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Characterization of a novel Obg-like ATPase in the protozoan *Trypanosoma cruzi* ☆

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ABSTRACT

We characterized a gene encoding an YchF-related protein, TcYchF, potentially associated with the protein translation machinery of *Trypanosoma cruzi*. YchF belongs to the translation factor-related (TRAFAC) class of P-loop NTPases. The coding region of the gene is 1185 bp long and encodes a 44.3 kDa protein. BlastX searches showed TcYchF to be very similar (45–86%) to putative GTP-binding proteins from eukaryotes, including some species of trypanosomatids (*Leishmania major* and *Trypanosoma brucei*). A lower but significant level of similarity (38–43%) was also found between the predicted sequences of TcYchF and bacterial YyaF/YchF GTPases of the Spo0B-associated GTP-binding protein (Obg) family. Some of the most important features of the G domain of this family of GTPases are conserved in TcYchF. However, we found that TcYchF preferentially hydrolyzed ATP rather than GTP. The function of YyaF/YchF is unknown, but other members of the Obg family are known to be associated with ribosomal subunits. Immunoblots of the polysome fraction from sucrose gradients showed that TcYchF was associated with ribosomal subunits and polysomes. Immunoprecipitation assays showed that TcYchF was also associated with the proteasome of *T. cruzi*. Furthermore, inactivation of the *T. brucei* homolog of TcYchF by RNA interference inhibited the growth of procyclic forms of the parasite. These data suggest that this protein plays an important role in the translation machinery of trypanosomes.

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1. Introduction

Many cellular processes including protein synthesis, vesicular traffic, intracellular transport, cell signaling and differentiation, involve an important group of proteins from the GTPase superfamily (Sprang, 1997a; Bourne et al., 1990). These proteins contain the P-loop NTPase fold (Saraste et al., 1990) and are known as P-loop GTPases (Leipe et al., 2002). The P-loop is characterized by the sequence motif $GX_4GK(S/T)$ (where X denotes any amino-acid), which ensures the correct positioning of the triphosphate moiety of a bound nucleotide.

P-loop GTPases have a common structural core domain – the G domain – which forms the guanine nucleotide-binding site. The G domain includes a number of highly conserved amino-acid motifs: the P-loop or G1 motif and three other conserved motifs – G2, G3 and G4. The guanine base is recognized by the G4 motif, the consensus sequence of which is NKXD. In addition to binding GTP/GDP, these elements mediate interactions with the Mg^{+2} cofactor and downstream effectors (Sprang, 1997b; Bourne et al., 1991; Vetter and Wittinghofer, 2001).

The binding and hydrolysis of GTP to generate GDP define two functional forms of the GTPase protein: the GTP-bound (active) and GDP-bound (inactive) forms. The transition between the active and inactive states is mediated by regulatory proteins that increase GTP hydrolysis (GAPs), stabilize the GDP-bound form or accelerate nucleotide exchange (GEFs) (Sprang, 1997b; Bourne et al., 1991; Takai et al., 2001).

P-loop GTPases have been well characterized and include four ancient subfamilies corresponding to the classical translation factors IF2/eIF5B, eIF-2/SelB, EF-Tu/EF-1α and eEF2/EF-G, all of which are widespread in all three superkingdoms (Bourne et al., 1990). Other conserved P-loop GTPases, such as Era (Ahnn et al., 1986) and Obg (Trach and Hoch, 1989), also have a universal phyletic distribution (Caldon et al, 2001; Leipe et al., 2002) and interact with rRNA and/or ribosomes (Meier et al., 1999, 2000; Sayed et al., 1999; Scott et al., 2000; Caldon et al., 2001; Wout et al., 2004). They may therefore also be broadly considered as translation factors, although their roles in bacterial metabolism remain unclear (Morimoto et al., 2002; Caldon and March, 2003). These GTPases,

☆ The nucleotide sequence of the *TcYchF* gene has been deposited in the GenBank database under GenBank Accession No. AY178827.

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including the four universal translation factor families, are now classified as members of the translation factor-related (TRAFAC) superfamily of GTPases (Leipe et al., 2002).

The Obg family has five well delimited subfamilies: Obg, DRG, YyaF/YchF, Ygr210 and NOG1. The first four of these subfamilies have a glycine-rich motif (GAxxGxGxGxxxl, where l is I, L or V) immediately after the G3 (walker B) motif (DXXG) (Leipe et al., 2002). All Obg subfamilies except the NOG1 subfamily have the $YXFXTX_5G$ motif (G2 motif or effector domain) containing a conserved threonine residue in the region between the Walker A (G1) and B (G3) motifs. Obg is essential for bacterial growth, chromosome partitioning and replication and the stress response (reviewed by Caldon and March, 2003). It is also associated with the ribosome and, specifically, with the ribosomal protein L13 (Scott et al., 2000). DRG and NOG1 are ubiquitous in Archea and Eukarya (Leipe et al., 2002). The eukaryotic NOG1 has been shown to be a nucleolar GTPase in yeast (Kallstrom et al., 2003) and in the African trypanosome *Trypanosoma brucei* (Park et al., 2001) and is required for biogenesis of the 60S ribosomal subunit (Jensen et al., 2003).

YyaF/YchF is ubiquitous in bacteria and eukaryotes and presents a remarkably high level of sequence conservation. However, the function of GTPases of this class remains unknown.

We describe here the characterization of a novel YchF-related protein in *Trypanosoma cruzi* (TcYchF). This protein may be part of a ribonucleoprotein complex associated with the translation machinery of *T. cruzi*, the hemoflagellated protozoan causing Chagas disease (De Souza, 2002).

2. Materials and methods

2.1. Trypanosome cultures

Epimastigotes of *T. cruzi* Dm28c were cultured in liver infusion tryptose (LIT) medium at 28 °C. The culture was initiated by adding 5×10^5–1×10^6 cells ml^{-1} and the parasites were harvested when the culture reached a cell density of 1–2 $\times 10^7$ cells ml^{-1} (log phase parasites).

Procyclic forms of *T. brucei* (Lister 427) strain 29-13, which constitutively expresses T7 RNA polymerase and tetracycline repressor, together with drug resistance markers, were grown in SDM-79 medium, supplemented with 10% FBS in the presence of G418 (15 μg/ml) and hygromycin (25 μg/ml) (Wirtz et al., 1999).

2.2. Identification and cloning of the TcychF gene

The *TcychF* gene was identified by sequencing expressed sequence tags (ESTs) from the epimastigote stage of the Dm28c clone of *T. cruzi*. The BLAST algorithm was used to search for sequence identity. A 0.7 kb EST containing the partial coding sequence of *TcychF* was used as a radioactive probe for the screening of a *T. cruzi* cDNA library in λgt11, to obtain clones containing the full-length TcYchF cDNA, as previously described (Huynh et al., 1985). The inserts of positive clones were amplified from lysates (2 μl) by PCR on reaction mixtures (100 μl) containing 10 pmol of primers λF (5′-TTGACACCAGACCAACTGGTAATG-3′) and λR (5′-GGTGGCGACGACTCCTGGAGCCCG-3′), a 200 μM of each dNTP, 1.5 mM $MgCl_2$, *Taq* DNA polymerase buffer (Invitrogen) and 2.5 units of *Taq* DNA polymerase (Invitrogen). The λF and λR primers were constructed from the sequences flanking the EcoRI cloning site in the λgt11 vector. The reaction mixtures were heated for 4 min at 94 °C, and then subjected to 35 cycles of denaturation at 92 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 2 min. The PCR products were inserted into the TOPO-TA vector (Invitrogen), sequenced and analyzed for the presence of the mini-exon sequence and/or a poly-A tail.

DNA sequences were analyzed using the EditSeq, Seqman and MegAlign programs from the Lasergene sequence analysis package (DNASTAR Inc.).

2.3. Production of the recombinant TcYchF

The GBPF (5′-ATGCCTCCCAAGAAGAAGGATGAGAAGTC-3′) and GBPR (5′-CTACTTCTTACCTCCTTTAGCGGCGTTA-3′) primers were used to amplify the coding region of *TcychF*, under the following conditions: 100 ng of total DNA from *T. cruzi* Dm28c, 10 pmol of primers GBPF and GBPR, 200 μM of each dNTP, 1.5 mM $MgCl_2$, *Taq* DNA polymerase buffer, 2.5 units of *Taq* DNA polymerase (Invitrogen). The reaction mixture was heated for 4 min at 94 °C, and then subjected to 35 cycles of denaturation at 92 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 2 min. The PCR-amplified DNA fragment was inserted into pTrcHis-TOPO (Invitrogen). The His_6-tagged TcYchF protein was purified in native conditions, using the Ni–NTA spin kit (Qiagen), according to the manufacturer's recommended protocol.

Alternatively, the *TcychF* gene was amplified by PCR using the primers GEXF (5′-ACG**AGATCT**TTATGCCTCCCAAGAAGAAG-3′) and GEXR (5′-GCC**GAATTC**CTACTTCTTACCTCCTTT-3′). BglII and EcoRI sites (in bold) were added to the 5′ end of the forward and reverse primers, respectively. The PCR product was digested with BglII and EcoRI and inserted into the pGEX3x vector (Smith and Johnson, 1988). The GST–TcYchF fusion protein was purified on a glutathione Sepharose 4B (Pharmacia Biotech) affinity matrix, according to the manufacturer's protocol.

The concentration of the His_6-tagged TcYchF and GST–TcYchF fusion protein was determined with the microBCA kit (Pierce).

2.4. Nucleotide hydrolysis assay

The hydrolysis of ATP and GTP was assessed using a colorimetric assay measuring Pi release (Lanzetta et al, 1979; Yim et al., 2003). The purified GST–TcYchF protein (4 μM) was incubated with various concentrations (2.5–50 mM) of ATP (Sigma) or GTP (Sigma) in 50 μl of binding buffer (50 mM Tris–HCl, pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 5% glycerol) for 60 min at 30 °C. GST was used in the control reactions. Reactions were stopped by adding 200 μl of freshly prepared malachite green reagent. This reagent contains 2 vol. of 0.0812% malachite green, 2 vol. of double-distilled water, 1 vol. of ammonium molybdate (5.72% in 6 N HCl) and 1 vol. of 2.32% polyvinyl alcohol. The mixture was incubated for 2 min at room temperature and 25 μl of 34% sodium citrate was then added to stop color development. The samples were read within 15 min, using a Star Fax-2100 Awareness plate reader with a 630 nm filter. Blanks containing the corresponding nucleotide concentrations in binding buffer plus malachite green reagent and citrate were subtracted from each sample. We determined the amount of enzymatically released Pi, by comparing samples with a standard curve, prepared with dilutions of a 500 μM KH_2PO_4 solution in binding buffer, over a range of inorganic phosphate concentrations from 0 to 5 nmol. We determined V_{max} and K_m, and a Michaelis–Menten equation was fitted to the data by non-linear regression analysis (GraphPad Prism version 3.00 for Windows; GraphPad Software, Inc.). Assays were carried out in triplicate. K_d values were calculated by curve fitting, using non-linear regression methods implemented in GraphPad Prism software.

2.5. Cloning and production of the recombinant GAPDH, the ribosomal proteins S7 and L26 and the proteasome regulatory non-ATPase subunit RPN10 of T. cruzi

The genes encoding the glycosomal GAPDH (Tc00.1047053506943.60) (39 kDa), ribosomal protein S7 (23.9 kDa)

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