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Identification and validation of *Trypanosoma cruzi*'s glycosomal adenylate kinase containing a peroxisomal targeting signal

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ABSTRACT

Adenylate kinases are key enzymes involved in cell energy management. Trypanosomatid organisms have the largest number of isoforms found in a single cell, constituting a major difference with the mammalian hosts. In this work we study an adenylate kinase, TcADK3, the only *Trypanosoma cruzi* protein harboring the putative peroxisomal (glycosomal) targeting signal, "-CKL". Parasites expressing GFP fused to TcADK3 showed a strong fluorescence in the glycosomes. The same result was obtained when the tripeptide "-CKL" was added at the C-terminus of the GFP, demonstrating that this signal is necessary and sufficient for targeting proteins to glycosomes. When this tripeptide was removed from the GFP-TcADK3 fusion protein, the fluorescence was re-localized in the cytoplasm. The CKL signal could be used for targeting foreign proteins to the glycosomes. This model also provides a useful tool to study glycosomes dynamics, morphology or number in living parasites in any stage of the life cycle.

1. Introduction

Trypanosomatid organisms compartmentalize important metabolic routes in a peroxisome-like organelle called glycosomes. Enzymes present in glycosomes and metabolic activities can differ during trypanosomatids life cycles. In this way they can adapt their metabolism to a wide range of environmental conditions and selection pressures. Glycosomes contain the first part of the glycolytic pathway, the pentose-phosphate pathway, β-oxidation of fatty acids, purine salvage, and biosynthetic pathways for pyrimidines, ether-lipids and squalenes (Michels et al., 2006).

The prediction of peroxisomal and glycosomal proteins is mainly based on "peroxisomal targeting signals" (PTS). Until today two main targeting sequences for peroxisomal proteins have been identified; the PTS1 which consists of a C-terminal tripeptide motif and the internal signal PTS2 which is typical of peroxisomal matrix proteins. The PTS2 is a bipartite signal found closely to the N-terminus of the protein. Furthermore there are only a few proteins that lack of canonical targeting sequences. It has been proposed that they enter the glycosomal matrix by coupling to other proteins which present PTS (Purdue and Lazarow, 2001). The nature of the PTS signal determines how proteins are going to translocate to glycosomes. Three transporters have been characterized, Pex5 which recognizes PTS1 signals; Pex7 which is a PTS2 recognition protein and Pex19.

Until today at the "Peroxisome Database" (Schluter et al., 2010), 2819 peroxisomal proteins have been predicted from different genome or proteome projects, including *Homo sapiens*, *Saccharomyces cerevisiae* and *Trypanosoma cruzi* glycosome. However, the peroxisomal localization has been experimentally confirmed only for a small proportion of those proteins.

According to mathematical models of glycolysis, in trypanosomatids one of the most relevant glycosomal enzymes on which rely the maintenance of the high-glycolytic flux are adenylate kinases (Bakker et al., 1997; Michels et al., 2006). This prediction has been recently confirmed in *Trypanosoma brucei* by knockdown experiments of the glycosomal adenylate kinase isoform (ADKD) using RNAi, demonstrating the essentiality of this enzyme (Ginger et al., 2005). Adenylate kinases (ADK, ATP:AMP phosphotransferases, EC 2.7.4.3) are involved in the homeostasis of adenine nucleotides by interconversion of constituents of the adenine nucleotide pool and duplicating the ATP energetic potential via its synthesis from ADP (Noda, 1973). Trypanosomatid parasites have complex life cycles involving morphologically distinct stages in a wide variety of environments. Maintenance of ATP homeostasis requires coordinated regulatory responses according to the availability of nutrients in the hosts. One of the enzymes required for energy management, involved in these adaptation processes, are adenylate kinases. Most organisms express one to three adenylate kinase isoforms; however, the presence of an expanded family of adenylate kinases seems to be a common feature of trypanosomatids.

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T. brucei has seven isoforms targeted to different subcellular structures, such as flagellum, glycosome, mitochondrion and cytoplasm (Ginger et al., 2005). According to the genomic data, *Leishmania major* presents six putative isoforms, and *T. cruzi* also has six putative isoforms targeted to different predicted subcelular localizations (Bouvier et al., 2006).

Considering that glycosomes are unique structures of the order Kinetoplastida where most of the cell energy metabolism takes place and their presence is essential for parasites viability, the study of an adenylate kinase isoform that maintains the nucleotide balance within this compartment as well as the signals for targeting proteins to this unusual organelle is a relevant scientific issue.

2. Materials and methods

2.1. Parasite cultures

Epimastigotes of the CL Brener strain were cultured at 28 °C in 25 cm^2 plastic flasks, containing 5 mL of LIT medium supplemented with 10% fetal calf serum, 100 U mL^{-1} penicillin, and 100 mg L^{-1} streptomycin (Camargo, 1964). Transfected parasites were maintained in the same medium containing 500 μg mL^{-1} G418.

2.2. Enzyme assays

A sample of 50 μg of protein fraction was added to the reaction mixture (100 mM Tris–HCl buffer pH 7.5, 20 mM glucose, 5 mM $MgCl_2$, 100 mM KCl, 2 mM dithiothreitol, 1 mM $NADP^+$, 5 units per mL hexokinase and 2 units per mL glucose-6-phosphate dehydrogenase) in a cuvette to a volume of 0.5 mL. After 5 min at 35 °C the reaction was started by the addition of a small volume of ADP to a final concentration of 10 mM, unless otherwise indicated. Adenylate kinase activity was calculated by measuring the increase in absorbance at 340 nm that accompanied the reduction of $NADP^+$ (Bouvier et al., 2006).

2.3. Fluorescence microscopy

Freshly grown trypanosomes samples were washed twice in PBS. After letting the cells settle for 30 min at room temperature onto poly-L-lysine coated coverslips, parasites were fixed at room temperature for 20 min with 4% formaldehyde in PBS, followed by a cold methanol treatment for 5 min. Afterwards, all the samples were treated with the primary hexokinase antibody for 1 h, followed by secondary antibody incubation anti-rabbit for 1 h. Slides were mounted using Vectashield with DAPI (Vector Laboratories). Cells were observed in an Olympus BX60 fluorescence microscope. Images were recorded with an Olympus XM10 camera.

2.4. Plasmid constructions and parasites transfection

The sequence coding for the full-length TcADK3 was fused to the 3′ end of the GFP gene present in the pTREX-GFP expression vector (Vazquez and Levin, 1999). TcADK3Δ^{CKL} and GFP^{CKL} were obtained by PCR amplification/modification of the wild type genes using the following primers: Full-length TcADK3-forward 5′-AAGC TTGAT GAATTTGTTGATCTTCGG-3′; TcADK3-reverse 5′-CTCGAGTCA GAG TTTACATTTTCGCTT-3′; and TcADK3Δ^{CKL}-reverse 5′-CTCGAGTC ATT TTCGCTTCGCTTTCC-3′. The constructions GFP^{CKL} and the control GFP^{SKL} containing the SKL signal from the *T. cruzi* malate dehydrogenase (GeneDB: Tc00.1047053511293.69) were obtained by annealing the following oligonucleotides: TcADK3-forward 5′-A GCTTGGA TCAGCTACGGAAAGCGAAGCGAAAATGTAAACT-3′; TcADK3-reverse 5′-CTAGAGTTTACATTTTCGCTTCGCTTTCCGTAGCTGATCCA-3′; and TcMDH-forward 5′-AGCTTTGCGAAGGGTGAGACGTTTGCGCGGTCA AAGTTGTAGC-3′; TcMDH-reverse 5′-TCGAGCTACAACTTTGACCGC GCAAACGTCTCACCCTTCGCAA-3′, digested with HindIII–NotI or XhoI and ligated to the pTEX-eGFP plasmid.

A total of 10^8 parasites grown in LIT medium at 28 °C were harvested by centrifugation, washed with PBS, and resuspended in 0.35 mL of electroporation buffer (PBS containing 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$). The cell suspension was mixed with 50 μg of plasmid DNA in 0.2 cm gap cuvettes (Bio-Rad Laboratories). The parasites were electroporated with a single pulse of 400 V, 500 μF with a time constant of about 5 ms. Stable cell lines were achieved after 30 days of treatment with 500 μg/mL G418 (Calbiochem) (Pereira et al., 2003).

2.5. Bioinformatics

Sequences from kinetoplastid genome projects were obtained at the TriTrypDB (http://tritrypdb.org/tritrypdb/). Analysis of the DNA sequence data were carried out using the software package Vector NTI v. 10 (Invitrogen Corporation). Online analyses were performed at the PeroxisomeDB (http://www.peroxisomedb.org/) (Schluter et al., 2010) and the NCBI (http://www.ncbi.nlm.nih.gov/).

3. Results and discussion

3.1. Cloning, overexpression and functional validation of TcADK3

Among the *T. cruzi* adenylate kinase family, TcADK3 is the natural candidate for a glycosomal isoform because it has a peroxisomal targeting signal PTS1 in the C-terminus and a high isoelectric point (9.7), a property of all glycosomal glycolytic enzymes (Misset et al., 1986). In addition, TcADK3 possesses all the consensus blocks of the adenylate kinase family; the "P-loop" (GxPGxGK) and the "long-LID" domain variant LLDGFPRT and YNPPK, characteristic of the non-cytosolic isoforms (Konrad, 1993). To test if TcADK3 is a *bona fide* adenylate kinase, the gene was cloned and overexpressed in *Escherichia coli*. The affinity purified recombinant protein showed the predicted molecular weight of 24 kD in SDS–PAGE. The functionality of the recombinant adenylate kinase was fully confirmed by standard enzymatic activity assays (Supplementary data, Fig. S1).

3.2. In silico analysis of TcADK3 targeting signal

A singular feature of TcADK3, is the putative glycosomal targeting signal, –CKL. Analyzing the complete *T. cruzi* proteome for PTS1-containing proteins,, 341 genes have been found (353 sequences including 12 pseudogenes), according to the amino acid pattern [STAGCN]-[RKH]-[LIVMAFY]$ (Prosite pattern: PS00342. The most frequent PTS1 was –SKL reaching about 7% of all sequences. Interestingly, the tripeptide –CKL was only present in TcADK3. In addition, TcADK3 orthologs in trypansomatids present other peroxisomal targeting sequences, with all *Leishmania* species harboring an –AKL signal and the African trypanosomes having a –SKL signal (Fig. 1A and Supplementary data, Table S1). It is worth to mentioning that a previous version of TcADK3 sequence (GenBank: AAS20417) was misannotated with a –SKL signal. This error is probably attributable to the low quality of the sequences, obtained from preliminary *T. cruzi* genomic data which was used for gene assembly.

3.3. TcADK3 C-terminal tripeptide is a glycosomal targeting signal

Considering the low-frequency of the –CKL among the PTS1s and the scarce information about the experimental validation of such signal, TcADK3 localization was studied using transgenic

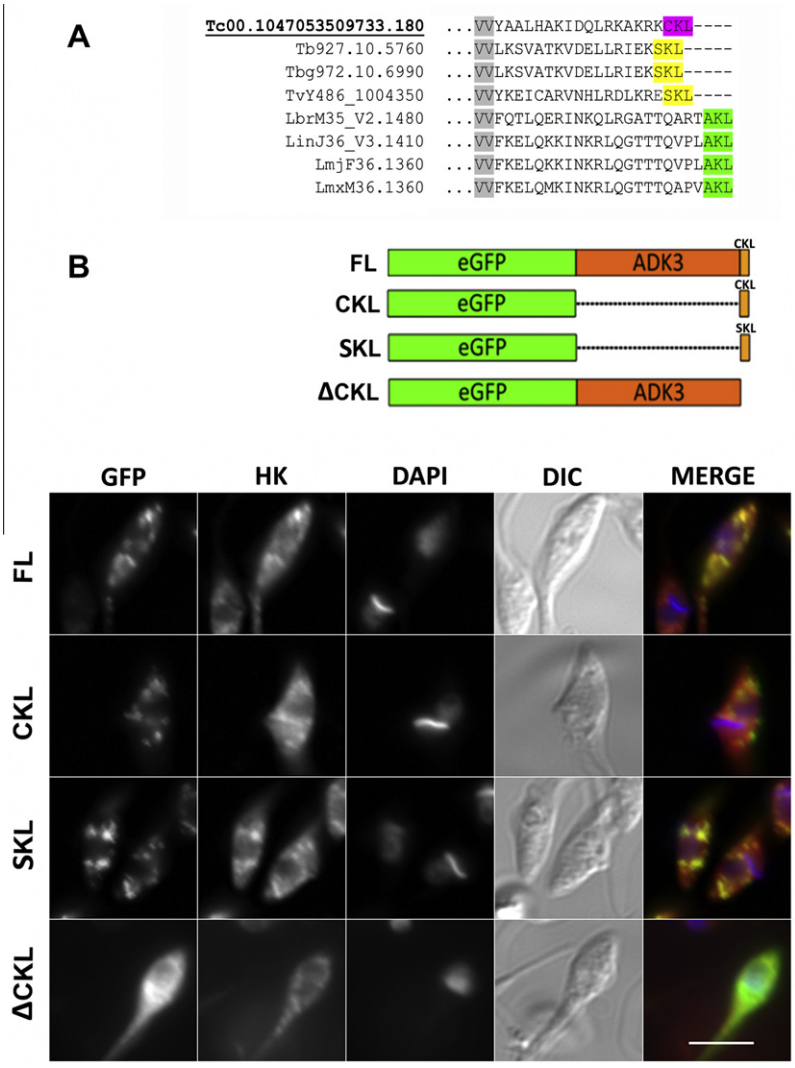

Fig. 1. Analysis of the glycosome targeting signal in TcADK3. (A) Sequence alignment of the C-terminal portion of trypanosomatids adenylate kinases named according the TritrypDB systematic IDs (Tc: *Trypanosoma cruzi*; Tb: *Trypanosoma brucei brucei*; Tbg: *Trypanosoma brucei gambiense*; Tv: *Trypanosoma vivax*; Lbr: *Leishmania braziliensis*; Lin: *Leishmania infantum*; Lmj: *Leishmania major* and Lmx: *Leishmania mexicana*). (B) Fluorescence microscopy of parasites expressing eGFP fused to TcADK3 (FL), eGFP fused to CKL (CKL), eGFP fused to SKL (SKL, from *T. cruzi* malate dehydrogenase), and eGFP fused to TcADK3Δ^{CKL} (ΔCKL). The columns show: GFP fluorescence from the fusion proteins (1), hexokinase immunofluorescence (2), DAPI (3), differential interference contrast (4), and the merged image (5). The upper panel shows a diagram of the mentioned plasmid constructions.

parasites and fusion proteins. Epimastigotes were transfected using three different plasmid constructions containing the complete TcADK3 fused to GFP, a truncated TcADK3 lacking the tripeptide –CKL fused to GFP, and only GFP but with the addition of the –CKL tripeptide in its C-terminus. Hexokinase antibodies and GFP fusion proteins to the PTS1 signal –SKL were used as control glycosomal markers. As Fig. 1B shows, overexpression of the GFP-TcADK3 construction clearly produced glycosomal fluorescence, demonstrating the glycosomal localization of TcADK3. The very low cytosolic background obtained suggests that the overexpression levels of TcADK3 were unable to saturate PEX5 which is the receptor responsible for recognition and protein translocation to glycosomes. The pattern observed was similar to the one obtained for the GFP-SKL fusion protein and it showed a co-localization with the glycosomal marker hexokinase. In contrast, a diffuse cytosolic fluorescence was observed in the parasites expressing the variant of GFPADK3 lacking the –CKL tripeptide. These results suggest that this tripeptide is not only responsible for TcADK3 glycosomal localization, but could also efficiently target other proteins (e.g. GFP-CKL), which is different to what has been reported previously for the –SKL sequence (Neuberger et al., 2003). Both the –CKL and the –SKL signal could be a new and simple way for targeting molecules to the 20 glycosomes, including compounds for therapeutic purposes. This parasite model also provides a very useful tool to study the glycosomes dynamics, morphology or number in living parasites along the life cycle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2012.01.020.

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