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Trypanosoma cruzi Tcp12^{CKS1} interacts with parasite CRKs and rescues the p13^{SUC1} fission yeast mutant☆

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Abstract

The complex mechanism of cell division in trypanosomatids is not completely fully understood. CRKs (cdc2-related kinases), Cyclins and CKSs (cdc2-kinase subunit) are involved in the progression through the cell cycle. The CKS proteins were first described as components of the cell cycle machinery in yeast and their action has been implicated in the regulation of CDK function. In the present work we identified Tcp12^{CKS1} a member of the CKS family in the parasite *Trypanosoma cruzi*. *TcCKS1* is expressed in the three forms of *T. cruzi*. By using anti-Tcp12^{CKS1} antiserum, protein kinase (PK) activities were immunoprecipitated. The PK activity level varies depending on the stage analyzed, being lower in trypomastigotes and thus suggesting that different stages have different CKS–CRK complexes. Moreover, these PK activities were inhibited by using Flavopiridol, a known CDKs inhibitor. Western blot analyses demonstrated that in the epimastigote stage, p12^{CKS1} stably interacts with TcCRK1 and TcCRK3. In addition, Tcp12^{CKS1} was able to rescue the p13^{SUC1} null mutant of *S. pombe*. The functional complementation between the CKS proteins of two evolutionary distant organisms supports the role of Tcp12^{CKS1} as a key regulator in *T. cruzi* cell cycle.

Keywords: *Trypanosoma cruzi*; CKS; CRKs; Cell cycle; Cyclins

1. Introduction

The parasitic protozoan *Trypanosoma cruzi* belongs to *Trypanosomatidae* family and is responsible for the human endemic Chagas disease. Trypanosomes have a complex life cycle that alternates between insect and mammalian hosts. In the infected mammalian host, circulating *T. cruzi* trypomastigotes, are taken up by an hematophagous vector when feeding. This infective, non-proliferating form in the insect's digestive tract differentiates to the rapidly dividing and non-infectious epimastigote form, which converts to metacyclic trypomastigotes in the vector hindgut. The metacyclic trypomastigotes deposited with the vector's feces can penetrate to the vertebrate bloodstream. Once there, this infective and non-proliferating form is able to invade host cells and differentiate to the dividing amastigote form. After a determined number of cell cycles, amastigotes differentiate to trypomastigotes that are released to the host bloodstream by cell lysis and can infect new cells or be taken up by feeding vectors to complete the life cycle [1,2].

The cell cycle regulatory mechanism is conserved throughout evolution, with homologs to CDKs (cyclin-dependent protein kinases) and cyclins found in a wide range of eukaryotes. The activity of CDKs is regulated by multiple mechanisms. At least four distinct post-translational mechanisms regulate CDKs function, including association with cyclins, binding to CKS proteins

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and by phosphorylation/dephosphorylation by specific kinases and phosphatases [3].

Trypanosomatids are classified as some of the most divergent eukaryotes [4], and a large gene family of CRKs and cyclins has been identified in these organisms [5,6]. However, despite the high amino acidic conservation in key regulatory positions compared with the mammalian CDKs, the regulation of CRK activity and the mechanisms that underlie the control of the cell cycle and the differentiation processes is still under investigation in these parasites.

Originally, CKSs were identified as suppressors of mutations in yeast's CDK1 genes [7]. CKS molecules are considered small conserved proteins that interact genetically and physically with CDKs and constitute a family of essential components of the CDK complex that regulate cell cycle progression [8]. The association of a CKS protein, in its monomeric state, with the CDK1–CycB complex permits the phosphorylation of the CDC25 phosphatase and the entry in mitosis [9]. In a similar way, the CDK1–CycB–CKS complex phosphorylates the sub-unit CDC27 of the anaphase promoting complex, which is crucial for the ubiquitination of cyclin B and further exit from mitosis [10]. Once cyclin B has been ubiquitinated, interaction between the CKS and the proteasome is necessary for actual proteolysis of the cyclin [11,12]. The human protein hCKS1 associates with SKP2, a subunit of the SCF-ubiquitin-ligase complex, and favors the ubiquitination of the CKI (cdk inhibitor) $p27^{Kip1}$ and thus the G1/S transition [13,14].

We have previously identified two CRKs (cdc2 related kinases) and three cyclins in the parasite *T. cruzi* [15,16]. To further investigate key components of the machinery of the cell cycle control of *T. cruzi*, we searched for the presence of a new CKS-family member. In this work we report the sequence of a CKS gene, called *TcCKS1*, and show that the protein encoded is capable of rescuing *Schizosaccaromyces pombe* $p13^{SUC1}$ mutant and of interacting with parasite CRKs. Moreover, these $p12^{CKS1}$ associated kinase activities varied depending on the stage analyzed, being higher in the actively replicative forms.

Taken together, our results demonstrate that $Tcp12^{CKS1}$ is part of the cell cycle control machinery of *T. cruzi*.

2. Materials and methods

2.1. Cellular cultures and protein preparations

T. cruzi epimastigotes from Tul-2 strain were cultured as previously described [15]. Metacyclic trypomatigotes were obtained by axenic culture under differentiating conditions. Amastigotes were obtained from Vero cell cultures as described [17].

Parasites protein extracts were prepared by resuspending parasites pellets in SK buffer with proteinase inhibitors (0.25 M sucrose, 5 mM KCl, 0.5 mM *N*-tosyl-L-lysine chloromethyl ketone (TLCK), 1 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride (PMSF), 25 U/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml trypsin inhibitor, 0.1 mM sodium orthovanadate and 10 mM sodium fluoride) followed by three to five freezing and thawing cycles. The complete rupture was confirmed by microscopic visualization. The protein extract was obtained as previously described [15]. Protein concentration in every fraction was assessed by Bradford method.

2.2. Cloning and sequencing of the T. cruzi CKS1 full length gene

EST TENG0820 was used as starting point since it shares high identity with LmCKS1 gene. Cloning of the gene was achieved in three steps (Fig. 1). Total RNA of *T. cruzi* epimastigotes was used in random hexamers-primed reverse transcription reaction mixture (Promega). The cDNA products were used in subsequent PCRs. A semi-nested PCR strategy was employed.

Step 1 (5′ UTR region): The sense primer (AACGCTATTATTGATACAGTTTCTGTACTATATTG) was designed taking advantage of the fact that trypanosomes bear the same 39-nt sequence (splice leader, SL) at the 5′ end of all mRNAs. The 3′ region of the EST was used to design primers II (GTAAAACATGTAGTGGCGCC) and IV (CCCAACCGACTGCTGAACG). Template cDNA and primers SL and II were used in a first PCR and an aliquot of this reaction was used as template for a second round of amplification with primers SL and IV. A specific amplification product was cloned in pGEM-Teasy (Promega) vector, amplified and sequenced. Step 2 (3′ and 5′ UTR regions): Based on the EST 5′ sequence, primers I (ACATGTCGGCGAGGGACTTC) and III (CCATTACCGACCCTGTGCAG) were designed. A polyT primer (PA) was used as antisense initiator. Template cDNA and primers I and PA were used in a first PCR and an aliquot of this reaction was used as template for a second round of amplification with primers III and PA. A specific amplification product was cloned in pGEM-Teasy (Promega) vector, amplified and sequenced (Fig. 1). Step 3 (complete gene) the partial fragments obtained in step 1 and step 2 were used to determine the sequence of the complete coding sequence (cds). Subsequently, primers encompassing 5′ ATGTCGGCGAGGGACTTC and 3′ CGCTACTCCGTTTTTGGCCGC regions of the cds were designed in order to amplify the full length gene from genomic DNA. The PCR products were cloned and sequenced.

2.3. Northern blot, Southern blot and pulse-field gel electrophoresis analysis

Genomic DNA for Southern blot analysis was purified from epimastigotes using DNAzol reagent, following the manufacturer's instructions (Molecular Research Center). Purified DNA (5 μg) was digested separately with the restriction enzymes EcoRI, EcoRV, PstI, PvuII and BamHI, electrophoresed on 0.7% agarose gel and transferred to nylon membrane (Hybond N+, Amersham). The blots were hybridized as described [15].

Total RNA from the different life stages of *T. cruzi* (epimastigote, trypomastigote, and amastigote) was obtained by using TRIzol reagent (Invitrogen) according to the manufacturer's