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Molecular architecture of leishmania EF-1α reveals a novel site that may modulate protein translation: A possible target for drug development

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Abstract

Elongation factor-1α plays an essential role in eukaryotic protein biosynthesis. Recently, we have shown by protein structure modeling the presence of a hairpin-loop of 12 amino acids in mammalian EF-1α that is absent in the leishmania homologue [D. Nandan, A. Cherkasov, R. Sabouti, T. Yi, N.E. Reiner, Molecular cloning, biochemical and structural analysis of elongation factor-1 alpha from *Leishmania donovani*: comparison with the mammalian homologue, Biochem. Biophys. Res. Commun. 302 (2003) 646–652]. As a consequence of this deletion, an exposed region is available on the main body of leishmania EF-1α. Here we report the generation of an anti-EF-1α antibody (DN-3) which bound selectively to the exposed region of leishmania EF-1α, with no reactivity with human EF-1α. In a leishmania cell-free protein translation system, DN-3 substantially inhibited protein translation. A similar inhibitory effect was observed when a specific peptide based on the exposed region was used in the cell-free protein translation assay. The application of structure-based *in silico* methods to identify potential ligands to target the exposed region identified a small molecule that selectively attenuated *in vitro* translation using leishmania extracts. Moreover, this small molecule showed selective suppressive effect on multiplication of leishmania in culture. Taken together, these findings identify a novel, exposed region in leishmania EF-1α that may be involved in protein synthesis and a potential site for drug targeting.

Keywords: Leishmania; Elongation factor-1α; SHP-1 activator; Translation; Drug-development

Protozoa of the genus *Leishmania* are causative agents of the leishmaniasis, a group of tropical and sub-tropical diseases endemic in 88 countries on 4 continents (http://www.who.int/tdr/diseases/leish/diseaseinfo.htm). In humans these organisms can cause a spectrum of diseases ranging from a relatively confined cutaneous form to progressive and fatal visceral disease. The visceral form of disease is usually caused by *Leishmania donovani*. According to WHO leishmaniasis affects 12 million people worldwide and two million new cases each year. Currently available drugs to treat leishmaniasis have high toxicity and only moderate levels of efficacy. Thus, there is an urgent need to identify novel drug targets or vaccine candidates to effectively control these devastating diseases.

Leishmania cycle between two distinct developmental stages. In the mammalian host, the organisms reside intracellularly as non-motile amastigotes within the phagolysomes of macrophages. Outside the mammalian host, they exist as extracellular flagellated promastigotes in the gut of their sandfly vector. Recent research has shown the potential for leishmania to modulate macrophage functions to their advantage [1–3]. Impairment of host signal transduction and alteration in cytokine profiles have been studied most actively. Interestingly, the phenotype of attenuated cell signaling in leishmania infected cells has been linked to activation of the host regulatory enzyme Src-homology 2 (SH2) domain containing protein tyrosine

Abbreviations: EF-1α, elongation factor-1α; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride.

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phosphatase-1 (SHP-1) [4,5]. Critical evidence to suggest a role for SHP-1 in disease pathogenesis includes the findings that SHP-1 activity was increased in leishmania-infected cells [4], and leishmania infection was attenuated in mice deficient in SHP-1 [5]. Recently, our search for a potential leishmania modulator of SHP-1 led to the identification of EF-1α as a potential virulence factor. Leishmania EF-1α was found to be a specific SHP-1 binding and activating protein that was able to recapitulate the deactivated phenotype of infected cells [6].

Eukaryotic EF-1α catalyzes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein synthesis. It is also involved in the capture of deacylated tRNA at the exit site and its delivery to aminoacyl tRNA synthase [7]. In addition to canonical function of EF-1α, a growing number of studies have identified novel functional properties of EF-1α unrelated to protein translation [8–12].

Despite the highly conserved nature of EF-1α, biochemical characterization of the leishmania protein revealed several distinct differences when compared to mammalian EF-1α [13]. Most importantly, a 12 amino acid deletion in the primary sequence of the leishmania sequence was identified [13]. The distinct properties of leishmania EF-1α suggested possible differences in protein folding. In fact, protein modeling based on the known crystal structure of yeast EF-1α showed significant differences when comparing the leishmania EF-1α with the human orthologue [13]. The single most striking difference was the absence of a hairpin loop from the leishmania sequence that corresponded to the 12 amino acid deletion. The crystal structure of EF-1α protein from yeast shows very close proximity of the 20 Å long hairpin loop to the main body of the protein [13]. Consequently, an exposed region is available in leishmania EF-1α that is protected by the hairpin loop in the mammalian protein. At present it appears that no known function has been assigned to this particular region in EF-1α.

In the present study we have established a cell-free protein translation system for *L. donovani* to investigate whether the exposed region of EF-1α is involved in protein translation. By targeting the exposed region with (i) an antibody, (ii) a peptide based upon the region, and (iii) a small molecule identified by an *in silico* design method, we observed selective inhibition of leishmania protein synthesis *in vitro*. In addition, *in silico* designed small molecule selectively attenuated multiplication of leishmania in culture. These findings identify a novel area in EF-1α that may modulate protein synthesis *in vivo*, and also identify EF-1α as a potential candidate for drug targeting.

Materials and methods

Reagents and chemicals. Medium M199, PBS, protease inhibitors (phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, chymostatin, pepstatin), hemin, adenosine, folic acid, amino acids, GTP, ATP, phosphocreatine, DTT, spermidine, creatine kinase, electrophoresis and Western blotting reagents were from Sigma Company, (St. Louis, MO). Epoxomicin was from Calbiochem Corp. (La Jolla, CA). Fetal bovine serum (FBS) was from Invitrogen Life Technologies (Burlington, Ont., Canada). [^{35}S]Methionine was from Perkin-Elmer Life Sciences (Woodbridge, Ont., Canada). Specific peptide (EKVRFIPIS) synthesis and rabbit polyclonal antibody development was carried out by Genemed Synthesis (South San Francisco, CA). Control peptide (KINERPVFY) was synthesized at NAPS (University of British Columbia, BC, Canada). Monoclonal EF-1α antibody was from Upstate Cell Signaling Solutions (Lake Placid, NY). ECL detection reagent was from Amersham Biosciences (Oakville, Ont., Canada). Compound A was from The National Cancer Institute ZIN database.

Cell culture. Leishmania donovani promastigotes and THP-1 cells were cultured *in vitro* as described [6].

Generation of polyclonal antibody against exposed region of leishmania EF-1α. Polyclonal antibodies were raised against a peptide (EKVRFIPIS, 186–194 aa) based upon the sequence of the exposed region of leishmania EF-1α [13]. Rabbit IgG was purified from pre-immune and immune sera using protein A–agarose.

Whole cell lysates for immunoblotting. Approximately 1×10^7 THP-1 cells were washed twice with phosphate buffered saline (PBS) and resuspended in 1 mL modified RIPA buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 0.15 M NaCl, and 0.25% sodium deoxycholate) containing protease inhibitors (1 mM leupeptin, 1 mM aprotinin, and 1 mM PMSF). Cell lysates were clarified by microcentrifugation at maximum speed for 10 min at 4 °C. Whole cell lysates of leishmania promastigotes were prepared essentially as described above for THP-1. Equal amounts of proteins from THP-1 cell and leishmania promastigote lysates were run on 10% SDS–PAGE gels and transferred to nitrocellulose membrane. Filters were blocked using 5% powdered skim milk in PBS followed by incubation with primary antibody. After washing, the blots were incubated with HRPO conjugated anti-rabbit and developed using the ECL detection system.

Immunoprecipitation. 5×10^8 leishmania promastigotes were lysed in ice cold lysis buffer (50 mM Tris, pH 7.4, 0.15 M NaCl, and 1% Triton X-100) containing protease inhibitors (1 mM leupeptin, 1 mM aprotinin, and 1 mM PMSF). After 45 min, insoluble materials were removed by centrifugation in a microcentrifuge at maximum speed for 10 min at 4 °C. The supernatant was saved and used for immunoprecipitation. Briefly, 500 μL of supernatant was transferred into a clean microcentrifuge tube and immune rabbit IgG (DN-3) was added at a dilution of 1:1250 and rotated at 4 °C for 16–18 h. Immune-complexes were recovered by protein A-sepharose at 4 °C for 1 h. After extensive washing, immune complexes were released by boiling agarose beads in Laemmli SDS–gel sample buffer. Samples were analyzed on a 7.5% SDS–polyacrylamide gel. After transfer to nitrocellulose, blots were probed with commercial monoclonal anti-EF-1α antibody. As a specificity control for immunoprecipitation an equal amount of pre-immune IgG was used.

Preparation of extracts from L. donovani for cell-free protein translation. Methodology used in this study was based upon work published by Duszenko et al. [14] with some minor changes. All procedures were carried out on ice. Briefly, 1×10^9 promastigotes of *L. donovani* were lysed in 1 mL hypotonic buffer containing a cocktail of protease inhibitors (10 mM 2-aminopurine, 2 mM dithiothreitol, 1 mM PMSF, 1 μM pepstatin, 1 μM leupeptin, 1 μM chymostatin, and 1 μM aprotinin), and proteosome inhibitor (1 μM epoxomicin), and homogenized in a tight-fitting Dounce homogenizer. Cell lysis was monitored by phase-contrast microscopy. The resulting homogenate was subjected to centrifugation at 12,000*g* at 4 °C for 6 min to remove unbroken cells, cell debris, mitochondria and nuclei. Prior to centrifugation, phosphate buffer, pH 7.4, was added to cell lysate to a final concentration of 20 mM. The resulting post-mitochondrial supernatant contained all the necessary components for monitoring ongoing peptide/protein synthesis by adding radiolabeled (L-[^{35}S]methionine).

In vitro cell-free protein translation. Assays were performed using 50 μL of post-mitochondrial supernatant supplemented with L-[^{35}S]methionine (20 kBq/μL); 100 μM each of all other amino acids, 160 mM potassium acetate, 2 mM $MgCl_2$, 150 mM GTP, 1 mM ATP, 10 mM phosphocreatine, 4 U creatine kinase, 2 mM DTT, 200 mM spermidine chloride, 1 mM PMSF, 1 μM pepstatin, 1 μM leupeptin, 1 μM chymostatin, 1 μM aprotinin, and 1 μM epoxomicin. The final reaction volume was 100 μL and

in vitro cell free protein translation was performed by incubation at 30 °C for 1 h unless otherwise specified. Reactions were terminated by placing reaction tubes under liquid nitrogen. For quantitation of radioactive methionine incorporation into newly synthesized peptides aliquots of 5 μL of each reaction tube were mixed with 450 μL NaOH (1 M) and 50 μL H_2O_2 (30%) and incubated at 37 °C to discharge aminoacyl-tRNAs, especially L-[^{35}S]Met-tRNA. Proteins were precipitated with TCA (20% final concentration) on ice for at least 3 h and precipitates were collected on glass fibre filters (Whatman GF/C) and washed 2 times with 10 mL TCA 8%, and once with 5 mL ethanol. Filters were dried at room temperature and bound radioactivity was recorded by liquid scintillation counting (Beckman Coulter L56500).

In vitro cell-free protein translation in the presence of DN-3 antibody and peptide based on the exposed region of leishmania EF-1α. In vitro translation assays were performed essentially as described above. For these experiments leishmania translation extracts (50 μL) were incubated on ice for 10 min in the presence of 2 μg of DN-3 IgG antibody or pre-immune IgG as control, before being supplemented with radiolabeled amino acid and other reaction components. Parallel assays were also performed in presence of peptide based on a sequence from the exposed area (EKVRFIPIS, specific peptide) of EF-1α or in the presence of a scrambled control peptide (KINERPVFY) both at 1 μM final concentration. Similar *in vitro* translation assays were performed essentially as described above using THP-1 cell translation extracts in the presence of DN-3 antibody as a control for specificity

Blocking of antigen binding site of DN-3 antibody prior to in vitro cell-free protein translation. In vitro translation assays were also carried out using DN-3 antibody that had been pre-incubated with immunizing peptide specific pep (DN-3*) in an attempt to neutralize the antibody reagent. These translation assays were performed essentially as described above. Pre-incubation with the specific peptide was done on ice for 20 min. Leishmania translation extracts were then incubated on ice for 10 min in presence of DN-3*, before being supplemented with radiolabeled amino acid and other reaction components. As a control, DN-3 antibody was pre-incubated in parallel with the scrambled control peptide. Two micrograms of DN-3 antibody and 0.1 μM of each peptide were used for these experiments.

In silico design method to screen for small compounds that specifically target leishmania EF-1α. These procedures involved a compound library generation, binding site prediction and small molecule docking. To create a database of drug-like compounds, we used The National Cancer Institute (NCI) ZINC database [15]. From 200,000 compounds in the database 20,000 met typical drug-likeness criteria. We applied the Alpha Site Finder function of the Molecular Operating Environment (MOE) package (Versión 2003.02, Montreal Chemical Computation Group Inc.) to identified potential cavities around the exposed region of Leishmania EF-1α. The cluster of spheres closest to the loop in three-dimensional space was selected as the binding site to the target. The Maestro program (Maestro, Schrödinger Inc., San Diego, CA, 2004) was used during protein preparation. Hydrogen atoms were added, no water molecules and ions were present in the two homology models. Protein and ligand charges were assigned by the OPLS molecular mechanics force field method. The Glide program (version 2.7, Schrödinger Inc., San Diego, CA, 2004) was used for running Ligand-receptor docking. Initially we docked the 20,000 compounds from our library to the leishmania EF-1α, then the top 200 scoring hits were docked against the human EF-1α. This narrowed our list to 20 compounds that showed the largest differences in docking scores between leishmania and human EF-1α. Out of this short-list compound CpA scored the highest and was obtained from NCI as a gift.

Results and discussion

Specificity of the polyclonal antibody against EF-1α

To examine the involvement of selectively exposed area of leishmania EF-1α in the protein translation, polyclonal antibody (DN-3) was raised against a peptide (EKVRFI-

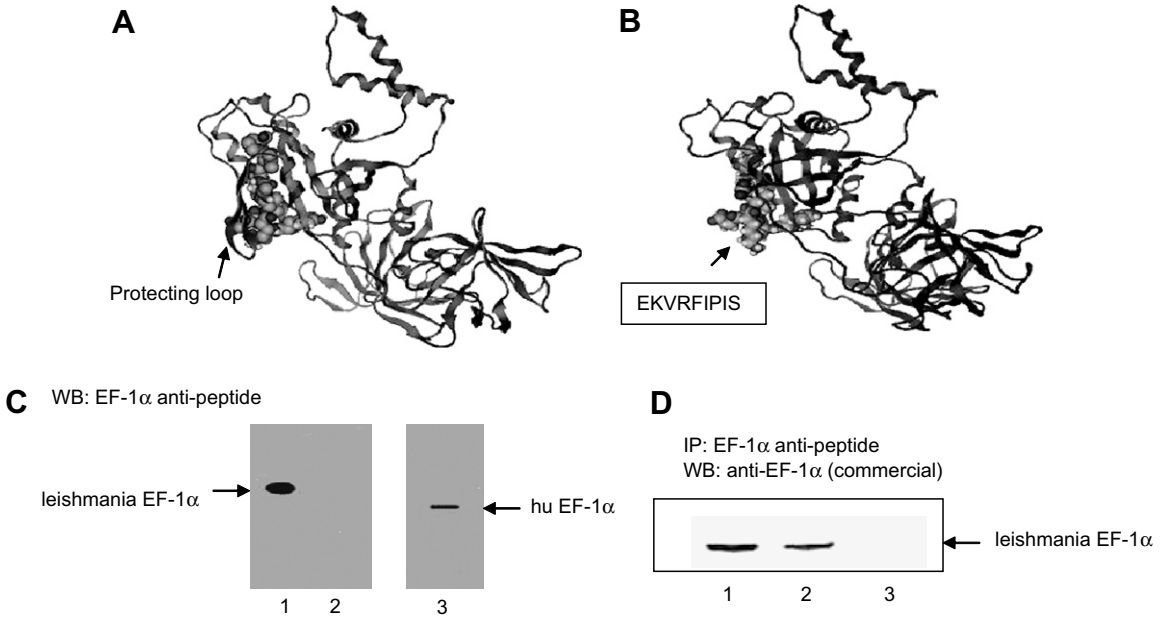

Fig. 1. Specificity of antibodies raised against the exposed region of leishmania EF-1α. Polyclonal antibodies (DN-3) were raised against a synthetic peptide based on the sequence (EKVRFIPIS) of exposed region of leishmania EF-1α (B) which is protected in human EF-1α (A). (C) Specificity of DN-3 was characterized by immunoblotting using whole cell lysates of leishmania promastigotes and human THP-1 cells. Lane 1, leishmania lysate and lane 2, THP-1 lysate. The presence of EF-1α in THP-1 extracts was confirmed by probing with commercial anti-EF-1α (lane 3). (D) DN-3 antibodies recognize native leishmania EF-1α. Triton X-100 soluble extracts from leishmania promastigotes were used for immunoprecipitation using DN-3 (lanes 1,2) or pre-immune IgG (lane 3). Immunoprecipitated proteins were separated by SDS–PAGE transferred to nitrocellulose and membranes were probed with leishmania EF-1α (commercial).

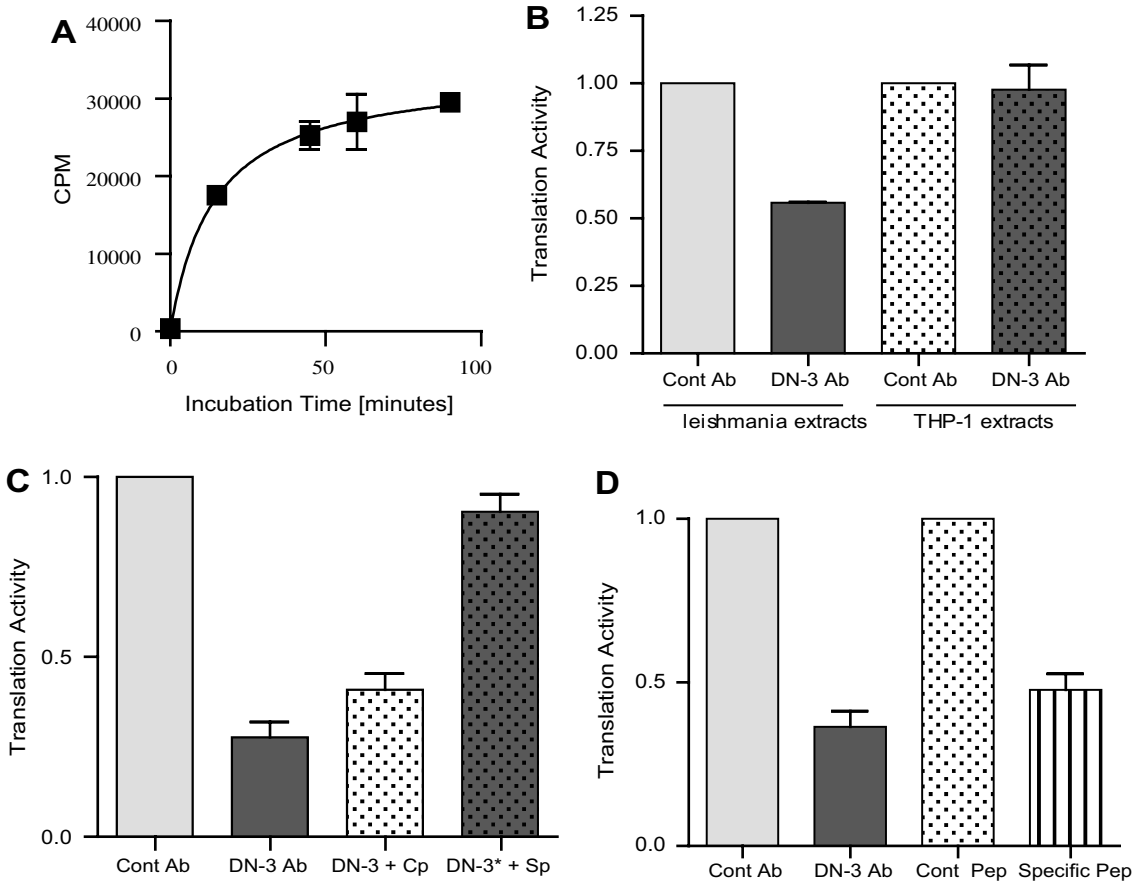

Fig. 2. (A) Time-course of incorporation of radiolabeled methionine into new proteins. Translation assays were performed using a post-mitochondrial supernatant containing all the components necessary to follow ongoing peptide/protein synthesis by adding radiolabeled amino acid (L-[^{35}S]methionine). *In vitro* translation was performed at 30 °C for indicated lengths of time. (B,D) Selective *in vitro* protein translation inhibition by DN-3 antibody and by a specific peptide. Leishmania translation extracts were incubated on ice for 10 min in the presence of 2 μg of DN-3 IgG or pre-immune IgG as control, before supplementation with radiolabeled amino acid and other reaction components followed by incubation for *in vitro* protein translation at 30 °C for 1 h. A specific peptide based on exposed area of leishmania EF-1α was used as potential inhibitor of *in vitro* translation. As a control a random peptide based on the same amino acid content of specific peptide was designed. Effect of DN-3 antibody on cell-free protein translation system in THP-1 cells. THP-1 translation extracts were separately incubated on ice for 10 min in the presence or absence of 2 μg of DN-3 IgG or pre-immune IgG as control, before supplementation with radiolabeled amino acid and other reaction components followed by incubation for *in vitro* protein translation at 30 °C for 1 h (B). (C) Mechanism of action of DN-3 antibody. Two micrograms of DN-3 antibody was pre-incubated with either 0.1 μM specific peptide (Sp) to produce preadsorbed antibody (DN-3*) or scrambled control peptide (Cp) on ice for 20 min. Leishmania translation extracts were then incubated on ice for 10 min in presence of DN-3*, before performing translation assay at 30 °C for 1 h.

PIS, 186–194 aa) based on the sequence of exposed region (Fig. 1A and B). Specificity of immune sera was investigated by immunoblotting using whole cell lysates of leishmania promastigotes and THP-1 cells. As shown in Fig. 1C and lane 1, antibody DN-3 reacted specifically to a protein of the correct size for leishmania EF-1α. On the other hand this antibody showed no reactivity with THP-1 cell lysates (lane 2). The presence of EF-1α in THP-1 extracts was confirmed by probing with commercial anti-EF-1α (Fig. 1C, lane 3). Taken together, these results demonstrated specificity of DN-3 towards leishmania EF-1α. The fact that DN-3 was able to recognize EF-1α in its native conformation, based on immunoprecipitation assays (Fig. 1D, lanes 1 and 2), allowed us to examine whether it had the capacity to inhibit *in vitro* protein synthesis in a leishmania cell free translation system.

Inhibition of in vitro translation by DN-3 antibody based on the exposed region of leishmania EF-1α

A cell free translation procedure was developed for leishmania based on published method for the related organism *Trypanosoma brucei* [14], with minor modifications as described in Materials and methods. Translation assays were performed at 30 °C for various lengths of time. As shown in Fig. 2A translation was active for at least 90 min. Specificity of *in vitro* translation was checked using cycloheximide, a specific eukaryotic translation inhibitor (data not shown). To address whether the exposed region of leishmania EF-1α was important for protein translation, leishmania translation extracts were incubated on ice for 10 min in the presence of 2 μg of DN-3 IgG or pre-immune IgG as a control, before being supplemented with radiola-

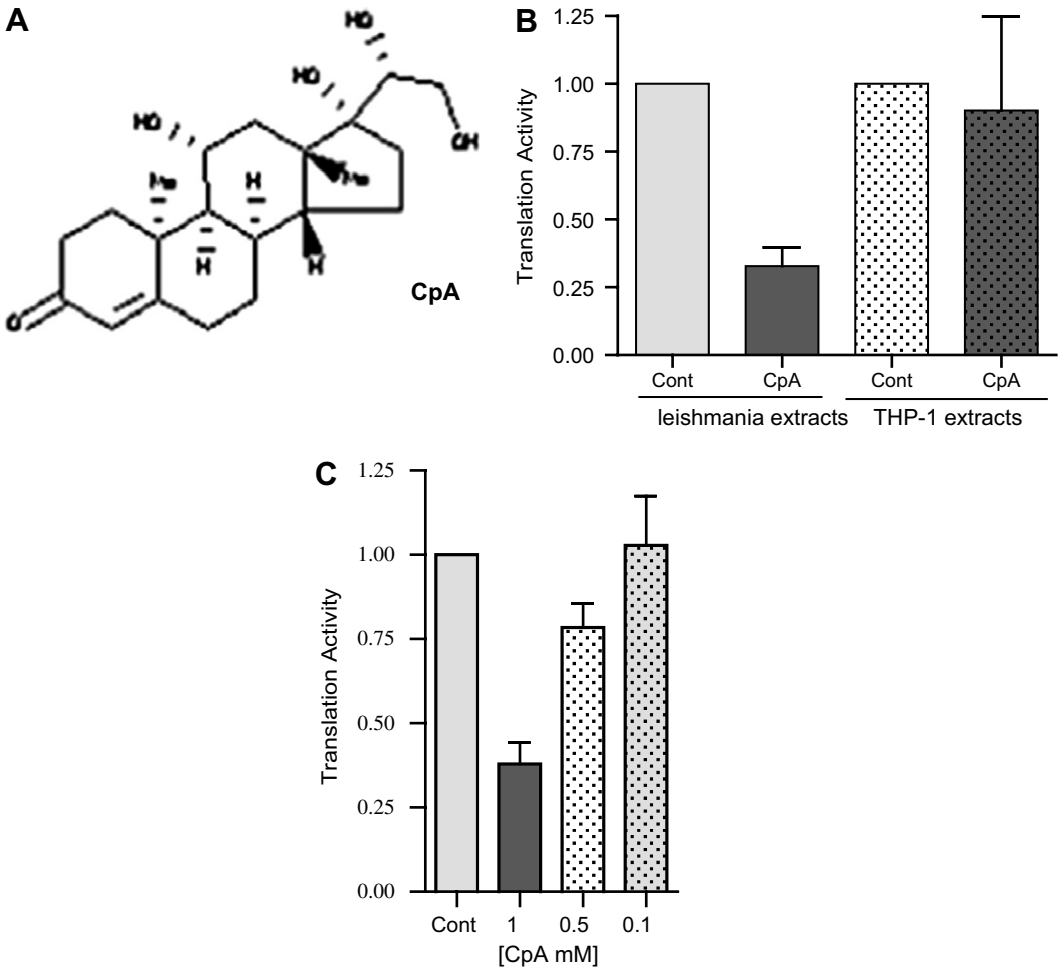

Fig. 3. Effect of compound CpA on cell-free protein translation system using leishmania and THP-1 cell extracts. Leishmania and THP-1 translation extracts were incubated on ice for 10 min in the presence or absence of indicated concentrations of compound CpA, before supplementation with radiolabeled amino acid and other reaction components. *In vitro* translation assays were performed as described in Fig. 2. (A) Chemical structure of compound CpA. (B) Effects of CpA on *in vitro* translation in leishmania and THP-1. (C) Concentration dependent inhibition of translation by CpA.

beled amino acid and other reaction components. As shown in Fig. 2B, DN-3 antibody significantly inhibited incorporation of L-[^{35}S]methionine into protein.

Mechanism of action of DN-3 antibody on in vitro cell-free protein translation

To strengthen the argument that DN-3 antibody was acting by interacting with the exposed region, we performed *in vitro* translation assays in the presence of DN-3 antibody that had been pre-incubated with the specific peptide (EKVRFIPIS, used for raising DN-3 antibody). We anticipated that DN-3 antibody would bind the specific peptide, and as a consequence antigen binding sites on DN-3 would be occupied and unavailable to bind EF-1α in the *in vitro* translation assay. As shown in Fig. 2C, the inhibitory activity of DN-3 was abrogated by pre-absorption with specific pep but not with the scrambled, control peptide. This argument also implies that the corresponding region in the mammalian protein which is shielded by the hairpin loop (missing from leishmania EF-1α) is also likely to be involved in protein translation.

Specific peptide designed based on exposed area inhibits in vitro cell-free protein translation

The antibody may have exerted an inhibitory effect either by inducing a conformational change in EF-1α, or by interfering with the binding of protein(s) to the exposed region that are required for protein translation to proceed. To address this question and to provide further evidence to support the importance of the exposed region in leishmania protein synthesis, a peptide EKVRFIPIS (specific peptide used for raising antibody) was used as potential competitive inhibitor of *in vitro* translation. As a control, a random peptide based on the same amino acid content of specific peptide was used. As shown in Fig. 2D, when compared to control peptide, addition of specific peptide significantly inhibited protein translation in a manner comparable to that of DN-3 antibody, supporting the conclusion that

DN-3 act by binding to the exposed of region EF-1α rather than via non-specific steric hindrance. The specific peptide seems to be a competitive inhibitor for component of the translational machinery interacting with exposed area of leishmania EF-1α. The effect of DN-3 antibody for inhibition of protein translation was selective as no effect of DN-3 antibody on *in vitro* translation was observed when extracts from THP-1 were used (Fig. 2B).

Selective inhibition of in vitro translation by a small chemical compound against the exposed region of leishmania EF-1α

Our findings of selective inhibition of protein translation using DN-3 antibody and a specific peptide identified the exposed region on leishmania EF-1α as a potential candidate target for drug development. To investigate further this possibility the 3D model of EF-1α was used to screen a large virtual compound library that led to a small number of hits. To examine whether the exposed region of leishmania EF-1α is suitable for drug targeting, structure based *in silico* methods were used to identify potential ligands to the exposed area of EF-1α. A potential lead compound identified in this manner was obtained from National Cancer Institute and tested in the *in vitro* translation assay. As shown in Fig. 3, compound CpA (Fig. 3B) significantly attenuated translation when added to leishmania extracts (>70%). Inhibition of translation was concentration dependent (Fig. 3C) and selective in that CpA did not significantly reduce translation activity in THP-1 cell extracts (Fig. 3B) providing strong support for the argument that the exposed region of leishmania EF-1α is an attractive drug target. Our data show that 1 mM compound A is required to achieve 70% inhibition of *in vitro* protein translation in leishmania without affecting translation in human THP-1 cells. Use of 1 mM compound CpA seems excessive; however it should be pointed out that EF-1α is an abundant protein [16]. It is also likely that some amount of compound A was consumed due to non-specific interactions with other components of *in vitro* translational system.

Activity of compound CpA against leishmania culture

As shown above, CpA selectively inhibited *in vitro* translation activity in leishmania extract. This prompted us to investigate whether CpA has effect on the multiplication of leishmania promastigotes in culture. Equal numbers of exponentially growing leishmania were distributed in a 24-well plate and incubated with CpA at a concentration of 0.5, 0.2, and 0.05 mM and multiplication was evaluated at 24, 48, and 72 h. As shown in Fig. 4A, compound CpA displayed a concentration-dependent suppressive effect on the multiplication of leishmania in culture. When treated with a concentration of 0.5 mM CpA, the multiplication of leishmania was severely affected (62–78% inhibition). This effect was selective as shown in Fig. 4B, CpA has no inhibitor effect on multiplication of THP-1 at concentrations which produced significant suppressive effect on leishmania. It is also possible that ineffectiveness of CpA on THP-1 cells could be lack of transport inside THP-1. However, hydrophobic nature of compound CpA should facilitate its transport across plasma membrane of THP-1. Taken together, these results show that CpA has potent anti-leishmanial properties with molecular selectivity.

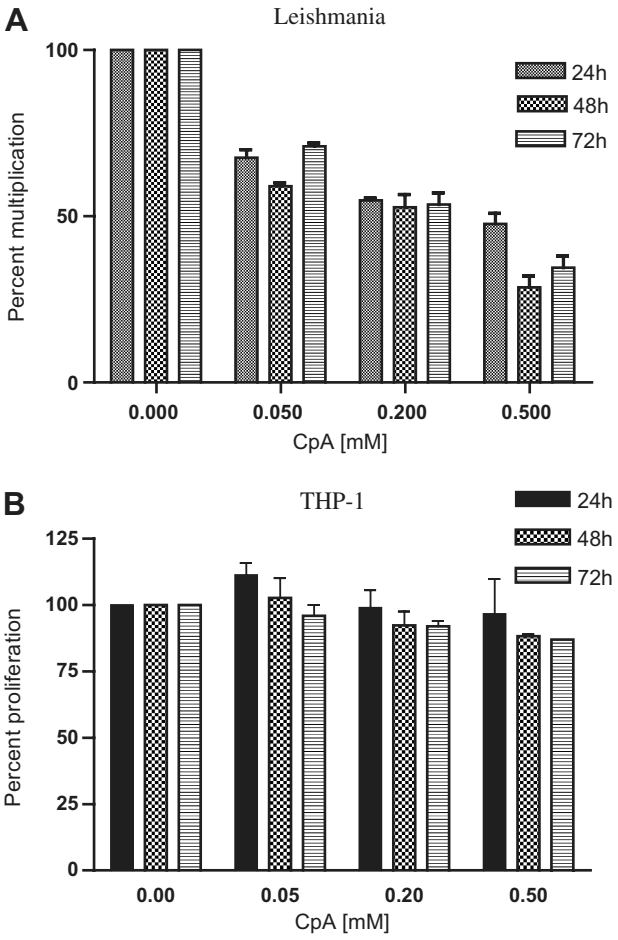

Fig. 4. Effect of compound CpA on multiplication of leishmania and THP-1 cells in culture. Equal number of leishmania [2×10^6 parasite/mL/well] (A) and THP-1 cells [0.2×10^6 cells/mL/well] (B) were separately distributed in a 24-well plate and treated with CpA at a concentration of 0.05, 0.2, and 0.5 mM. Cell numbers were determined at the indicated period of time using a hematocytometer and trypan blue staining for assessment of cell viability.

In summary, the results reported herein, have made a novel observation that an exposed region within leishmania EF-1α appears to be functionally important in modulating protein translation, at least in leishmania. Previous studies from this laboratory [6] provided evidence that leishmania EF-1α is involved in pathogenesis. The role of leishmania EF-1α as a potential virulence factor provides an additional basis—beyond its role in translation—for examining this protein as a candidate drug target. Given that EF-1α is critical for cell survival, even partial inhibition of translation by targeting this region would likely have substantial impact the viability of leishmania. Moreover, this study represents an example, how recent tools including molecu-

lar modeling, *in silico* protein structure analysis and docking of virtual chemical compounds can be combined with more traditional biochemistry to identify potential drug target.

Acknowledgments

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