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*Tb*ENF is an essential *Tb*TFIIB-interacting trypanosomatid-specific factor

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ABSTRACT

Trypanosoma brucei, the causative agent of African Sleeping Sickness, is replete with unique biochemistry, including unusual features of gene transcription. The parasite also contains over 4500 non-annotated genes, representing novel biochemistry yet to be explored. Using tandem affinity purification (TAP)-tagged *Tb*TFIIB, we identified and subsequently confirmed, one of the non-annotated *T. brucei* proteins, Tb11.02.4300, as a *Tb*TFIIB-interacting protein. The 49 kDa protein is nuclear and essential for parasite variability as determined by RNA interference studies; hence, the nomenclature *T. brucei* Essential Nuclear Factor (*Tb*ENF). *Tb*ENF is shown to interact with DNA in a sequence-independent fashion under the conditions examined. Furthermore, *Tb*ENF bears motifs associated with many eukaryotic transcription factors, such as a glutamine-rich region and a leucine zipper, yet *Tb*ENF is specific to trypanosomatids making it a potentially attractive therapeutic target. Taken together, our results suggest a role for *Tb*ENF in trypanosome gene transcription.

1. Introduction

Parasitic protozoan trypanosomatids are the causative agent of world-wide diseases, including Chagas' Disease, Leishmaniasis, and African Sleeping Sickness, afflicting millions [1]. *Trypanosoma brucei*, transmitted through the tsetse fly, causes human African Sleeping Sickness. The parasite also infects livestock, causing significant negative economic impact in sub-Saharan Africa. Treatments for the disease are limited, often toxic, and resistance can be problematic. New therapeutics are needed.

Trypanosomes have many unusual biochemical features which may serve as possible therapeutic targets, including transcription. RNA polymerase (RNAP) II-dependent gene transcription and the regulation of gene expression differ significantly from the process in higher eukaryotes. Protein-coding genes are transcribed polycistronically and are processed into mature individual mRNA through the *trans*-addition of a short capped Spliced Leader (SL) RNA and the addition of a poly(A) tail at the 5′ and 3′ ends of the transcript, respectively [2,3]. The regulation of gene expression is hypothesized to occur mainly post-transcriptionally and, in some cases, has been found to occur at the level of translation [4–6]. Recent work also indicates that at least some transcriptional control is exercised at the level of chromatin remodeling in the parasites [2,7].

Canonical RNAPII promoter elements for protein coding genes appear lacking. Indeed, the small nuclear SL RNA gene contains the only known RNAPII-dependent gene promoter in trypanosomes [8]. The transcriptional initiation of protein coding genes is not well understood, although evidence is accumulating that transcription initiates at regions of the genome where clusters of open reading frames switch from one strand to another (strand-switch regions) [9,10].

Several of the basal transcription factors are either present in divergent forms or appear absent. For example, the trypanosomatid general transcription factor TFIIB is divergent in its sequence [11,12] and the crystal structure reveals the presence of extra helices and loops speculated to participate in trypanosome–specific protein interactions [13]. The composition of *Tb*TFIIH was examined and found to contain essential subunits unique to the parasite, while lacking the cyclin-activating kinase [14,15]. The basal factors TFIIF and TFIIE appear either absent or are not readily discernable in the genome [16]. An extremely divergent form of the mediator head module was identified in trypanosomes and shown to be essential in small nuclear gene transcription [17]. The trypanosome small nuclear RNA-Activating Protein complex (SNAPc), involved in small nuclear RNA gene transcription, harbors one unique subunit and lacks others compared to its homologs in higher eukaryotes [18,19]. *T. brucei* TATA binding protein (TBP)/*Tb*TBP-related factor 4 (TRF4) is unique as several key DNA-interacting amino acids are not conserved in the trypanosome homologs [20]. Finally, the carboxyl terminal domain

Abbreviations: RNAP, RNA polymerase; SL, spliced leader; ENF, Essential Nuclear Factor; TAP, Tandem Affinity Purification; RNAi, RNA interference.

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(CTD) of the largest subunit of *Tb*RNAPII lacks the canonical heptad repeats of YSPTSPS, which in other eukaryotes orchestrate and coordinate the factors involved in the various stages of transcription [21,22]. The observed divergence of the factors characterized thus far suggests transcription in trypanosomatids, in part, does not mirror the process in other eukaryotes. The unique properties of the characterized parasite proteins suggest novel, uncharacterized proteins may play roles in trypanosomatid transcription.

Currently, more than 4500 *T. brucei* gene products are non-annotated (http://tritrypdb.org/tritrypdb); among these are potential candidate proteins for roles in transcription. This pool of non-annotated proteins may also represent a host of targets for much needed therapeutics to treat the diseases inflicted by these obligate parasites. To seek out novel transcription-related proteins in trypanosomatids, we utilized a tandem affinity purification (TAP)-tagged *Tb*TFIIB and identified interacting proteins through mass spectrometry. Among the interacting proteins is one that harbors several hallmarks of known transcription factors, yet is specific to trypanosomatids. The protein, Essential Nuclear Factor 49 kDa (*ENF*), strongly interacts with *Tb*TFIIB, is essential for parasite viability, and binds tightly to DNA.

2. Materials and methods

2.1. TAP-tagged TbTFIIB generation and purification

To generate a carboxyl-terminal tandem affinity purification (TAP)-tagged *Tb*TFIIB, the TFIIB gene (GenBank ID: 7083113) was amplified from *T. brucei* genomic DNA and inserted into the pLEWIII plasmid using the BamHI and HindIII sites. All primer sequences may be found in the Supplemental Tables 1 and 2. The N-terminal TAP tag was obtained from pJM26 (a kind gift from the Bellofatto laboratory, [18]) through BamHI digestion and insertion into the pLEWIII derivative containing the *Tb*TFIIB gene. The resulting construct was verified by DNA sequencing and named pTAP-*Tb*TFIIB. Ten μg of NotI-linearized pTAP-*Tb*TFIIB was transfected into procyclic *T. brucei* cell line 29-13 [23], following the electroporation protocol outlined in [24]. TAP-*Tb*TFIIB expression was induced through the addition of 500 ng/mL tetracycline to the media for 24 h.

TAP-*Tb*TFIIB was purified from 2 L of parasites grown to 1.5×10^7 cells/mL based on the protocols of [18] and [25]. Nuclear extract was first prepared [26], nucleic acids were removed through ammonium sulfate precipitation, and the resulting proteins resuspended in immunoglobulin G (IgG) binding buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% NP-40, 1 mM DTT, 10% glycerol, containing PMSF, pepstatin, and leupeptin). The proteins were applied to an IgG sepharose column, and after extensive washing, the column was equilibrated into tobacco etch virus (TEV) cleavage buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 0.1% NP-40, 1 mM DTT, 5% glycerol). Protein was eluted from the column following digestion by 100 units of TEV protease at 4 °C for 2 h. The eluted proteins diluted into calmodulin binding buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1% NP-40, 10% glycerol, and 1 mM DTT), were applied to calmodulin resin (Sigma). After extensive washing, the proteins were eluted with calmodulin elution buffer (calmodulin binding buffer containing 5 mM EGTA). Eluted proteins were concentrated, separated by 10% SDS–PAGE, and analyzed by MALDI-TOF and Q-TOF mass spectrometry at the Center for Advanced Proteomics Research (UMDNJ-New Jersey Medical School, Newark, NJ).

2.2. Glutathione-S-transferase (GST)-tagged TbENF generation and purification

To generate recombinant amino-terminal GST-tagged *Tb*ENF, the *Tb*ENF gene was amplified from *T. brucei* genomic DNA and inserted into pGEX-6P-1 (GE Healthcare) at the XhoI site. The resulting construct was verified by DNA sequencing and named pGST-*Tb*ENF. Recombinant GST-*Tb*ENF was expressed in *Escherichia coli* BL21 cells and purified to approximate homogeneity using glutathione agarose according to the manufacturer's guidelines. PreScission protease (GE Healthcare) was used to remove the GST tag and the resulting recombinant *Tb*ENF protein was used to generate custom polyclonal antibodies in rabbits (Lampire Biologicals, Pipersville, PA).

2.3. Verification of the TbENF–TbTFIIB interaction

To verify the interaction between *Tb*TFIIB and *Tb*ENF, recombinant GST-*Tb*ENF or GST and *T. brucei* 427 whole cell extract, prepared as described in [27], were used. Ten μg of recombinant GST-*Tb*ENF purified to homogeneity was bound to 35 μL of glutathione sepharose and mixed with 700 μg of whole cell extract. The reaction, 300 μL total volume in pulldown buffer (150 mM sucrose, 20 mM potassium glutamate, 10 mM HEPES-KOH, pH 7.9, 2.5 mM $MgCl_2$, 1 mM EDTA, and 2.5 mM DTT containing protease inhibitors), was incubated with rotation at 4 °C for 30 min. The beads were then washed extensively with 150 mM sucrose, 10 mM HEPES-KOH, pH 7.9, 2.5 mM $MgCl_2$, 1 mM EDTA, 0.2% NP-40, 2.5 mM DTT, and either KCl ranging from 0.3 to 0.9 M or potassium glutamate ranging from 0.2 to 0.4 M. After the stringent wash, the interacting proteins were analyzed by Western blot analysis using *Tb*TFIIB, *Tb*TBP, and *Tb*RNAPII antibodies (kind gifts from the Bellofatto laboratory). In every case, a control reaction carried out under identical conditions using GST was performed in parallel with the GST-*Tb*ENF pulldown reaction. Pulldown reactions in which the whole cell extract was treated with DNase were carried out under identical conditions using a 0.7 M KCl wash except that the extract was pretreated with 2 units of DNase at room temperature for 15 min. The DNase was determined to be active at room temperature in whole cell extract using plasmid DNA.

2.4. TbENF RNA interference studies and examination of transcript levels

To target *Tb*ENF by RNA interference, a 309 bp region corresponding to nucleotides 22-329 of the open reading frame was amplified from *T. brucei* genomic DNA and inserted into p2T7-177 [28] to yield p*Tb*ENF-RNAi. *Tb*ENF-RNAi was transfected into cell line 29-13 through electroporation and clonal cell lines generated by limiting dilution. Production of double-stranded RNA was induced by the addition of 1 μg/mL of tetracycline to the SDM-79 media daily. Parasite growth was monitored over 8 days. Daily, 8×10^6 cells were removed from culture, resuspended in Laemmli buffer [29], and *Tb*ENF levels monitored by Western blot using custom rabbit polyclonal antibody against *Tb*ENF (serum was used at dilution of 1:800) and goat anti-rabbit-IgG conjugated with alkaline phosphatase used at 1:20,000.

To measure transcript levels, RNA was isolated from 1×10^8 parasites using Trizol reagent. The RNA samples were DNase-treated and 0.5 μg RNA was used in a reverse transcription reaction (Finnzyme Phusion RT-PCR kit used according to the manufacturer's instructions) using gene-specific primers for Tb11.02.4300, the spliced leader RNA intron region, and U6 snRNA (Supplemental Table 3). The resultant cDNA was used as template in PCR reactions (20 cycles) and the products visualized on 8% polyacrylamide gels stained with SYBR green. The relative amounts of cDNA used