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Characterization of the *Trypanosoma cruzi* Cdc2p-related protein kinase 1 and identification of three novel associating cyclins☆

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Abstract

Several Cdc2p-related protein kinases (CRKs) have been described in trypanosomatids but their role in the control of the cell cycle nor their biological functions have been addressed. In *Trypanosoma cruzi* two CRKs have been identified, *Tz*CRK1 and *Tz*CRK3. In this work we further characterize *T. cruzi* CRK1 and report the identification of three novel associating cyclins. We demonstrate that CRK1 levels and localization do not vary during the cell cycle, and show that it is localized in the cytoplasm, discrete regions of the nucleus, and is highly concentrated in the mitochondrion DNA (kinetoplast), suggesting a putative control function in this organelle. Using purified anti-CRK1 IgGs, we immunoprecipitated from the soluble fraction of *T. cruzi* epimastigote forms a protein kinase activity which is not inhibited by CDK inhibitors. In addition, we co-precipitated with p13Suc1p beads a kinase activity that was inhibited by the CDK inhibitor flavopiridol and olomoucine. Lastly, using the yeast two-hybrid system we identified three novel cyclin-like proteins able to associate with *Tz*CRK1, and demonstrate that two of these cyclins also bind the *T. cruzi* CRK3 protein, indicating that these two CRKs are cyclin-dependent kinases.

Keywords: *Trypanosoma cruzi*; Cdc2p-related kinases; Cyclins; Cell cycle; Kinetoplast; Two-hybrid system

1. Introduction

A family of protein kinases termed cyclin-dependent kinases (CDKs) control the major transitions between the phases of the cell cycle [1]. Cellular CDK levels tend to remain in constant excess throughout the normal cell cycle, and regulation of catalytic activity is primarily post-translational. CDK activity is mainly regulated by association with cyclin proteins, phosphorylation of conserved amino acids and binding with inhibitory polypeptides named CDK inhibitors (CKIs). Cyclins are positive regulatory subunits and constitute multi-protein families in yeast and metazoans. Homology among cyclins is often limited to a relatively conserved domain of about 100 amino acids, the cyclin box, which is responsible for CDK binding and activation [1]. Cyclins were originally defined as proteins whose levels oscillated during the cell cycle, but currently, admission to the cyclin family of proteins requires cyclin-box homology and demonstrable interaction with any CDK [2]. In addition, a single cyclin can sometimes interact with multiple CDKs, and CDKs can associate with

☆ *Note:* Nucleotide sequence data reported in this paper have been submitted to the GenBank™ database with accession numbers, *TzCYC4*, AF237588, *TzCYC5*, AF237589, and *TzCYC6*, AF237587.

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many different cyclins. For example, CDK2 can associate with cyclins A and E, and cyclin A can also bind CDK1.

Many protein kinases are closely related to the CDKs, but relatively few have been shown to possess cyclin-dependent activity. In the parasite *Trypanosoma cruzi* we have isolated two Cdc2p-related kinase genes, *TzCRK1* and *TzCRK3* [3]. *T. cruzi* is the parasitic protozoan that causes Chagas disease. This trypanosomatid undergoes a complex series of morphogenetic changes throughout its lifecycle in both, an insect vector and a vertebrate host. When feeding on the blood of an infected vertebrate host the hematophagous vector ingests circulating trypomastigotes, non-proliferative and infectious forms. In the insect digestive tract trypomastigotes differentiate to rapidly dividing and non-infectious epimastigote forms which then convert to metacyclic trypomastigotes in the vectors hindgut. After their inoculation into the vertebrate host, metacyclics invade cells where they transform and divide in the host cells cytoplasm as amastigotes. Completed their dividing cycle, amastigotes transform to trypomastigotes that can infect new cells or be released into the bloodstream and taken up by feeding vectors to complete the cycle [4].

In a previous work, we showed that *T. cruzi* CRK1 is a 33 kDa protein equally expressed in all life cycle stages of the parasite and demonstrated its association with mammalian cyclins [3]. CRK1 homologues have been described in the trypanosomatids *Leishmania mexicana*, *Lmm*CRK1 [5], *Trypanosoma brucei*, *Tb*CRK1 [6] and *Trypanosoma congolense*, *Tc*CRK1 (accession # Z30312). CRK1 proteins have been tested for their ability to complement *cdc2/CDC28* mutations in yeast, but under the assayed conditions none of them could complement the Cdc2p/CDC28 protein function [3,5]. Gene disruption experiments in *L. mexicana* indicated that *LmmCRK1* was essential to the promastigote form [7].

Other CRK protein coding genes have been described in the trypanosomatids *T. cruzi*, *TzCRK3* [3], *L. mexicana*, *LmmCRK3* [8], *Leishmania major*, *LmajCRK3* [9], *T. brucei*, *TbCRK2* and *3* [6] and *Crithidia fasiculata*, *CfCRK4* [10]. The Leishmania, *T. brucei* and *T. cruzi* CRK3 proteins have been tested for their ability to complement different *Schizosaccharomyces pombe cdc2* and/or *Saccharomyces cerevisiae CDC28* temperature sensitive mutants ([6,8,9]; Gómez et al., unpublished results). Only *Lmaj*CRK3, was shown to restore Cdc2p/CDC28 activity. These limited results suggest that, at least for trypanosomatids and yeast, cross-species complementation may not be a very powerful tool to establish functional homologies between proteins [9].

The only cyclins identified in trypanosomatids were isolated from the parasite *T. brucei*, *Tb*CYC1-3 [11,12]. *Tb*CYC2 and *Tb*CYC3 have been recently isolated for their ability to complement *S. cerevisiae* G1 cyclins and it was shown that *Tb*CYC2 could associate with *Tb*CRK3 [12]. Whether *Tb*CYC1 and 3 can form a complex with a protein kinase remains to be clarified.

Several kinetoplastids genes encoding proteins with homology to known cell cycle polypeptides have been isolated, but little is known about their function and role in cell cycle and differentiation events. In addition, although in a previous work we have shown that *T. cruzi* CRK1 could bind mammalian cyclins [3], no trypanosomatid cyclin able to associate with CRK1 proteins has been identified. Thus, it is not clear if these proteins are bona fide cyclin-dependent kinases. In an attempt to enlighten the role of Cdc2p-related protein kinases (CRKs) in trypanosomatids we decided to further characterize the CRK1 protein from the parasite *T. cruzi*. Using this kinase as bait in a two-hybrid screen we identified three novel trypanosomatid cyclins. We also studied the *Tz*CRK1 expression and localization throughout the amastigote cell cycle and characterized its endogenous kinase activity. In addition, we describe a CRK activity able to associate with the yeast p13Suc1p protein.

2. Materials and methods

2.1. Cellular cultures and protein preparations

Axenic *T. cruzi* amastigotes, CA-I/72 cloned stock, were maintained by serial passages at 27°C in a brain heart-tryptose culture medium (BHT medium) supplemented with 10% heat inactivated fetal bovine serum [13,14]. Amastigote cultures were synchronized as described [15]. *T. cruzi* epimastigotes from Tul 2 strain were cultured as described previously [16].

Epimastigotes protein extracts were prepared by resuspending the 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 phenylmethyl-sulphonyl fluoride (PMSF), 25 U ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 2 μg ml^{-1} trypsin inhibitor, 0.1 mM sodium orthovanadate and 10 mM sodium fluoride), followed by 3–5 freezing and thawing cycles. The complete rupture was confirmed by microscopic visualization. The protein extract was fractionated by differential centrifugation as described [16].

The L40 yeast strain [17] was grown at 30°C in rich medium or in synthetic minimal medium with appropriate supplements.

2.2. Electron microscopy and immunogold localization

Approximately 10^8 parasites were washed twice with PBS (2000 × *g*, 10 min 4°C). Cell pellets were fixed in 2% paraformaldehyde, 0.05% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4, for 2–4 h at 4°C. Axenic amastigotes were then cryoprotected, frozen, sectioned, and immunolabeled using ultrathin cryosections and immunogold labeling techniques as described previously [4,18,19]. IgG antibodies were purified as described [20]. A specific rabbit polyclonal anti-*Tz*CRK1 serum [3] (dilution 1:100–1:200), purified anti-*Tz*CRK1 IgGs (dilution 1:100–1:200), and goat anti-rabbit IgG-10 nm gold-labeled antibody (dilution 1:20) were used. Pre-immune rabbit serum (dilution 1:20) was used for specificity control. Photographs were taken at 31 500 × in a Philips CM10 electron microscope. To facilitate quantification of gold particles, magnification was identical in all electron microphotograph prints.

The cellular distribution of *Tz*CRK1-gold label in parasites including cytoplasm, nucleus and kinetoplast was calculated by counting the number of gold particles in 15 cells and is expressed as percentage of total gold particles per cell. The density of immunogold labeled-*Tz*CRK1 in the cell cytoplasm, nucleus, kinetoplast and mitochondrion was estimated from the number of gold particles per square micron. Cellular and subcellular areas were measured using NIH image 1.62.

The distribution of *Tz*CRK1 within the nucleus was quantified in 16 cells by counting the number of gold

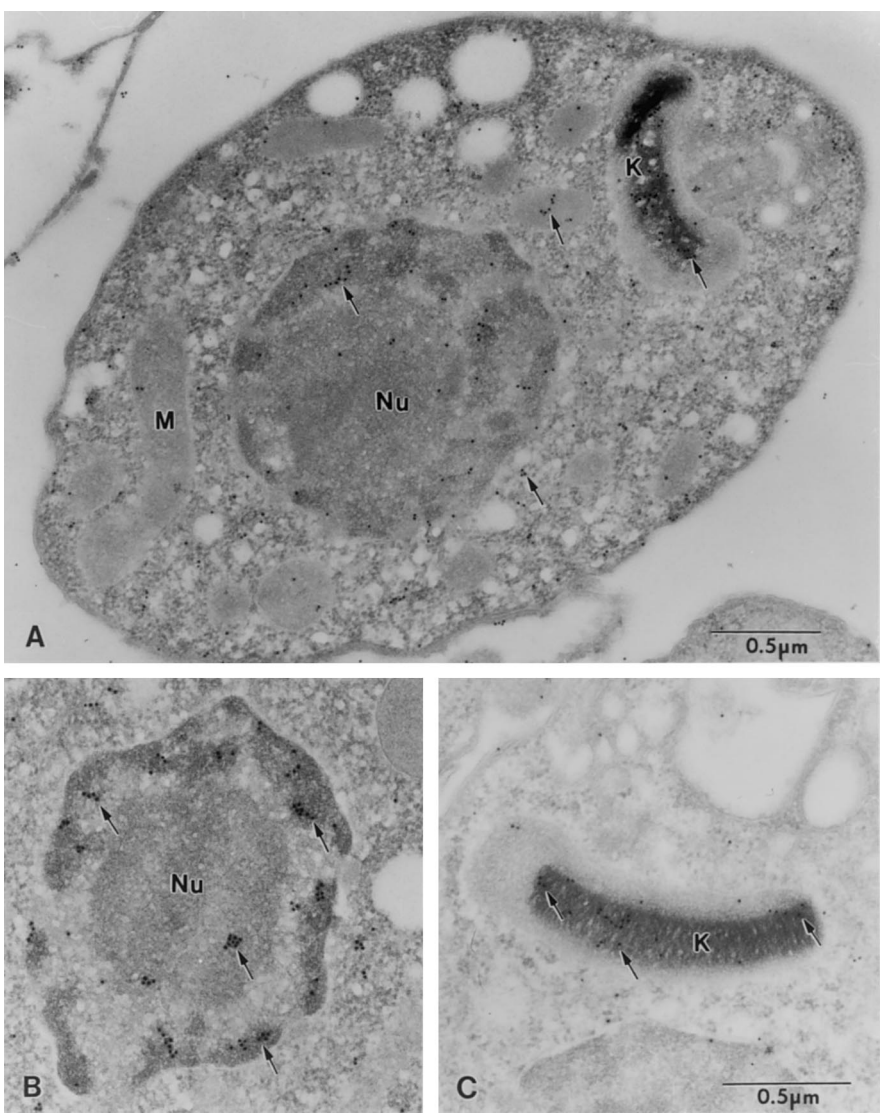

Fig. 1. Localization of *Tz*CRK1 in *T. cruzi* axenic amastigote: amastigotes were fixed in 2% paraformaldehyde, 0.05% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4. The fixed cells were then cryoprotected, frozen, sectioned and immunolabeled with purified anti-*Tz*CRK1 IgG antibodies (dilution 1:100–1:200), and goat anti-rabbit IgG-10 nm gold-labeled antibody (dilution 1:20). Pre-immune rabbit serum (dilution 1:20) was used for specificity control. Photographs were taken at 31 500 × in a Philips CM10 electron microscope: (A) amastigote cell, (B) nucleus and (C) kinetoplast. Arrows indicate gold particles: M — mitochondrion; Nu — nucleus; K — kinetoplast.

particles in the condensed chromatin and non-chromatin areas of the nucleus (Fig. 1(B)). For these studies, the mitochondrion was separated into two distinct areas: the kinetoplast, restricted to the area containing the mitochondrial DNA (kDNA), and the mitochondrion per se. Quantification of gold-particles was similarly performed by selecting equivalent areas of the kinetoplast and mitochondrion (Fig. 1(C)). Statistical analyses were performed with Microsoft Excel software.

2.3. Immunoprecipitations, p13Suc1p-agarose co-precipitations and kinase assays

One hundred micrograms of *T. cruzi* epimastigote soluble fractions (S_{100}) were preclarified with protein A-agarose (Gibco-BRL) and incubated with anti-*Tz*CRK1 purified IgGs, normal rabbit serum (NRS) purified IgGs or p13Suc1p-agarose beads (Calbiochem). The p13Suc1p-agarose complexes and proteinA-agarose precipitated immunocomplexes were washed four times with PBS and incubated with the corresponding kinase assay buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA, 5 mM $MnCl_2$, 0.5 mM sodium fluoride, 0.4 mM sodium orthovanadate and 5 μCi [γ-^{32}P]ATP), or resuspended in 1 × Laemmli's buffer for Western blot analysis. The histone H1 kinase buffer contained 0.1 mg ml^{-1} of histone H1 (Calbiochem) with 50 μM of unlabeled ATP, and the Rb kinase assay had 0.025 mg ml^{-1} of a purified GST-retinoblastoma protein (GST-Rb) [3,21], and 10 μM of unlabeled ATP. Reactions were performed at 30°C for 30 min in a total volume of 30 μl and stopped with 5 × Laemmli's buffer. Samples were analyzed by 12% SDS-PAGE, stained with Coomassie Blue R-250, and exposed to an autoradiographic film. The ability of p13Suc1p to co-precipitate a kinase activity was tested by incubating p13Suc1p-agarose beads with *S. cerevisiae* protein extracts.

2.4. Inhibition assays

Immunoprecipitated *Tz*CRK1, 1 μg of recombinant GST-*Tz*CRK1 [3] or p13Suc1p precipitates were incubated with different CDK inhibitors: flavopiridol, olomoucine and roscovitine (Calbiochem). The inhibitors were dissolved in DMSO as indicated by the manufacturer. Increasing concentrations up to 1 mM for roscovitine and olomoucine, and 0.5 mM for flavopiridol, were tested. Enzyme sources without the addition of inhibitors were used as positive controls to compare the effect of the inhibition. The CDC28 kinase activity co-precipitated with p13Suc1p-agarose beads from *S. cerevisiae* protein extracts was used to test quality of the inhibitors.

2.5. Library construction and two-hybrid system screening

A two-hybrid system was used to identify proteins that interact with *Tz*CRK1. A library of hybrid proteins between a nuclear localized VP16 activation domain and random *T. cruzi* epimastigote cDNA fragments was constructed in the pVP16 plasmid according to a procedure by Hollenberg et al. [17]. Briefly, purified polyA + *T. cruzi* epimastigote RNA was random primed and the double stranded cDNA was synthesized with a GIBCO-BRL kit (Rockville), according to the manufacturer instructions. A double stranded adapter was obtained by annealing oligonucleotides P1, 5′-ATCCTCTTAGACTGCGGCCGCTCA-3′ and P2, 5′-phosphate-TGAGCGGCCGCAGTCTAAGAG-3′. This adapter was ligated to the cDNA ends and the cDNA was amplified by PCR using oligonucleotides P1 as a primer. DNA fragments bigger than 300 bp were gel purified and ligated into the *Not* I site of the pVP16 vector.

The *S. cerevisiae* L40 strain was used as the reporter yeast in the two-hybrid screen. This strain contains two integrated reporter genes, yeast *HIS3* and bacterial l*acZ*, under the control of the Gal1 minimal promoter fused to multimerixed *LexA* binding sites. The full length *TzCRK1*, a truncated version of *TzCRK1* (nucleotides 181–901) (t-*TzCRK1*) and *TzCRK3* genes were subcloned in the pBTM116 plasmid (trp +) in fusion with the *LexA* DNA binding domain gene [22]. These constructs were used to transform the L40 strain. Western blot analyses using anti-*Tz*CRK1 or anti-LexA antibodies (Clontech) confirmed the expression of the fusion proteins, LexA-*Tz*CRK1, LexA-t-*Tz*CRK1 and LexA-*Tz*CRK3. L40 yeast transformed with p*LexA-TzCRK1* and p*LexA*-t-*TzCRK1* did not activate the *HIS3* and *lacZ* reporter genes, being unable to grow in the absence of histidine and were white when tested for β-galactosidase (β-gal) activity. The p*LexA-TzCRK3* yeast transformants grew in the absence of histidine, so 10 mM of 3-aminotriazole (3-AT) was added to the selective medium.

L40 yeast expressing LexA-*Tz*CRK1 were transformed with 300 μg of the *T. cruzi* epimastigote cDNA library. The transformants were grown for 16 h in synthetic medium lacking leucine and tryptophan to maintain selection for the plasmids, and to obtain efficient expression of the *HIS3* reporter gene. The transformants were selected for histidine protrotrophy in yeast synthetic medium lacking tryptophan, leucine, uracil, histidine and lysine. Histidine positive colonies were collected within 3–5 days post-transformation and assayed for β-galactosidase activity on nitrocellulose filters. The library vectors of the his + /β-gal + colonies were selectively recovered by virtue of the yeast *LEU2* gene, present in pVP16, to complement a leuB mutation

present in the *Escherichia coli* HB101 strain. The plasmids were analyzed with different restriction enzymes and re-grouped. To identify false positives, one plasmid of each group was used to transform L40 yeast containing the pBTM116-lamin vector [23]. Library positive DNA plasmids were sequenced with the sense primer 5′pVP16, 5′-GGAATTGACGAGTACGGT-3′ and the antisense M13 Forward oligonucleotides, 5′-GTAAAACGACGGCCAGT-3′. The *LexA* fused open reading frames were defined using the ORF program (anonymous, NCBI) and the sequences were compared with the BLAST program [24] against different databases.

2.6. Identification of the cyclins full length sequences

To obtain the *TzCYC4* and *TzCYC5* complete sequences, a genomic λ FIX II library (gift from Dr. Edson Rondinelli, UFRJ, Brazil) was used. The screenings were carried out using the fragments identified in the two-hybrid screen as probes. These were radiolabeled with the Prime a gene labeling system (PROMEGA) according to the instructions provided by the manufacturer. Positive clones were isolated, phage DNA purified and sequenced. The sequencing primers for the different groups were designed based on the already known sequences: cyc5-a (5′-AAAAGGGCATGCTGCGATAA-3′), cyc5-c (5′-GTGAGACCGACGAGCAGTTC-3′) and cyc5-e (5′-TGGAATGAGTGAGACACATACC-3′) were used to obtain the 5′ sequence and the cyc5-b (5′-GGAGGTGGAGACATGGGCGGAGT-3′) and cyc5-d (5′-GGAGGTGGAGACATGGGCGGAGT-3′) to obtain the 3′ end of the *TzCYC5* gene. For *TzCYC4*, the sequencing primers designed were: cyc4-a (5′-GGG-AAGACGATGGGAGTGAA-3′) and cyc4-c (5′-AGCCACCGTAGTTGCCG-3′) for the 5′ end, and cyc4-b (5′-CGGGTTGCATCACTCGGG-3′) and cyc4-d (5′-ACGAGTGATAGTGTCACGATGG-3′) for the 3′ sequence.

The *TzCYC6* 5′ sequence and its splice leader acceptor site were obtained by RT/PCR followed by a heminested PCR using the spliced leader sequence (SL), present in all kinetoplastids mRNAs [25], as sense primer, 5′-AACGCTATTATTGATACAGTTTCTGTACTATATTG-3′, and oligonucleotides designed from the group 6 sequence as antisense primers, cyc6-a (5′-ATACTCAGCACGAAGCACTC-3′) and cyc6-b (5′-AGTAGGAGTACTTGGCAATACG-3′). Total *T. cruzi* epimastigote RNA was used in random hexamers-primed reverse transcription reactions. The cDNA products were used as templates in PCR amplifications using the SL and cyc6-a primers. An aliquot of this reaction was used in a heminested PCR using the SL and cyc6-b primers. The obtained products were subcloned and sequenced.

Table 1
Subcellular distribution and concentration of *Tz*CRK1 in *T. cruzi* axenic amastigotes

	Distribution (%)[a]	Density[b]
Cell	100	15.7 ± 3.4[h]
Cytoplasm[c]	60.2 ± 6.4	11.0 ± 2.1[i]
Nucleus	16.4 ± 3.9	16.8 ± 4.5
Kinetoplast (kDNA)[d]	23.6 ± 6.1	125.7 ± 41.2[i]
Mitochondrion[e]	–	6.8 ± 2.1[i]
Background[f]	–	1.2 ± 0.8[i]
Pre-immune serum[g]	–	1.9 ± 0.6[i]

[a] Percentage of gold labeled *Tz*CRK1 per cell ($n = 16$ cells).
[b] Immunogold labeled *Tz*CRK1-particles per μm^2 ($n = 16$ cells).
[c] Cytoplasm includes mitochondrion with the exclusion of nucleus and kinetoplast.
[d] Kinetoplast is only mitochondrial DNA (kDNA) region.
[e] Mitochondrion excludes kDNA area.
[f] Extra-cellular areas in EM-sections.
[g] Control with pre-immune, normal rabbit serum.
[h] Values are mean ± S.D.
[i] Differences are significant ($P < 0.01$) with respect to total cell density.

The 5′ sequence and splice leader acceptor site for *TzCYC4* and *TzCYC5* were confirmed following the same procedure as for *TzCYC6*. The oligonucleotides employed in the sequencing reaction were used as antisense primers, cyc4-a and cyc4-c; cyc5-c and cyc5-e.

3. Results

3.1. Subcellular localization of TzCRK1

The general morphology of a *T. cruzi* axenic amastigote and the distribution of immune-gold label using an anti-*Tz*CRK1 serum are shown in Fig. 1A and Table 1. The distribution of gold-labeled *Tz*CRK1 was approximately ten times higher with the specific hyperimmune antiserum (1:200 dilution) than with pre-immune serum (dilution of 1:20) (Table 1). Over 98% of the gold particles in immune-stained sections localized to cells and was indicative of specific labeling while less than 2% non-specific background localized to extracellular areas (Fig. 1A and Table 1). Sixty per cent of total *Tz*CRK1 gold-particles were evenly distributed throughout the cytoplasm while the remaining was partitioned between the nucleus (16%) and the kinetoplast (24%).

The amount and density of gold particles was determined in several subcellular compartments. *T. cruzi* presents a single mitochondrion extending throughout the cytoplasm that contains condensed DNA (kDNA), a.k.a. the kinetoplast, a structure distinguishable from the rest of the mitochondrion [4]. The distribution of gold particles within these two regions showed that the kinetoplast had around 10-fold higher density of la-

beled-*Tz*CRK1 than the cytoplasm. Most *Tz*CRK1-label in the mitochondrion (93%) was clustered in the kinetoplast while the rest of the mitochondrion had significantly lower label than the cell (Fig. 1C).

The density of *Tz*CRK1 within the nucleus was similar to that of the cell, except that 86% of the label was associated to chromatin (Fig. 1B). *Tz*CRK1-labeling of other cellular structures such as the flagellar pocket and the Golgi apparatus were similar to that of the cytoplasm (data not shown).

3.2. TzCRK1 levels and localization do not vary during the cell cycle

Synchronized *T. cruzi* amastigote forms were used to study the localization and levels of *Tz*CRK1 protein during the cell cycle. Amastigote samples were collected at 4 h intervals during the first 32 h of amastigote cultures and prepared for immunoelectronmicroscopy as described in Section 2. The distribution and levels of gold-labeled *Tz*CRK1 did not vary in the different analyzed samples indicating that *Tz*CRK1 is a constitutive protein which localization and levels do not oscillate significantly during the cell cycle of amastigote forms.

3.3. Characterization of the endogenous TzCRK1 activity

In order to further characterize the *Tz*CRK1 protein, the ability of the soluble immunoprecipitated kinase to phosphorylate the exogenous substrate histone H1 and retinoblastoma protein was tested. Purified anti-*Tz*CRK1 IgG antibodies were used to precipitate the soluble *Tz*CRK1 protein. As a negative control normal rabbit serum (NRS) purified IgGs were used. The immunocomplexes were incubated with the different kinase buffers and resolved by SDS-PAGE. Fig. 2 shows that the anti-*Tz*CRK1 antiserum can immunoprecipitate a kinase/s which have the ability to phosphorylate the CDK substrates histone H1 and retinoblastoma protein. These results are in accordance with previous published assays where we show that a recombinant GST-*Tz*CRK1 protein had histone H1 and retinoblastoma kinase activities [3].

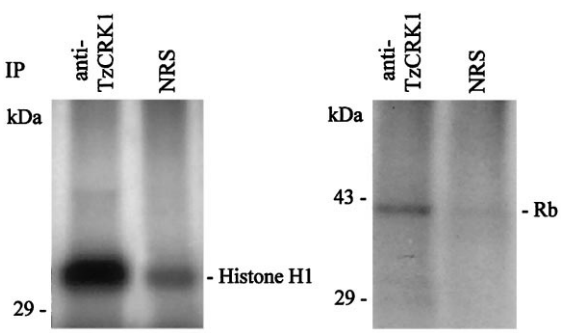

Fig. 2. Activity of the endogenous *Tz*CRK1 protein: 100 μg of *T. cruzi* epimastigote soluble fractions (S_{100}), were preclarified with protein A-agarose and incubated with anti-*Tz*CRK1 purified IgGs (IP TzCRK1) or normal rabbit serum (IP NRS) purified IgGs. The proteinA-agarose precipitated immunocomplexes were incubated with histone H1 or Rb kinase buffers. Reactions were stopped with 5 × Laemmli's buffer, analyzed by 12% SDS-PAGE and exposed to an autoradiographic film.

Over the last years, considerable effort has been made to develop chemical CDK inhibitors (CKIs) to block cell cycle progression. Some inhibitors have remarkable selectivity and can differentiate the human CDK family into two subfamilies: (1) CDK1, CDK2 and CDK5 and (2) CDK4/6. Three chemical inhibitors, olomoucine, roscovitine and flavopiridol have shown selectivity for CDK1 and CDK2 proteins. Using purified recombinant proteins it was reported that roscovitine had IC_{50} values of 0.65 μM and 0.7 μM for CDK1 and CDK2 respectively, and >100 μM for CDK4. In the case of flavopiridol, the IC_{50} was 0.4 μM for CDK1 and CDK2 and it had no effect on CDK4. Olomoucine had an IC_{50} value of 7 μM for both CDK1 and 2 proteins, and >1000 μM for CDK4 [26].

In the present study the effect of these three inhibitors on the kinase activity of *Tz*CRK1 was assayed. The recombinant GST-*Tz*CRK1 protein [3] and soluble immunoprecipitated *Tz*CRK1 were incubated with the different inhibitors and tested for kinase activity. Under the conditions of the assay, the CKIs did not inhibit the kinase activity of immunoprecipitated *Tz*CRK1 or the recombinant protein (data not shown).

3.4. Identification of an epimastigote p13Suc1p binding kinase activity that can be inhibited by CKIs

The Cdc2p and CDK2 proteins from many species can associate with the *S. pombe* p13Suc1p protein [27]. Although in vivo, only a small percentage of the Cdc2p kinase is associated with the constitutively present p13Suc1p, it has been shown that these two proteins have a very high affinity in vitro [28–30]. Because of this property p13Suc1p-agarose beads have frequently been used for the purification of Cdc2p proteins or Cdc2p-related proteins from a wide variety of organisms. In *L. mexicana*, a SUC1 binding CRK (SBCRK) has been purified using this technique [5]. To identify a *T. cruzi* SUC1 binding CRK, epimastigote soluble protein fractions were incubated with p13Suc1p-agarose beads. In all experiments, protein A-agarose was used as a negative control. The kinase activity of the co-precipitated proteins was analyzed using histone H1 as a substrate. Fig. 3 lanes 1 (protein A) and 2 (w/o inhibitor) show that a kinase activity able to phosphorylate the assayed substrate associated with the p13Suc1p protein (lane 2).

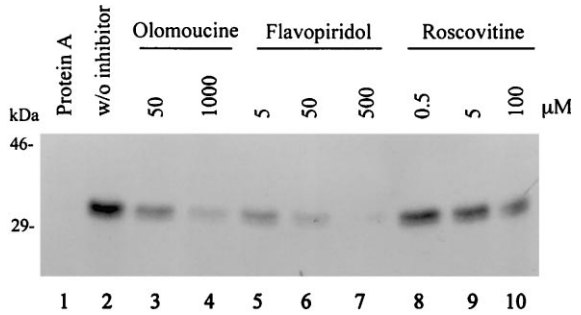

Fig. 3. *T. cruzi* has a p13Suc1p CRK that is inhibited by CKIs: 100 μg of *T. cruzi* epimastigote soluble fractions (S_{100}), were preclarified with protein A-agarose and incubated with p13Suc1p-agarose beads (lanes 2–10) or protein A-agarose (lane 1). The precipitated proteins were incubated with histone H1 kinase buffer and the indicated concentrations of olomoucine, roscovitine and flavopiridol. Reactions were stopped with 5× Laemmli's buffer. Samples were analyzed by 12% SDS-PAGE, stained with Coomassie Blue R-250 and exposed to an autoradiographic film.

To study the nature of this kinase, the p13Suc1p-associated proteins were analyzed by Western blot using anti-*Tz*CRK1 and anti-PSTAIRE antibodies (Santa Cruz). The PSTAIRE antiserum has been used to analyze CRKs in many species, and specifically recognizes protein kinases of the Cdc2p family. In a previous work, we have shown that these antibodies can recognize in Western blot assays a recombinant *Tz*CRK1 protein [3]. These antisera failed to detect any protein in the p13Suc1p elutes (data not shown). To control the quality of the p13Suc1p-agarose beads, these were incubated with a *S. cerevisiae* protein extract. The associating proteins were analyzed by Western blot using the anti-PSTAIRE antiserum. This antiserum detected a 34 kDa protein in the p13Suc1p elute, showing that the p13Suc1p beads are capable of binding the *S. cerevisiae* Cdc2p homologue, CDC28 (data not shown). These results suggest that *Tz*CRK1 can not bind p13Suc1p and that the CRK protein that associates with p13Suc1p is not recognized by this PSTAIRE antiserum.

To further characterize the p13Suc1p precipitated kinase, the CKI inhibitors, flavopiridol, olomoucine and roscovitine were incubated with *T. cruzi* p13Suc1p-agarose precipitates. As shown in Fig. 3 flavopiridol and olomoucine could inhibit the kinase activity associated with p13Suc1p beads. In the case of roscovitine, a slightly inhibition effect can be observed with the tested concentrations. Only when very high concentrations of the inhibitor were used, (>0.1 mM) the activity of the kinase was highly inhibited (data not shown). These results indicate that *T. cruzi* has a Cdc2p-related kinase that associates with p13Suc1p and is inhibited by CKIs, and show that this parasite has a CRK with properties similar to CDK1 and CDK2 proteins.

3.5. Isolation of TzCRK1-interacting proteins

A *T. cruzi* epimastigote cDNA library was screened for plasmids expressing proteins that interact with *Tz*CRK1 using a two-hybrid system. L40 yeast expressing LexA-*Tz*CRK1 were transformed with the cDNA library and selected in a medium lacking histidine. A total of 95 colonies were collected and tested for β-galactosidase activity. Seventy-four his+ colonies were blue or light blue and were considered positives in this initial screen. Restriction enzyme analyses of the recovered cDNA plasmids revealed the existence of 21 different cDNA inserts and the 74 positive plasmids were subsequently organized in 21 groups (Table 2). To identify non-specific interactions, L40 yeast containing the pBTM116-lamin vector were transformed independently with the recovered plasmids and assayed for their ability to grow in the absence of histidine and for expression of β-galactosidase. Only one colony was his+/β-gal+ with LexA-lamin suggesting a non-specific interaction with *Tz*CRK1, and was discarded. The nucleotide sequences of the remaining clones were determined and comparative analyses of the sequences carried out (Table 2). Of the 21 different cDNA groups, 13 were false positives, corresponding to *T. cruzi* ribosomal genes, sequences encoding polypeptides with less than 5 amino acids or plasmid vectors with no insert. The 8 remaining groups (groups 1–8) possessed LexA-fused open reading frames (ORFs) coding for putative interacting proteins and were considered authentic positives of the two-hybrid screen (Fig. 4A). Together, these 8 groups represented 66% of the his+/β-gal+ colonies (49/74) (Table 2). As further proof, the positive plasmids were each independently transformed into L40 yeast lacking a pBTM116-*Tz*CRK1 plasmid. As expected, his+ transformants were not found confirming that both the expressed trypanosome cDNA and *Tz*CRK1 were required for transcription activation.

3.6. Analyses of the positive clones: identification of three novel cyclins

Sequence analyses of groups 2, 3, 4 and 6, using the ORF and BLAST programs, identified three ORFs with homology to the PREG protein family described in *Neurospora crassa* (Accession # Q06712), *Picea mariana* (Accession # AAC32127) *Arabidopsis thaliana* (Accession # AAC27476) and *S. pombe* (Accession # CAA16850), *S. cerevisiae* PHO85 cyclins, PCL7 (Accession # NP012214), PCL6 (Accession # NP010980) and PHO80 (Accession # P20052), and the *T. brucei* cyclin 2 protein (*Tb*CYC2) [12]. The proteins belonging to the PREG family have a cyclin box domain but no CDK-like regulated protein has been identified.

The cDNAs from groups 3 and 4 are different fragments coding for the same gene where the insert of

group 3 extends from nucleotide (nt) 119 to nt 753 of group 4. The insert of group 4 comprised 1251 bp and did not have an ATG initiation codon nor a stop codon. The plasmids in group 2 have a 664 bp insert. Sequence analyses showed that this is also a 5′ and 3′ end truncated cDNA. These fragments were used as probes to screen a genomic library to obtain the complete sequences of both genes. In addition, the 5′ end and the trans-splicing acceptor site were confirmed by sequencing a fragment obtained by RT/PCR using the splice leader sequence as 5′ primer and 100% identical oligonucleotides as antisense primers.

The gene identified using the insert of group 4 as probe has 2391 bp encoding a protein of 796 amino acids. Fig. 5A shows the deduced amino acid sequence of this gene compared with different proteins. The homologies showed to be restricted to the cyclin box domain. Amino acid sequence analyses using the BLAST program revealed 36% identity and 61% similarity with *Tb*CYC2, 35% and 58% with *Ath*PREG and 25% and 48% with *Sc*PHO80. This protein was named *Tz*CYC4 since it had no significant homology with the other trypanosomatid cyclins. The fact that his+/β-gal+ colonies were obtained with the group 3 plasmids, and the fact that its coding sequence has homology with cyclin box domains indicate that this fragment includes a cyclin box and suggests that this is the domain involved in the interaction with *Tz*CRK1.

The positive clones resulting from the library screening using the insert of group 2 as probe presented an ORF of 2277 bp. The sequence of this gene had 30% identity and 52% similarity with the cyclin box domain of *A. thaliana* PREG protein and 36% identity and 53% similarity with *S. cerevisiae* PCL6 (Fig. 5B). This gene does not have significant similarity with any of the cloned trypanosome cyclins so it was named *T. cruzi* cyclin 5 (*Tz*CYC5).

The group 6 insert is 620 bp in length with a stop codon at position 459 (Table 2). Sequence comparisons showed that it was a 5′ end truncated cDNA. The complete sequence was obtained by RT/PCR followed by a heminested PCR using the splice leader sequence as 5′ primer and 100% identical oligonucleotides as antisense primers. The full length ORF was shown to be 612 bp encoding a polypeptide of 203 amino acids. Once again the homology was restricted to the cyclin box domain. The identity and homology values obtained with the BLAST program were 46% and 66% with *Ath*PREG and 30% and 52% with *Sc*PHO80 (Fig. 5C). When comparing this sequence with *Tz*CYC4 the homology is also restricted to the cyclin box domain with an identity of 35% (Fig. 5C). The amino acid homology with *T. brucei* cyclin 2 is restricted to a fragment of 139 amino acids. Within this region, they present 60% identity and 80% similarity. This protein was tentatively named *T. cruzi* cyclin 6 (*Tz*CYC6).

The ORFs present in the groups 1, 5, 7 and 8 sequences have no homology with known proteins when compared with several databases. Table 2 shows the approximate insert size and, if present, the stop codons position.

Table 2
Yeast two-hybrid system screen results[a]

Group	Number of clones	Approximate insert size	bp to stop codon	Name
1	10	800 bp	246	
2	15	667 bp	NP	*TzCYC5*
3	2	637 bp	NP	*TzCYC4*
4	10	1251 bp	NP	*TzCYC4*
5	5	1100 bp	189	
6	1	620 bp	459	*TzCYC6*
7	4	700 bp	148	
8	2	1600 bp	NDY	
Total	49			
Groups	Number of clones	Type of false positives		
9 to 21	25	*T. cruzi* ribosomal genes No inserts Coding of polypeptides with less than 5 amino acids		

[a] The L40 reporter yeast strain was transformed with p*LexA-TzCRK1* plasmid and an epimastigote cDNA library. The transformants and interacting proteins were selected in media lacking his, trp, leu, lys and ura. The his+ colonies were tested for β-gal activity and the library plasmids of the his+/β-gal+ colonies were recovered and divided in 21 groups based on restriction enzyme analyses. At least one clone of each class was sequenced and analyzed using the ORF and BLAST programs. The table indicates from left to right: group number (1–21); number of independent clones in each group; approximately insert size in bp; position, if present, of a stop codon, and the name given in this work. ORF — open reading frame, NP — not present, NDY — not determined yet.

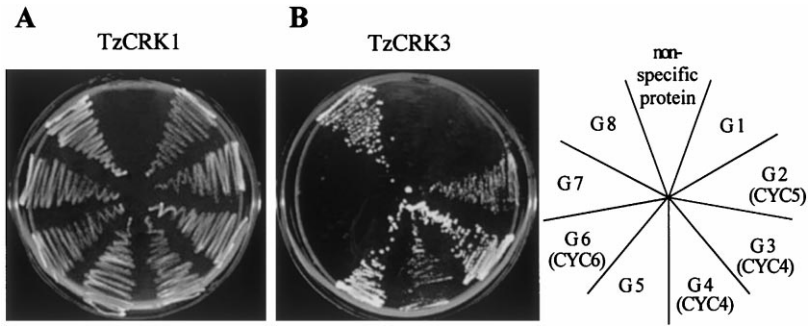

Fig. 4. Interaction of positive clones encoded proteins with *T. cruzi* CRK proteins: L40 yeast was transformed with pBTM116-*TzCRK1* ((A) TzCRK1) or pBTM116-*TzCRK3* ((B) TzCRK3). The transformants were re-transformed with the positive clones belonging to groups 1–8 (G1–G8) and with a pVP16 vector expressing a non-related protein, and selected on minimal media lacking tryptophan, leucine, uracil and lysine. The colonies transformed with both plasmids were streaked on plates lacking histidine and grown for 3 days at 30°C (*Tz*CRK1 and *Tz*CRK3).

3.7. TzCRK1 cyclin box binding domain is necessary for its association with the novel cyclins

In an attempt to determine if the cyclin box binding domain of *Tz*CRK1 was necessary for the interaction with the proteins identified in the two-hybrid screen, the 5′ region of *TzCRK1*, which includes the PCTAIRE domain, was deleted from the construct. This truncated fragment (t-*TzCRK1*) was subcloned in the pBTM116 vector and transformed into L40 yeast. Western blot analyses using the *Tz*CRK1 antiserum and anti-LexA monoclonal antibodies (Clontech) showed that the full-length *Tz*CRK1 protein and its truncated form were equally expressed (data not shown). L40 yeast expressing LexA-t-*Tz*CRK1 were transformed with the different positive clones (*TzCYC4*, 5, 6 and groups 1, 5, 7 and 8). None of the transformants could grow in the absence of histidine indicating that the truncated CRK1 could not associate with the proteins encoded by the plasmids of groups 1 to 8. These results suggest that the N-terminal domain of *Tz*CRK1 is important for the interactions observed in this system.

3.8. TzCRK3 interacts with TzCYC4 and 5 but not with TzCYC6

To study the specificity of the interaction between the proteins expressed by the positive clones and *Tz*CRK1, their capacity to associate with *T. cruzi* Cdc2p-related kinase 3 (*Tz*CRK3) was analyzed [3]. The *TzCRK3* gene was subcloned in the pBTM116 vector and used to transform L40 yeast. These yeast were independently transformed with the different positive plasmids (*TzCYC4*, 5, 6 and groups 1, 5, 7 and 8) and tested for β-galactosidase activity and their ability to grow in the absence of histidine. Under the conditions of the assay it was found that *Tz*CRK3 could interact with *Tz*CYC4, *Tz*CYC5 and the proteins expressed by the plasmids of groups 5 and 8, and could not interact with *Tz*CYC6 and the proteins expressed by the plasmids 1 and 7 (Fig. 4B).

4. Discussion

In this work we characterized the Cdc2p-related protein kinase 1 from the parasite *T. cruzi* and showed the identification of three novel cyclins.

Anti-*Tz*CRK1 antibodies were used in immunoelectronmicroscopy studies to determine the localization of *Tz*CRK1 in *T. cruzi* amastigote forms. The results showed that 60% of total *Tz*CRK1 gold-particles were distributed in the cytoplasm, 16% in the nucleus and the remaining 24% in the kinetoplast. When the distribution of gold particles was analyzed, the kinetoplast had around 10-fold higher density of labeled-*Tz*CRK1 than the cytoplasm. The kinetoplast is a distinguishable structure in the mitochondrion containing the mitochondrial DNA, termed kinetoplast DNA (kDNA). This DNA consists of a network of numerous intercatenated minicircles (0.5–2.8 kb) and maxicircles (20–37 kb). Genes for ribosomal RNA and conventional mitochondrial proteins are encoded by the maxicirles. Unlike mitochondrial DNA, kDNA replicates once in the cell cycle. Although nuclear and kinetoplast DNA synthesis initiate at about the same time, the kinetoplast S-phase ends earlier, and as a consequence the kinetoplast cycle has a longer cytokinesis [31]. Interestingly, the timing of these events is similar to the G1/S and G2/M yeast cell cycle checkpoints [32] but the mechanisms that regulate and coordinate both the nuclear and kinetoplast checkpoints are unknown. If a general machinery regulates both cycles, at least the same proteins are expected to be in both compartments as well as in the cytoplasm. In this work we have showed that Cdc2p-related kinase 1 from *T. cruzi* is localized in the kinetoplast, nucleus and cytoplasm. This result and the fact that *Tz*CRK1 has homology with CDK proteins suggest the existence of a general molecular mechanism involving *Tz*CRK1, that might control the nuclear and kinetoplast cycles, or, if each cycle possesses its own machinery, *Tz*CRK1 could participate in their correct coordination. Although *Tz*CRK1 does not

have a kinetoplast localization signal, proteins which lack mitochondrial targeting presequences have been found in the kinetoplast [33,34].

The anti-*Tz*CRK1 purified IgGs were also used to immunoprecipitate *Tz*CRK1 from epimastigote soluble fractions. The immunoprecipitate could phosphorylate both histone H1 and the Rb protein as was shown for the recombinant *Tz*CRK1 [3]. The CKIs olomoucine, flavopiridol and roscovitine did not inhibit these activities. The mechanism of action for each of these inhibitors was shown to be competitive inhibition of ATP binding [26] suggesting that the structure of the ATP binding domain of *Tz*CRK1 might be different from the corresponding domains of human CDK1 and CDK2.

On the other hand, p13Suc1p bound to agarose could precipitate a kinase which was not recognized, in Western blot analysis, by anti *Tz*CRK1 antibodies. Although this methodology is not conclusive to test the association of p13Suc1p with *Tz*CRK1, this result is in accordance with previous published experiments, which showed that the *L. mexicana* *Tz*CRK1 homologue, *Lmm*CRK1, could not bind p13Suc1p beads [5]. In addition, the fact that the p13Suc1p associated kinase was inhibited by olomoucine and flavopiridol supports evidence that suggest that *Tz*CRK1 is not the kinase

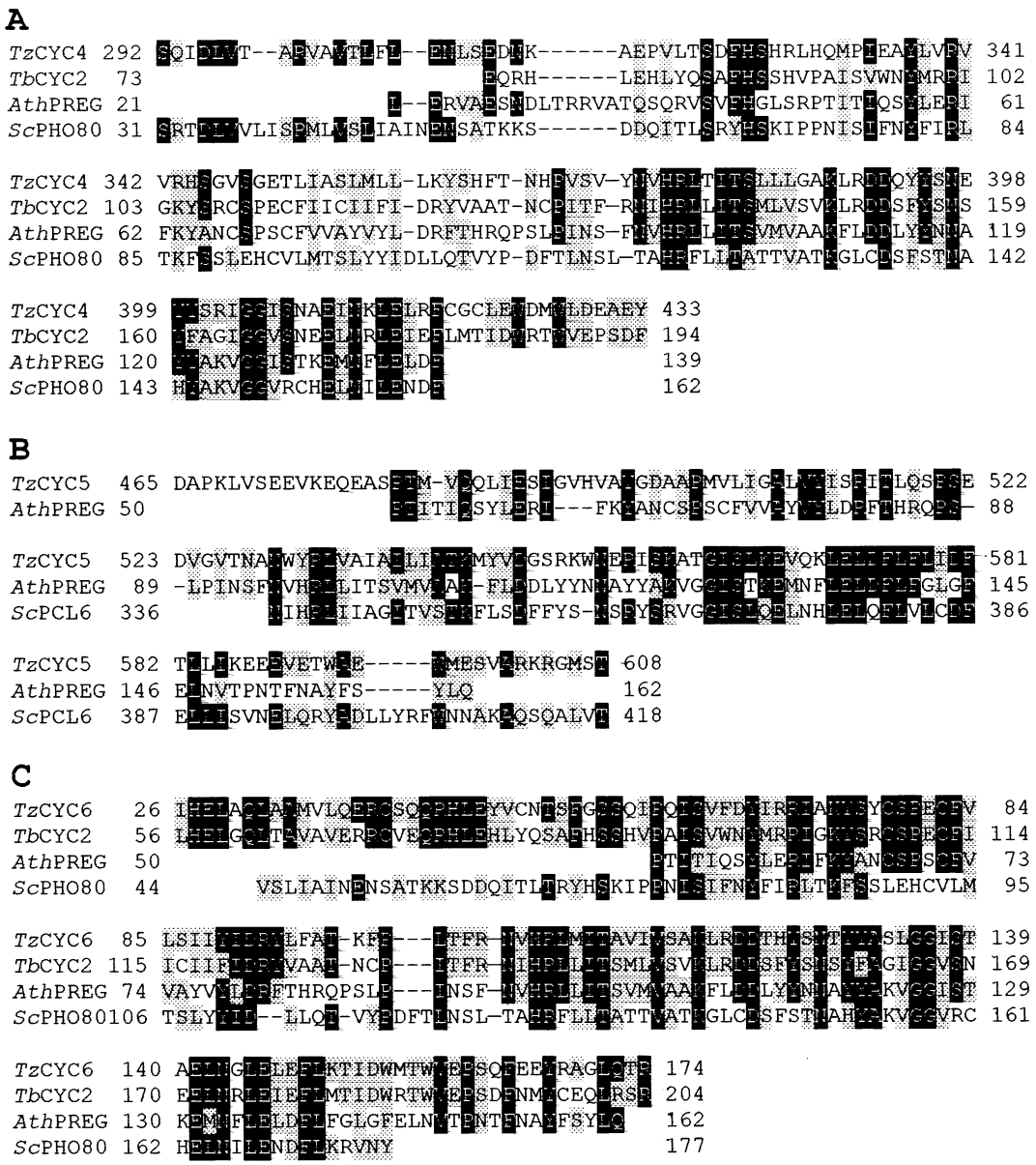

Fig. 5. Amino acid sequence alignment of (A) *Tz*CYC4, (B) *Tz*CYC5 and (C) *Tz*CYC6: The alignment was performed using BLAST [24]. Identical aminoacids are highlighted with a black background and positions with conserved substitutions in one or more sequences are shaded in gray. Ath — *Arabidopsis thaliana*; Sc — *Saccharomyces cerevisiae*; Sp — *Schizosaccharomyces pombe;* Tb — *Trypanosoma brucei*; Tz — *Trypanosoma cruzi.*

that interacts with p13Suc1p. In *L. mexicana*, a SUC1 binding CRK (SBCRK) was identified using p13Suc1p-agarose beads [5], and it was shown that *Lmm*CRK3 could bind p13Suc1p [8]. Also, inhibition experiments established that SBCRK and a recombinant *Lmm*-CRK3 protein could be inhibited by olomoucine [8]. This evidence suggests that at least *T. cruzi* CRK3 could be the kinase associated to p13Suc1p.

To gain a broader understanding of how *Tz*CRK1 is regulated and enlighten its role in trypanosomatids cell biology, a two-hybrid screen was performed using *Tz*CRK1 as bait. Seven different open reading frames were identified that codify for putative interacting proteins. Three of the 7 ORFs presented homology with cyclin like proteins and were named *Tz*CYC4, *Tz*CYC5 and *Tz*CYC6.

The predicted amino acid sequences of the three *T. cruzi* identified cyclins have homology with the cyclin box domains of PREG proteins from different organisms and PHO85 cyclins from *S. cerevisiae*. The *N. crassa* PREG is a cyclin-like protein, which participates in the repression of genes involved in phosphate metabolism [35]. This protein has identity with the *S. cerevisiae* PHO80 cyclin, that forms a complex with the PHO85 CDK and regulates the acid phosphatase transcription by phosphorylating the PHO4 transcription factor [36]. PHO85 participates also in processes different to phosphate metabolism. When associated with different cyclins it has roles in cell cycle control, glycogen metabolism and actin cytoskeleton regulation. Consistent with its multifunctional nature, ten genes encoding known or putative PHO85 cyclins have been identified [2,37]. When comparing the amino acid sequences of *Tz*CRK1 and PHO85 the identity is not different to the one with other CDKs, so no putative function can be deducted, but the fact that *Tz*CRK1 can bind at least 3 cyclin-like proteins suggests that it could participate in a variety of cellular processes including or not cell cycle control.

Two of these cyclins, *Tz*CYC4 and *Tz*CYC5, can also interact in a two-hybrid system with *Tz*CRK3, suggesting that these two proteins can regulate the activity of two different kinases, property shared by various cyclins. *Tz*CRK3 can also associate with the proteins encoded by the plasmids of groups 5 and 8, suggesting that these proteins could be involved in a general CRK regulatory pathway. Studies remain to be done to establish the nature of these proteins. On the other hand, we show that *Tz*CYC6 and the polypeptides encoded by groups 1 and 7 can only interact with *Tz*CRK1, suggesting an specific role for this cyclin.

The predicted amino acid sequence encoded by *Tz*-CYC6 has a high amino acid identity within a 139 region with *T. brucei* cyclin 2. *Tb*CYC2 associates with *Tb*CRK3 and not with *Tb*CRK1 [12]. On the other hand, *Tz*CYC6 can interact with *Tz*CRK1 and not with *Tz*CRK3. Although the high similarity observed in the 139 amino acid region, these two proteins associate with different CRKs. When the complete *Tz*CYC6 and *Tb*CYC2 amino acid sequences were compared, the N- and C-terminal domains did not have significant similarity. Also, *Tz*CYC6 presents a longer C-terminal domain, while *Tb*CYC2 has a longer N-terminal region. It is possible that the differences observed in these domains are responsible for the different binding specificities.

Even though the interaction between the novel cyclins and CRK1/CRK3 provides strong evidence that these kinases are CDKs, further experimentation is required to confirm this hypothesis. These results evince that trypanosomatids have a complex CRK-cyclin network similar to other eukaryotic organisms.

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