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Short communication

TcNDPK2, a *Trypanosoma cruzi* microtubule-associated nucleoside diphosphate kinase

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ABSTRACT

Nucleoside diphosphate kinases (NDPKs) are enzymes required to preserve the intracellular nucleoside phosphate equilibrium. *Trypanosoma cruzi* has four putative nucleoside diphosphate kinases with unidentified biological roles and subcellular localization. TcNDPK2 has an N-terminal domain (DM10) with unknown function, which defines a subgroup of NDPKs distributed in a wide variety of organisms. Digitonin extraction demonstrated that this isoform is distributed in detergent soluble and insoluble fractions. Fluorescence microscopy showed that TcNDPK2 alone or fused to GFP was localized in cytoskeleton and flagella. TcNDPK2 was also detected by Western blot in purified polymerized tubulin and flagellar samples. In parasites expressing DM10 fused with GFP, the fluorescence was localized in cytoskeleton and flagellum with an identical pattern to TcNDPK2. This constitutes the first report that could give insights on the role of DM10 domains in NDPKs and also the identification of the first *T. cruzi* peptide that contains a microtubule association domain.

Nucleoside diphosphate kinases (NDPKs) are enzymes involved in energy metabolism; these proteins assist in the maintenance of the nucleoside triphosphate (NTP) balance in the cell. It has been proposed that NDPKs participate in processes other than NTPs homeostasis and have been found in different subcellular compartments such as cytosol, nucleus, mitochondria and flagellum [1–4]. Within one subgroup of the NDPK family there are variants defined by the presence of a single N-terminal sequence, called DM10 domain (*Drosophila melanogaster* 10, InterPro ID IPR006602 and SMART ID smart00676), preceding the catalytic site, however, the function of this domain remains unknown (see: Supplementary Data, Fig. S1). A second family of proteins, different from NDPKs, which also have DM10 domains is exemplified by *Chlamydomonas* Rib72, which consists of three repeated DM10 regions followed by a C-terminal domain containing two EF-hands that are predicted to bind Ca^{2+} [5]. The mammalian ortholog, Efhc1, is a component of the protofilament ribbons within the doublet microtubules of the flagellar axoneme and it has been associated with the disease called "juvenile myoclonic epilepsy" [6].

Nucleotide generation and homeostasis is essential inside subcellular compartments. Trypanosomatids have a highly compartmentalized metabolism; therefore, they have a large number of enzymes related to nucleoside metabolism [7–9]. *Trypanosoma cruzi* has four predicted NDPK isoforms in the genome named TcNDPK1–4 [9–11]. While TcNDPK1 is similar to canonical NDPKs, TcNDPK2 shows an N-terminal extension similar to a DM10 domain, which is also present in TcNDPK3 (see: Supplementary Data, Fig. S1). TcNDPK4 has a truncated "NDPK domain" (NDPk7B, NCBI systematic ID: cd04412) in addition to N- and C-terminal extensions [9]. Two of these isoforms, TcNDPK1 and TcNDPK2, were previously cloned and biochemically characterized, presenting different kinetic parameters and regulation mechanisms. TcNDPK2 is expressed in all parasite stages, epimastigotes, bloodstream trypomastigotes and amastigotes [9]. Noteworthy, NDPKs were detected in sea urchin sperm flagella and mammalian sperm axoneme [12,13]. Other NDPK, named p61 or RSP23, has been detected tightly associated with the *Chlamydomonas* axoneme. This NDPK has calmodulin-binding motifs and its activity is stimulated by Ca^{2+} [4].

In this work, we focused our study on the nucleoside diphosphate kinase 2 (TcNDPK2) from the parasite *T. cruzi*. The subcellular localization of NDPK activity in *T. cruzi* epimastigotes was previously determined by digitonin extraction followed by biochemical assays [9]. About 20% of the enzymatic activity remains in the pellet at high digitonin concentrations, indicating the presence of NDPK isoform(s) associated with a digitonin insoluble cell structure. To test if TcNDPK2 was present in the digitonin insoluble fraction, new extraction experiments were carried out followed by Western blot analysis. The extraction pattern of TcNDPK2 was

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compared with four different markers: glutamate dehydrogenase (GDH, cytosol), hexokinase (HK, glycosomes), paraflagellar rod protein 2 (PAR2, flagella) and aspartate amino transferase (AAT, mitochondria) [14–17]. TcNDPK2 was detected in the supernatant between 0.2 and 5 mg mL^{-1} digitonin concentrations. However, a significant amount of enzyme remains in the digitonin insoluble fractions up to 5 mg mL^{-1} of digitonin. The obtained TcNDPK2 extraction pattern was completely different from those obtained for all markers and suggested that it could be partially localized in a digitonin insoluble cell structure such as the cytoskeleton or flagella (see: Supplementary Data, Fig. S2).

Further studies of TcNDPK2 subcellular localization analysis were performed using fluorescence microscopy techniques and transgenic parasites. Cytoskeletons from epimastigotes expressing a TcNDPK2-GFP fusion protein produced a whole-cell fluorescence pattern including flagella, but significantly increased in the periphery of the cell body with a "horseshoe-like" distribution similar to subpellicular microtubules (Fig. 1A and Supplementary Data, Fig. S3). By co-localization analysis with β-tubulin the observed pattern could be assigned to cytoskeleton microtubules (Fig. 1A–D). In addition, when cytoskeletal microtubules were differentially depolymerized by calcium treatment, the fluorescence signal could be observed in flagella, distributed parallel to the paraflagellar rod (Fig. 1E–H). The expression of TcNDPK2-GFP fusion protein was also verified by Western blot in whole-cell and flagella-enriched samples (see: Supplementary Data, Fig. S4). Samples extracted from control parasites expressing GFP, showed no fluorescence signal (data not shown). On the other hand, immunofluorescence using wild-type parasites and anti-TcNDPK2 antibodies, showed a very similar pattern than TcNDPK2-GFP expressing parasites (Fig. 1I and A).

To confirm the results obtained by immunofluorescence microscopy, *T. cruzi* polymerized tubulin was purified from epimastigote cells using Taxol-GTP or GTP only, as polymerizing agents, and analyzed by Western blot using anti-TcNDPK2 and anti-β tubulin antibodies. PAR2 and GDH were used as flagellar and cytosolic markers. As Fig. 2A shows, TcNDPK2 co-sedimented with polymerized tubulin (lanes Taxol P and GTP P). The GDH marker showed the lack of cytosolic contamination in the obtained pellets and the PAR2 marker revealed the absence of intact flagella in the original samples. To further investigate the putative association of TcNDPK2 and tubulin, Western blot analyses were carried out using whole cell extract or tubulin and flagella-enriched fractions obtained by NP-40/NaCl extractions from *T. cruzi* epimastigotes. As Fig. 2B shows a single band corresponding to TcNDPK2 was detected in total extract (E), supernatants S1–S3 and flagella-enriched (F), but mainly in the supernatant S3 which corresponds to soluble-depolymerized tubulin and associated proteins according to the β-tubulin marker. Reinforcing these results, cytoskeletal and flagella-enriched fractions have a clearly detectable NDPK enzyme activity (data not shown).

To determinate if the N-terminal sequence of TcNDPK2 containing a DM10 domain is responsible for the cytoskeletal and flagellar localization, epimastigote cells were engineered to express the N-terminal peptide (88 aa) of TcNDPK2 fused with GFP (DM10-GPF). After parasite selection, immunofluorescence analysis of control parasites (GFP) showed that GFP is homogenously distributed (data not shown), while parasites harboring the N-terminal TcNDPK2 extension fused to GFP have a strong fluorescence signal in cytoskeleton and flagella (Fig. 1K–M and P). The presence of the fusion protein in parasites extract was also confirmed by Western blot analysis (see: Supplementary Data, Fig. S4). In addition, control parasites, transfected with TcNDPK2 (ΔDM10) – GFP fusion plasmid, produced a weak fluorescence signal with a pattern similar to wild-type TcNDPK2 (Fig. 1N and O), probably by the formation of heteropolymers between TcNDPK2 (ΔDM10) – GFP and the endogenous wild type TcNDPK2.

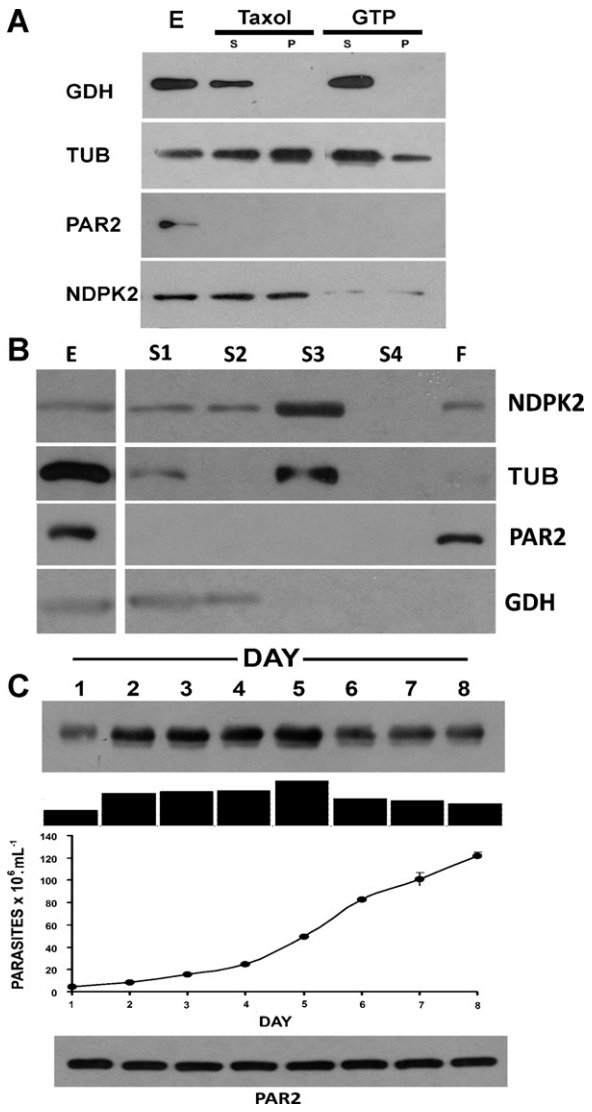

Fig. 1. *TcNDPK2 detection in polymerized tubulin samples.* (A) Whole extract (E), polymerized tubulin using Taxol-GTP or GTP only (P) and the remaining supernatants (S) were analyzed by Western blot using anti-TcNDPK2, anti-GDH (cytosolic contamination control), anti-β-tubulin (TUB) and anti-PAR2 antibodies (flagellar contamination control). (B) Whole extract (E), fractions obtained by successive extraction with NP-40 (S1–S2) and NP-40/NaCl (S3–S4); or flagella-enriched samples (F) were analyzed by Western blot using the same antibodies described above (panel A). TcNDPK2 antiserum was obtained from BALB/c mice immunized with *T. cruzi* recombinant TcNDPK2. *TcNDPK2 expression along the parasite growth curve* C. Parasites from the same culture flask were harvested at different days of culture between days 1 and 8. An equal number of cells were analyzed for the detection of TcNDPK2 by Western blot with anti-TcNDPK2 antibodies (upper panel). Band intensities were normalized using anti-PAR2 antibodies, quantified and represented using a bar graphic. Parasite number in each day of culture growth was also represented graphically.

In order to study TcNDPK2 regulation during epimastigote culture growth phases, parasite extract samples from the 1st to the 8th day of culture were analyzed by Western blot and band densitometry. Fig. 2C shows that TcNDPK2 expression increases continuously during the logarithmic growth phase up to day 5. After that, the expression decreases during the early stationary phase. Values were normalized using PAR2 as a loading control (Fig. 2C, bottom). The observed variation of TcNDPK2 during the growth curve is similar to those reported for arginine kinase and adenylate kinase, other *T. cruzi* phosphotransferases [7,18].