. T. brucei have a single flagellum that is attached to the cell surface through a flagellum attachment zone, which is composed of intracellular filamentous proteins and microtubules (31). Flagellar assembly plays a role in organelle segregation and in cell morphogenesis. The flagellum is attached to the cell body through membrane-bound glycoproteins, and several of these contain long poly(Pro) tracts including FLA1 and FLA2 (which both contain 9 proline stretches). FLA1 is expressed in PF parasites, whereas FLA2 is the homolog expressed in the BSF. Knock-out of FLA1 in procyclic trypanosomes has been reported to lead to detached flagella (31), which interestingly is also a phenotype that we observed after knockdown of eIF5A in both BSF and PF parasites.

An additional specialized function for eIF5A in T. brucei is likely to be in the translation of the T. brucei surface coat proteins VSGs and EPs. VSGs are expressed in the bloodstream form of the parasite life cycle and form a dense surface coat. T. brucei contain more than 1000 VSG genes, which permit the antigenic variation that allows the parasite to evade the host adaptive immune system (32). During the insect stage, VSGs are silenced, and the parasite expresses a dense coat of procyclins. This switch is dependent on the rapid degradation of VSGs and subsequent expression of procyclins. Interestingly, cysteine peptidases have been shown to be instrumental in the degradation of VSG during surface coat exchange (46). A large number of cysteine peptidases containing more than 8 consecutive prolines were identified in T. brucei, providing a potential role for eIF5A in expression of peptidases involved in VSG degradation during switching. Additionally, we found several atypical VSG proteins within the set of poly(Pro) proteins.

In analyzing the T. brucei proteome, we have identified promising candidates for the future study of eIF5A function in translation of endogenous proteins including several candidates that may lead to a specialized role or demand for eIF5A in the parasite. The requirement for eIF5A for the expression of potentially up to 15% of T. brucei proteins suggests that inhibitors of hypyusination will have potent and rapid effects on parasite cell growth. Thus, if druglike molecules that show good selectivity over the human enzyme can be identified, DHS could be a promising target for future drug discovery in not just T. brucei but in other eukaryotic pathogens.

Author Contributions—M. A. P. conceived and coordinated the study and wrote the paper. S. N. and S. R. designed, performed, and/or analyzed experiments shown in Figs. 2–5. L. K. and N. V. G. designed, performed, and analyzed experiments shown in Fig. 6. Q. L. provided technical help for Figs. 3 and 4. S. N. and C. L. designed, performed, and analyzed experiments shown in Fig. 7. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgment—We thank Anthony J. Michael for critical reading of the manuscript.
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