Experimental Parasitology 132 (2012) 537–545

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Functional characterization of *Tc*CYC2 cyclin from *Trypanosoma cruzi*

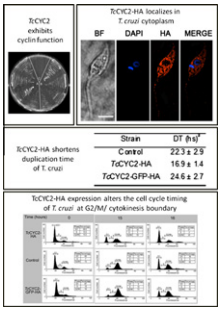
Mariana Potenza [a,*], Sergio Schenkman [b], Marc Laverrière [c], María Teresa Tellez-Iñón [a]

[a] Instituto de Investigaciones en Ingeniería Genética y Biología Molecular "Dr. H.N. Torres", INGEBI-CONICET, Vuelta de Obligado 2490, C1428ADN Buenos Aires, Argentina
[b] Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, Rua Pedro de Toledo 669 6andar, SP 04039-032, São Paulo, Brazil
[c] Instituto de Investigaciones Biotecnológicas, UNSAM-CONICET, Av. General Paz 5445, Ed 24y19, 1650 San Martín, Buenos Aires, Argentina

HIGHLIGHTS

- The cyclin *Tc*CYC2 from *Trypanosoma cruzi* was characterized.
- *Tc*CYC2 gene complemented G1-cyclin activity in yeast.
- Recombinant *Tc*CYC2-HA expression shortened the cell cycle of *T. cruzi* epimastigotes.
- *Tc*CYC2-HA expression altered duplication time and morphology of *T. cruzi*.
- *Tc*CYC2-HA was expressed mainly in the cytoplasm of *T. cruzi*.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 19 February 2012
Received in revised form 3 September 2012
Accepted 4 September 2012
Available online 13 September 2012

Keywords:
Cell cycle
Cyclin
Hydroxyurea
Trypanosoma cruzi

ABSTRACT

In eukaryotes, an oscillating network of protein kinase activities drives the order and timing of the cell cycle progression. Complexes formed by cyclins associated to cyclin-dependent kinases (CDKs) are the central components of this network. Cyclins act as the activating subunits and their abundance is regulated by different mechanisms in order to promote or prevent kinase activity. Protein synthesis, proteasomal degradation and/or differential subcellular compartmentalization modulate cyclin expression levels along the cell cycle. We describe in this work the characterization of *Trypanosoma cruzi* Cyclin 2 (*Tc*CYC2), which contributes to a better understanding of the cell cycle regulation in this protozoan parasite. We found *Tc*CYC2 exhibited cyclin function in a yeast complementation assay and over-expression of hemagglutinin tagged *Tc*CYC2-HA rendered shorter duplication times and smaller cell sizes in the epimastigote form of the parasite. Analysis of synchronized cultures showed that over-expression of *Tc*CYC2-HA altered the timing epimastigotes pass through G2/M boundary or cytokinesis. Taken together, our results showed that *Tc*CYC2 is a functional cyclin whose over-expression modifies the dynamics of the cell cycle as well as the morphology of epimastigote forms of *T. cruzi*, suggesting it plays an important role in the cell cycle regulation machinery.

1. Introduction

Trypanosoma cruzi and the related kinetoplastids *Trypanosoma brucei* and *Leishmania* spp. are pathogenic protozoan parasites which have early branched in the eukaryotic lineage (Body et al., 2010; Simpson et al., 2006). These parasites have complexes life cycles that allow the passage from invertebrate to vertebrate hosts and *vice versa*, causing disease. In humans, *T. cruzi, T. brucei* and *Leishmania* spp. cause Chagas disease, Human African Trypanosomiasis and Leishmaniasis, respectively. To assure survival, these parasites alternate between dividing and non-dividing forms. This is exerted by a tight cell cycle regulation which allows continuous adaptation to multiply in the two different hosts (Fenn and Matthews, 2007; Tyler and Engman, 2001).

In eukaryotes, the cell cycle is a process in which a plethora of interconnected events occur in unidirectional manner to ensure the generation of two new daughter cells. These sequential steps

* Corresponding author. Fax: +54 11 4786 8578.
E-mail address: potenza@dna.uba.ar (M. Potenza).

0014-4894/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.exppara.2012.09.002

are driven by the kinase activity of different cyclin–CDK complexes, which are active at different stages along the cell cycle (Bloom and Cross, 2007; Weingartner et al., 2003). Phosphorylation of key targets by cyclin–CDK complexes triggers several downstream events necessary for proper cell cycle progression. Cyclins are synthesized or recruited when they are required to activate particular CDKs, and then degraded or relocated when this activity needs to be abolished (Parry et al., 2003). Although the preference of particular cyclins for specific CDKs is still under revision (Coudreuse and Nurse, 2010; Hochegger et al., 2008), it is known that specificity is required for the correct cell cycle phase progression (Bloom and Cross, 2007). In budding yeast, distinct cyclin isoforms activate a single CDK (Cdc28) at different phases of the cell cycle. While specific G1-type cyclins are involved in initial bud formation and spindle pole duplication, S-type cyclins promotes DNA replication. By contrast, mitotic cyclins establish mitotic spindle orientation allowing chromosome segregation (Mendenhall and Hodge, 1998).

In trypanosomatids, the mechanisms that regulate cell cycle are still partially understood. Several cyclins, cyclin related kinases (CRKs) and other proteins involved in the cell cycle control have been characterized (Hammarton et al., 2007). Ten cyclins (CYC2–CYC11) were identified in *T. brucei* (Hammarton, 2007), being nine of them well characterized (Gourguechon et al., 2007; Gourguechon and Wang, 2009; Li and Wang, 2003; van Hellemond et al., 2000). Furthermore, downregulation of the PHO-like CYC2 and the mitotic-type CYC6 was shown to induce cell cycle arrest (Hammarton et al., 2003, 2004; Li and Wang, 2003). A divergent mitotic cyclin from *Leishmania* genus, with no orthologous genes in *T. cruzi* or *T. brucei*, has been also characterized (Banerjee et al., 2006). From these studies, it can be predicted that some mechanisms that govern the cell cycle machinery in these protozoa follow a similar pattern when compared to higher eukaryotes, taking cyclins and CRKs major roles in cell cycle progression. However, several differences are present in the cell cycle regulation of these pathogens. A clear dissociation exists between cytokinesis and mitosis regulation in *T. brucei* (Kumar and Wang, 2006; Ploubidou et al., 1999; Tu et al., 2006), where cell division can be successfully completed even in absence of nuclear duplication and segregation (Hammarton et al., 2003; Tu and Wang, 2004). In addition, several rounds of DNA replication and organelle duplication can be performed in a single cell without cellular division (Jetton et al., 2009; Kumar and Wang, 2006; Lillico et al., 2003; Ngo et al., 1998). On the other hand, some cyclin-CRK complexes show differences in response to regulatory signals that modulate kinase activity. CyclinA-CRK3 in *Leishmania mexicana* is active even in the absence of phosphorylation on residue Thr178 (Gomes et al., 2010), while this phosphorylation is required to activate some human CDKs (Kaldis, 1999). Also, canonical CDK inhibitors are absent from the genomes of trypanosomatids (Hammarton, 2007).

Previously, we reported the identification of three coding sequences from a cDNA library of *T. cruzi*, which were able to associate to *Tc*CRK1 or *Tc*CRK3, two protein-related kinases capable of binding human cyclins (Gómez et al., 1998). Using the *Tc*CRK1 isoform as bait in a yeast two-hybrid system, the identified putative *T. cruzi* cyclins were named *Tc*CYC2, *Tc*CYC4 and *Tc*CYC5 (published in Gómez et al., 2001, as TzCYC4, TzCYC5 and TzCYC6 and then renamed in agreement with their *T. brucei* and *Leishmania* homologues). We determined that *Tc*CYC2 did not interact with *Tc*CRK3, suggesting a preferential kinase binding (Gómez et al., 2001), while the *T. brucei* homologue *Tb*CYC2 it does with *Tb*CRK3 (Gourguechon et al., 2007). *Tb*CYC2 was also found to be essential for proper progression through G1 phase of the cell cycle (Hammarton et al., 2004; Li and Wang, 2003).

In the present report, we further characterized *TcCYC2* in *T. cruzi*. We show here that *TcCYC2* exhibited cyclin function by rescuing the cell cycle arrested phenotype in a G1-defficient *Saccharomyces cerevisiae* mutant. We also analyzed the phenotype of epimastigote parasites that over-express different *Tc*CYC2 tagged proteins. We found *Tc*CYC2-HA over-expression modified parasite morphology, as well as shortened the cell cycle.

2. Methods

2.1. Bioinformatic analysis of T. cruzi cyclin sequences

Protein coding sequences of putative cyclins in *T. cruzi* CL Brener genome were extracted from The Kinetoplastid Genome Resource (http://www.tritrypdb.org). Analysis of DNA and protein similarity were performed at the NCBI Blast server (Altschul et al., 1990), and sequences were aligned using the NTI Vector 10 Advance software (Informax Inc. Bethesda, MD, USA). Phylogenic analysis was performed at http://www.phylogeny.fr (Dereeper et al., 2008), on the base of a MUSCLE alignment (Edgar, 2004).

2.2. Parasite cultures

T. cruzi epimastigote forms from CL Brener clone and TcI lineage were used. Both strains were typified by spliced-leader gene polymorphisms (Burgos et al., 2010), and classified according to the recommended DTU designation (Discrete Typing Units) as TcVI and TcI, respectively (Zingales et al., 2009). Epimastigote cultures from both strains were grown at 28 °C in liver infusion tryptose medium (LIT) supplemented with 10% Fetal Bovine Serum (FBS, Natocor, Argentina) plus 10 U/ml penicillin and 10 mg/l streptomycin.

2.3. DNA sequencing and gene expression analysis of TcCYC2

TcCYC2 coding sequence was amplified from CL Brener and TcI *T. cruzi* strains by PCR as follow: 200 μl of exponentially growing epimastigote cultures were pelleted, washed once with PBS and resuspended in 10 μl of nuclease free water (New England Biolabs). Parasites were lysed by two freeze–thaw cycles, centrifuged at 10,000*g* and 1 μl of supernatant was used in standard PCR reaction using primers CYC21 and CYC22 (Table 1), and Pfu DNA polymerase (Fermentas). PCR products were purified and sequenced for further analysis.

Total RNA was isolated from *T. cruzi* TcI epimastigotes using Total RNA Extraction (Real Biotech Corporation), according to manufacturer's instructions. Purified RNA was then visualized by agarose gel electrophoresis and quantified using NanoDrop™ (Thermo Scientific, USA). cDNA synthesis was performed using 1 μg of RNA with M-MLV reverse transcriptase (Promega, USA) and oligo-dT as primer in a 20 μl reaction. 1 μl of 1:10 dilution of the RT-reaction and primers CYC21 and CYC22 (Table 1) were used for the PCR-step. Negative controls (to discard contaminant DNA) were performed with RNA samples without reverse transcription. PCR products were loaded onto agarose gel, bands excised and then purified for sequencing and analysis.

Table 1
Oligonucleotides used in this study.

Oligo	Sense	Sequence 5′ → 3′
CYC21	Forward	GCG GAATTC ATG AGT CGT GCG CGT GC
CYC22	Reverse	GCG GTCGAC CTG TGG GGT GTC CCG CT
CYC23	Forward	GCGGGATCCATGAGTCGTGCGCGTGC
CYC24	Reverse	CGGTCGACTCACTGTGGGGTGTCC
CYC25	Reverse	AAC GTC GTA GGG GTA CTG TGG GGT GTC
CYC26	Reverse	TCGAGTCAAGCGTAATCCGGAACGTCGTAGGGGTA

2.4. Cloning of TcCYC2 and yeast complementation assay

The *TcCYC2* gene was amplified from TcI *T. crzui* DNA by PCR using CYC23 and CY24 primers, which introduced *BamHI* and *XhoI* restriction sites, respectively (Table 1). The PCR product was cloned in pZeRO 2.1 vector (Invitrogen), sequenced and then sub-cloned in yeast vector pRS416 (kindly provided by Dr. Jeremy Mottram, University of Glasgow, UK), in which expression is under MET25 promoter (URA3, (Mumberg et al., 1994). For complementation assays, a DL1 *S. cerevisiae* strain was used (*MATα ade1 his2 leu2–3, 112 trp1a ura3, GAL1::cln2*) in which the three G1 cyclin genes *CLN1*, *CLN2*, and *CLN3* are inactivated (Lew et al., 1991). This mutant also carries the expression of *CLN2* gene under GAL1 promoter, which is repressed by glucose and induced by galactose, allowing DL1 strain to grow on galactose-based medium but arresting cells in G1 in a glucose-based medium. Yeast cells were grown overnight in YEPG medium (2% w/v peptone, 1% w/v yeast extract, 2% w/v galactose), at 30 °C and then transformed with pRS416-*TcCYC2* by electroporation. As positive and negative controls, *T. brucei* cyclin 2 cloned in pRS416 (pGRS416-*TbCYC2*) and the empty vector were used, respectively. Transformants were selected by growing cells in selective medium (ura^- gal^+) at 30 °C for 48 h. Complementation assays were then performed after streaking transformed yeasts in medium containing glucose for 48–72 h.

2.5. Parasite transfections

TcCYC2 sequence was isolated from pZERO-*TcCYC2* vector and tagged at its C-terminal with the hemagglutinin epitope HA (YPYDVPDYA), using forward primer CYC21 and reverse primers CYC25 and CYC26 in two consecutive rounds of PCR. The resulting *TcCYC2-HA* fragment was sequenced and cloned in the expression vector pTREX (Vazquez and Levin, 1999). *TcCYC2* was also isolated from pZERO-*TcCYC2* by PCR using primers CYC21 and CYC22 and then cloned in pTREX-Omni vector (kindly given by Dr. León Bouvier, Instituto Lanari, Argentina). This vector holds the green fluorescence protein sequence (GFP) in frame with the HA-tag (GFP-HA), resulting in *Tc*CYC2-GFP-HA. Empty and recombinant vectors (pTREX, pTREX-*TcCYC2-HA* and pTREX-Omni-*TcCYC2*), were transfected separately in TcI *T. cruzi* epimastigotes as follows: 1×10^7 parasites were collected for each transfection, washed twice in phosphate buffered saline (PBS) and resuspended in 350 μl of electroporation buffer (PBS 1×, 0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 272 mM sucrose, pH 7.2). This suspension was transferred into a 2 mm gap electroporation cuvette (BioRad) and incubated on ice with 10 μg of each plasmid DNA, for 15 min. One pulse of 400 V, 500 μF was applied using a GenePulser electroporator (BioRad) followed by incubation on ice for 5 min. Subsequently, the cells were transferred to a flask containing 5 ml of LIT media with 10% FBS. Selection was initiated after 24 h by addition of 250 μg/ml geneticin (G418, Novagen). The drug concentration was increased to 500 μg/ml 72 h later. Control cells died after 3 weeks, and experiments were performed after 8 weeks under continuous selection of G418.

2.6. Western blot analysis

To detect recombinant protein expression in *T. cruzi* transfected parasites, 1×10^7 epimastigotes were harvested by centrifugation, resuspended in 5× SDS–PAGE loading buffer and parasite total proteins resolved by SDS–PAGE. Gels were transferred to nitrocellulose membranes and detected using anti-HA mouse monoclonal antibody (clone 16B12, Covance) and horseradish peroxidase-conjugated goat anti-mouse IgG (Calbiochem). Both antibodies were used at dilution 1:1000 in 3% bovine serum albumin in PBS (BSA–PBS) and detected with ECL™ chemiluminescence kit (GE Healthcare), according to the manufacturer's instructions.

2.7. Immunofluorescence microscopy

Exponential growing cultures of TcI *T. cruzi* epimastigotes transfected with pTREX, pTREX-*Tc*CYC2-HA or pTREX-Omni-*Tc*CYC2 were spun down, washed once in PBS and allowed to attach on poly-L-lysine coated slides. Cells were fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS and washed twice with PBS. After blocking slides in 2% BSA–PBS, fixed parasites were incubated with anti-HA antibody, washed three times with PBS and then incubated with goat anti-mouse Alexa Fluor 594 antibody (Molecular Probes). Each antibody was diluted 1:1000 with 1% BSA-PBS and incubations were made for 1 h at room temperature. Slides were mounted in ProLong Gold antifade reagent (Molecular Proves), containing 10 μg/ml of 4′,6′-diamino-2-phenylindole (DAPI). Cells were observed in an Olympus IX-71 inverted fluorescence microscope. Serial images of 0.2 μ (Z-increment) were acquired with a Hamamatsu R2 camera driven by CelÎR software (Olympus) and then processed by blind deconvolution using Autoquant X 2.1 software (Media Cybernetics).

2.8. Hydroxyurea-induced synchronization and flow cytometry analysis

Transgenic TcI *T. cruzi* epimastigotes (transfected with plasmids pTREX, pTREX-*TcCYC2-HA* or pTREX-Omni-*TcCYC2*) were synchronized with hydroxyurea (HU) as previously described (Galanti et al., 1994). Briefly, 4×10^6 parasites/ml in exponential growth were transferred to LIT media containing 10% FBS and 20 mM HU, and incubated 24 h at 28 °C. HU was removed by centrifugation (1000*g* for 10 min), followed by three washes with cold PBS and the parasites were resuspended in fresh LIT lacking HU. Samples were taken at every hour spanning at least one complete round of the cell cycle and analyzed by flow cytometry as follows: 6×10^6 parasites from each sample point were washed in PBS and fixed with 500 μl of 70% ethanol in PBS at 4 °C overnight. Fixed parasites were washed with PBS and incubated for 30 min at 37 °C with 500 μl of staining solution containing 2 mM EDTA, 10 μg/ml of DNAse-free RNase A and 20 μg/ml propidium iodide (Invitrogen) in PBS. Samples were analyzed in a flow cytometer (FACSCalibur, BD Biosciences) using the CellQuest software. The fluorescence intensity, which is proportional to DNA content of the parasites, was plotted against cell count for each point to monitor the progression of cell cycle. Experiments were independent quadruplicates.

To compare the cell size at G1, S and G2/M phases of the cell cycle between different *T. cruzi* transfectants, non-synchronized epimastigotes were fixed, stained with propidium iodide as above and then analyzed by flow cytometry. Each of the three peaks representing the different phases of the cell cycle were gated and plotted together as number of counts in function to forward scatter (FSC) for each population, as described in (Hammarton et al., 2004). It was assumed in this assay that the light scattered from a cell population at small angles is proportional to cellular size.

2.9. Population doubling time for T. cruzi strains

To calculate doubling times, TcI *T. cruzi* epimastigotes (transfected with plasmids pTREX, pTREX-*TcCYC2-HA* or pTREX-Omni-*TcCYC2*) were grown in the same batch of LIT supplemented with 10% FBS. Four independent and identical dilutions of each transfected cultures were prepared and parasite counted every 24 h for a 2 weeks period using a Neubauer chamber. Points at the early exponential phase of growth were used to calculate the population doubling time of each culture using the algorithm provided by

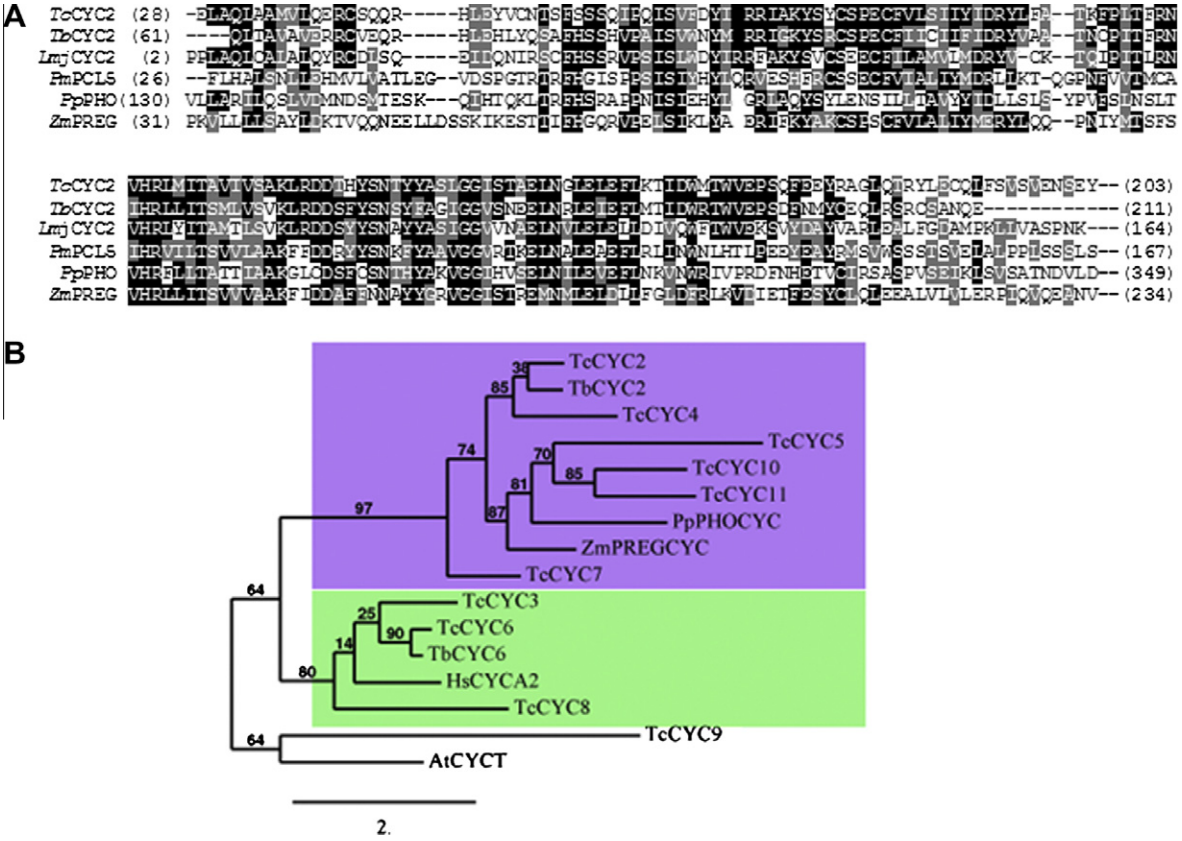


Fig. 1. Panel A: multiple sequence alignment of *Trypanosoma cruzi Tc*CYC2 cyclin box domain (TcCLB.511727.199) and those from other organisms: *Tb*CYC2 (*Trypanosoma brucei* CYC2, Tb11.01.5660), *Lmj*CYC2 (*Leishmania major* CYC2-like cyclin, LmjF.32.0820), *Pp*PHO (*Pichia pastoris* PHO80/85 cyclin, CCA40233.1), *Zm*PREG (*Zea mays* PREG-like cyclin protein, ACG36976.1), *Pm*PCL5 (*Perkinsus marinus* putative G1/S-specific cyclin PCL5, XP_002788088). Amino acids are colored as follows: white for different residues, black for identical residues, gray for similar and conserved residues. Panel B: phylogenetic tree for the *T. cruzi* putative cyclins. Sequences included are as follow: *Tc*CYC2 and the remaining set of *T. cruzi* cyclin sequences (*Tc*CYC3 to *Tc*CYC11: TcCLB.506711.30, TcCLB.508385.30, TcCLB.504021.10, TcCLB.511025.120, TcCLB.506945.270, TcCLB.509507.49, TcCLB.511727.260, TcCLB.511903.300, TcCLB.503551.20), human cyclin A (*Hs*CYCA, AAM54042.1), maize PREG-like cyclin (*Zm*PREGCYC; ACG36976.1), yeast PHO-like cyclin from *Pichia pastoris* (*Pp*PHOCYC; CCA40233.1) and cyclin T from *Arabidopsis thaliana* (*At*CYCT, NP_193695.2). Background colors indicate mitotic (light green), and non-mitotic (violet) clusters. Branch support values are shown as percentages, %. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

http://www.doubling-time.com/compute.php, which uses an exponential curve fitting method with least squares (http://www.mathworld.wolfram.com/LeastSquaresFittingExponential). Mean values and standard deviation were calculated using SigmaPlot 12.0 software (Jandel Scientific, San Rafael, CA).

3. Results

3.1. TcCYC2 belongs to the non-mitotic cyclin group and is transcribed in T. cruzi TcI lineage

Searches in the integrated TriTryp database (http://www.tritrypdb.org) showed that *TcCYC2* is annotated as a unique sequence (TcCLB.511727.199) in the CL Brener *T. cruzi* genome. Its coding sequence presents a cyclin box domain similar to plants (*Zea mays* and *Arabidopsis thaliana*) and yeast (*Pichia pastoris*) PREG/PHO-like proteins (Fig. 1A), which are regulators involved in phosphate uptake in some organisms (Huang et al., 2007; Kang and Metzenberg, 1993). *TcCYC2* showed the highest identity with Cyclin2 from *T. brucei*, CYC2-like cyclin from *Leishmania major* and the putative G1/S-specific cyclin PCL5 from the protozoan *Perkinsus marinus* (52%, 47% and 34% of identity, respectively). The phylogenetic relationship between *Tc*CYC2, the remaining cyclin sequences coded in the *T. cruzi* genome and some used in the multiple alignment is shown in Fig. 1, panel B. This phylogeny supports the existence of two main groups; mitotic and non-mitotic cyclins, in which *Tc*CYC2 is clustered with the PREG/PHO-like proteins and *Tb*CYC2 (G1-type cyclin) from *T. brucei*.

Then, we confirmed *TcCYC2* is actively transcribed in *T. cruzi* TcI lineage by RT-PCR and sequencing analysis. A single amino acid change was observed in the theoretical translated messenger as compared to the CL Brener annotated protein coding sequence (184 Serine by Alanine), although this residue change falls out of the predicted cyclin box domain.

3.2. TcCYC2 complements a yeast G1-cyclin deficient strain

To determine whether the *TcCYC2* gene from TcI *T. cruzi* lineage encodes a functional cyclin, the *S. cerevisiae* DL1 strain was transformed with pRS416-*TcCYC2* recombinant vector. As a positive control, we used the *T. brucei* CYC2 (pRS416-*TbCYC2*), which was previously used for efficiently replacing of the G1 cyclin deficiency present in DL1 strain (van Hellemond et al., 2000). After selection on uracil lacking but glucose containing plates, only colonies transformed with pRS416 carrying either *TcCYC2* or *Tb*CYC2 genes grew properly, whereas the colonies transfected with the empty vector did it poorly (Fig. 2). It demonstrated that *TcCYC2* gene from *T. cruzi* (as it was also showed for *Tb*CYC2) is able to complement the cyclin deficiency and therefore it encodes a functional cyclin protein.

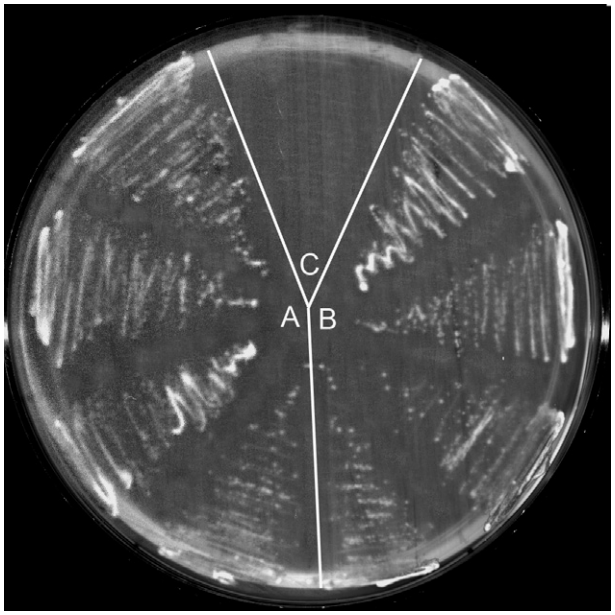


Fig. 2. Complementation assay of *Tc*CYC2. Functional complementation of a *S. cerevisiae* deficient G1 cyclin strain with *Tc*CYC2 from *T. cruzi*. Several clones of DL1 *S. cerevisiae* strain transformed with *Tc*CYC2 were found to overcome G1 arrest of mutant *S. cerevisiae*, indicating functional complementation. Inset letters: (A) *Tc*CYC2-pRS416; (B) *Tb*CYC2-pRS416; (C) empty vector pRS416.

3.3. TcCYC2 localizes in the cytoplasm of T. cruzi and does not vary along the cell cycle

To investigate the sub-cellular localization of *Tc*CYC2 along the cell cycle of TcI *T. cruzi* epimastigotes, we transfected parasites with two different constitutive expression vectors containing the *TcCYC2* gene fused to the HA or GFP-HA tags. After selection of transgenic parasites with geneticin, we analyzed protein expression by western blot and performed immunofluorescence microscopy using the monoclonal anti-HA antibody (Fig. 3). We found that exogenous *Tc*CYC2-HA or *Tc*CYC2-GFP-HA are mainly localized in the cytoplasm (panels A and B in Fig. 3), and this is maintained along the different phases of the cell cycle.

3.4. TcCYC2-HA over-expression in T. cruzi alters duplication time and morphology

In order to infer the specific function of *Tc*CYC2, we analyze the effect of the over-expression of this protein in *T. cruzi* epimastigotes from TcI lineage. We measured the duplication time of parasites carrying the pTREX, pTREX-*TcCYC2-HA* or pTREX-Omni-*TcCYC2* vectors. The doubling times for these cultures were calculated at the early exponential growth phase (Table 2). Epimastigotes overexpressing *Tc*CYC2-HA protein showed a shorter duplication time (16.9 ± 1.4 h), compared to both control (pTREX) and expressing *Tc*CYC2-GFP-HA (pTREX-Omni-*TcCYC2*) cultures (22.3 ± 2.9 h and 24.6 ± 2.7 h, respectively).

To further study the impact of *Tc*CYC2-HA and *Tc*CYC2-GFP-HA over-expression in terms of cell morphology, the size of parasites was analyzed by forward scatter (FSC), a parameter that is related to particle volume when a cell population is studied (Fig. 4). Culture samples at exponential growth were analyzed and the cell volume compared among the different transgenic parasites at G1, S or G2/M phases (Fig. 4). We found that the relative volume of *Tc*CYC2-HA over-expressing epimastigotes was smaller than control and *Tc*CYC2-GFP-HA over-expressing cells, at any phase of the cell cycle (panels B–D in Fig. 4). On the contrary, the *Tc*CYC2-GFP-HA cell population presented almost identical volumes as the control parasites (carrying pTREX empty vector).

3.5. Over-expression of TcCYC2-HA protein in epimastigotes modifies the length of the cell cycle

In order to analyze which phase of the cell cycle could be affected by the over-expression of *Tc*CYC2-HA, we studied the behavior of synchronized cultures through time. Transgenic *T. cruzi* cultures were chemically synchronized with the addition of HU to arrest the cells at G1 phase. After washings to remove HU from the growth media, cells were let to start a new round through the cell cycle, which for TcI lineage it takes around 22 h. Culture samples were taken at every hour and the DNA content was analyzed over time (Fig. 5). We found that synchronized epimastigotes peaked at S and G2/M phases around the same time (6 and 14 h, respectively) for the three transgenic TcI cultures studied, but differences appeared at time parasites pass through G2/M boundary and finished cytokinesis. The percentage of cells finishing cytokinesis increased earlier for *Tc*CYC2-HA over-expressing parasites and as a consequence, a higher number of cells at G1 phase could be counted (Fig. 5, inner tables in histograms at times 15 and 16 h). For TcCYC2-HA over-expressing epimastigotes, more than 60% of the culture reached the next G1 phase, meanwhile for the control or over-expressing *Tc*CYC2-GFP-HA cultures these percentages were around 26–38%.

4. Discussion

In the present investigation, we performed a functional analysis of *Tc*CYC2 cyclin protein. We verified the transcription of *TcCYC2* gene by RT-PCR in our working *T. cruzi* TcI lineage. The presence of this gene was recently identified by the shotgun sequencing of the *T. cruzi* Silvio X10/1 genome, a representative strain belonging to TcI lineage (Franzen et al., 2011). In addition, we confirmed that *TcCYC2* sequence is a functional cyclin as it was able to restore the growth arrest in DL1 *S. cerevisiae* mutant strain, allowing yeast to progress through the cell cycle. This result does not necessary means that *Tc*CYC2 is specific for G1 phase, because this *S. cerevisiae* strain could be rescued either by non-mitotic or mitotic cyclins, as it was showed for G1 and mitotic cyclins CYC2, CYC3 and CYC6 from *T. brucei* (van Hellemond et al., 2000; Hammarton et al., 2003), as well as by mitotic cyclins from mammalians and plants (Day et al., 1996; Lew et al., 1991). One characteristic of trypanosomatid cyclins is that their genomes do not code for those archetypal G1 cyclins found in other eukaryotes, and the same can be found in some plant genomes (Wang et al., 2004). In such cases, a re-distribution of cyclins roles along the different phases of the cell cycle is observed (Gourguechon et al., 2007) and therefore, restriction of *Tc*CYC2 activity other than G1/S checkpoint should be not discarded. Interestingly, *Tc*CYC2 cyclin box presents high similarity with those PREG and PHO-like cyclins from yeast and plants. Results obtained with the *T. brucei* CYC2 homologue showed that this protein did not present the PHO-like function by yeast complementation assays (Gourguechon et al., 2007), suggesting that it is not involved in the phosphate metabolism of the parasite. Although specific experiments must be performed for *Tc*CYC2 from *T. cruzi*, this isoform may exhibit similar characteristics.

To better characterize the *Tc*CYC2 in the context of the parasite, we constitutively over-expressed this protein tagged to HA epitope in TcI *T. cruzi* epimastigotes. We found *Tc*CYC2-HA is located throughout the cytoplasm of parasites with no nuclear signal at any phase of the cell cycle. It has been shown that some cyclins isoforms regulate their activity by relocation to distinct sub-cellular compartments in a cell cycle dependent manner (Miller and Cross,

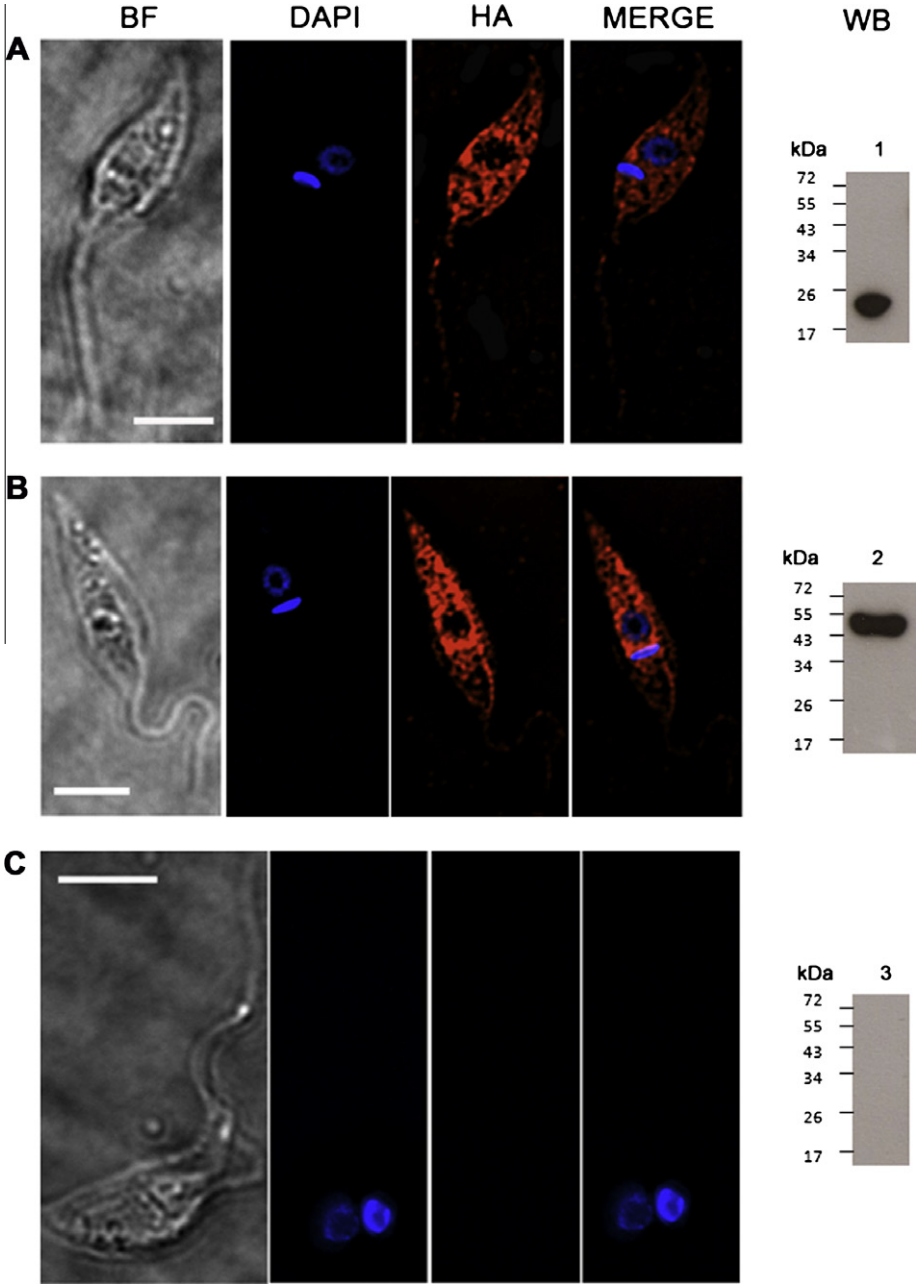


Fig. 3. Localization of *Tc*CYC2 in *T. cruzi*. An anti-HA antibody was used to detect the expression and localization of tagged *Tc*CYC2. Panels A and B: parasites over-expressing *Tc*CYC2-HA or *Tc*CYC2-GFP-HA, respectively. Panel C: parasites transfected with the empty pTREX vector. BF, bright field; HA, anti-HA antibody staining; DAPI, 4′,6′-diamino-2-phenylindole staining of the nuclei and kinetoplast. *Scale bar* is indicated in the bright field panel, 7 μm. WB, western blot analysis of whole-cell extract from each epimastigote cultures. Lanes 1 and 2: parasites over-expressing *Tc*CYC2-HA (24 kDa) and *Tc*CYC2-GFP-HA (53 kDa), respectively. Lane 3: control parasites transfected with pTREX only. The band signals appeared at expected sizes.

2001; Pines and Hunter, 1991), but these mechanisms do not seem to occur in our transgenic cultures which could be attributed to a failure in the routing of this over-expressed, tagged protein or simply to the absence of this mechanism for *Tc*CYC2.

On the other hand, we studied the phenotype of *Tc*CYC2-HA over-expressing cells synchronizing epimastigotes with hydroxyurea. The typical behavior of a synchronized *T. cruzi* culture follows a general rule and is reproducible within the same strain (Galanti et al., 1994; Santori et al., 2002). In our case, synchronized epimastigotes pass through all phases of the cell cycle starting from G1 upon HU release. The S phase usually peaks after 6 h; meanwhile the G2/M boundary can be seen around 14 h. After 22 h, most cells have undergone cytokinesis entering again into a new G1 phase to start another round of DNA duplication and subsequent cell divi-

Table 2
Population doubling times of *T. cruzi* transfected epimastigotes. Doubling times (DT) were calculated ($n = 4$) for each population. Control: parasites transfected with empty pTREX; *Tc*CYC2-HA or *Tc*CYC2-GFP-HA: parasites over-expressing *Tc*CYC2-HA or *Tc*CYC2-GFP-HA fusion proteins, respectively.

Strain	DT (h)[a]
Control	22.3 ± 2.9
*Tc*CYC2-HA	16.9 ± 1.4
*Tc*CYC2-GFP-HA	24.6 ± 2.7

[a] Mean ± standard deviation.

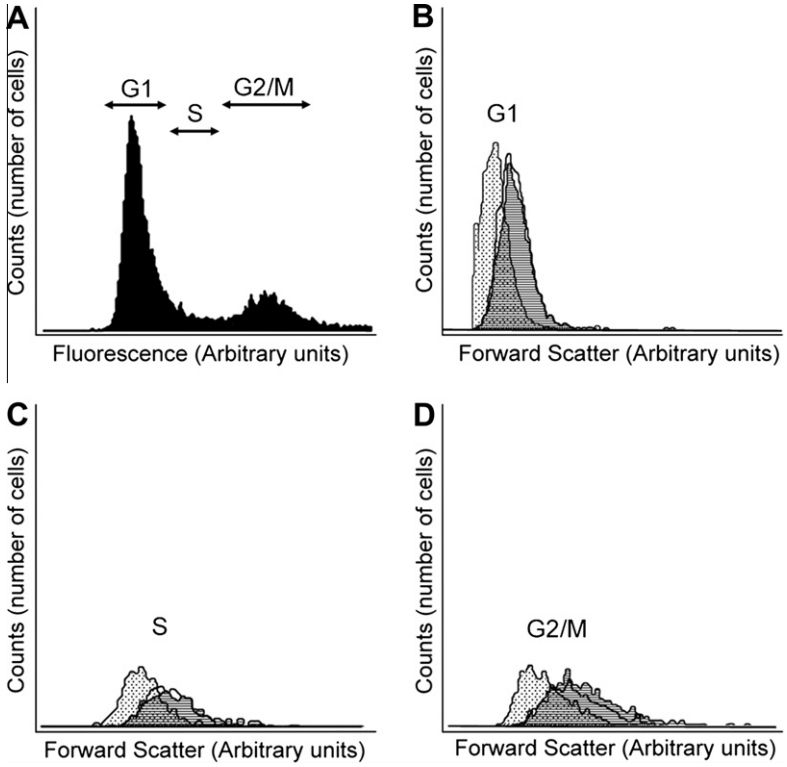


Fig. 4. Cell size analysis of *T. cruzi* epimastigotes over-expressing *Tc*CYC2 fusion proteins. Forward scatter were used to compare cell volume between strains over-expressing *Tc*CYC2-HA, *Tc*CYC2-GFP-HA or carrying pTREX empty at each phase of the cell cycle. Panel A: schematic representation for each gated zone. Double-headed arrows indicate the phase of the cell cycle that was taken for further analysis. Panel B: forward scatter plotted against counts (cell number). Dotted curve area under the line represents the volume of epimastigote cells over-expressing *Tc*CYC2-HA. Lined and white curve areas represent parasites transfected with pTREX vector only or over-expressing *Tc*CYC2-GFP-HA fusion protein, respectively. Panels C and D: analysis of cell volume for the three different transgenic cultures at S and G2/M phases, respectively.

sion. We found that upon release of the HU blockade, both control and over-expressing *Tc*CYC2-HA epimastigotes undergo the typical lag period until the cultures reach S phase and G2/M boundary at practically same times. However, *Tc*CYC2-HA over-expressing cells pass through mitosis or cytokinesis in a much shorter time than control parasites, even though the three strains successfully undergo cell division. This was evidenced by the fact that after 15 h, there was an increased number of cells in G1 for over-expressing *Tc*CYC2-HA as compared to control parasites. Furthermore, we found that the doubling time of over-expressing *Tc*CYC2-HA epimastigotes was shorter in comparison to control parasites in asynchronous cultures. Both phenotypes, shorter cell cycle and doubling time, were abolished with the addition of a GFP protein to the C-terminal site of *Tc*CYC2. This could be due to a steric hindrance produced by insertion of the GFP, which could alter the proper *Tc*CYC2 structural conformation and therefore avoiding protein–protein interactions between this cyclin and its binding partners. There are some reports describing impairment of protein function by addition of GFP or another fluorescent molecule to the C-terminal domain (Han et al., 2005; Ossenbuhl et al., 2006; Thomas and Maule, 2000; Verweij et al., 2011), but to corroborate this in our system, more experiments are required. Despite this observation, it is important to take into account that site-directed mutagenesis is particularly poorly informative in cyclins, where it has been showed that the overall cell structure is necessary for the function and localization rather than few key residues (Bendris et al., 2011).

It is necessary to point out that besides inhibiting the enzyme ribonucleotide reductase (Berglund and Sjöberg, 1979), HU can also cause DNA damage by ion-dependent base oxidation, which was showed in an *in vitro* assay (Sakano et al., 2001). In addition, HU induces double strand breaks in cells starting mitosis. It is interesting that *T. brucei*, rather than being arrested at G1 phase (Mutomba and Wang, 1996), could be induced to synchrony in S phase using low concentrations of HU (Chowdhury et al., 2008; Forsythe et al., 2009), suggesting that different check points operate in both organisms. In eukaryotes, some complexes formed by cyclins-CDKs need to be deactivated if DNA damage occur, in order to stop the progression of the cell cycle until the DNA is repaired (Gillis et al., 2009; Guo et al., 2000; Pontano and Diehl, 2009). If HU treatment also causes DNA damage in epimastigote cells, it would be a possibility that G2/M DNA damage checkpoint is affected or bypassed by the over-expression of *Tc*CCY2-HA. This could promote progression of cell cycle even if DNA repair is not complete, contrary to the case of cells with physiological levels of *Tc*CYC2. The latest case opens an interesting question to further investigate the involvement of *Tc*CYC2 in DNA damage and repair.

In addition, we found that epimastigotes over-expressing *Tc*CYC2-HA were smaller in size compared to control parasites, at each phase of the cell cycle in asynchronous cultures. The smaller size was not found when fusing the GFP protein in *Tc*CYC2-GFP-HA over-expressing epimastigotes, indicating that this later protein could be inactive. Correlations between cell size and cyclin expression have been found in other organisms. In *Drosophila*, it was demonstrated that over-expression of cyclin E isoform truncated the G1 phase and decreased the cell size (Datar et al., 2000). Moreover, overproduction of cyclin G produces small cells whereas shortage produces large cells (Faradji et al., 2011). Interestingly, it was demonstrated in *T. brucei* that *Tb*CYC2 gene is necessary to proper morphology of procyclic forms (Gourguechon et al., 2007; Hammarton et al., 2004). It remains to be answered whether the *Tc*CYC2 is directly regulating cell size, or it is involved in the cross-talk between cell size and cell cycle checkpoints.

Taken together, our results suggest that *Tc*CYC2-HA over-expression alters the parasite size and cell cycle. It is worth men-

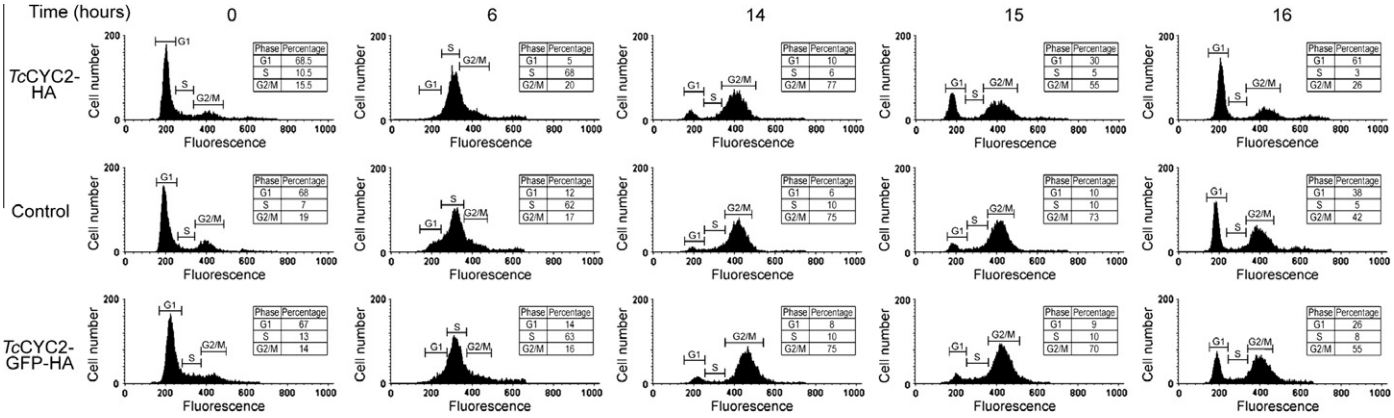


Fig. 5. Flow cytometry analysis of HU-synchronized *T. cruzi* epimastigotes over-expressing *Tc*CYC2 proteins. *T. cruzi* cells transfected with different plasmid constructions were arrested at G1 phase and analyzed by FACS. The figure shows a representative experiment with samples taken at time 0 h (G1 phase peak), 6 h (S phase peak), and 14–16 h (boundary G2/M and cells entering to G1 phase after cytokinesis). Control: parasites transfected with pTREX only. *Tc*CYC2-HA and *Tc*CYC2-GFP-HA: parasites over-expressing these corresponding fusion proteins. Histograms represent the DNA content (fluorescence) of each sample culture at different times. The histogram inset show the percentage of counts gated in G1, S or G2/M phases. Remaining counts were excluded for the analysis because it represents duplets or apoptotic cells.

tioning that this phenotype could be due to the presence of a protein which physiological levels of expression is lower, as the proteomic evidence seems to demonstrate. Using this approach, there is still no published data mentioning the detection of *Tc*CYC2 (or any other cyclin) in *T. cruzi* (Atwood et al., 2005; Ayub et al., 2009; Cordero et al., 2009; Ferella et al., 2008; Kikuchi et al., 2010; Paba et al., 2004a,b; Sant'Anna et al., 2009; Sodré et al., 2009; Ulrich et al., 2011). These findings support the idea that cyclins are present in the cell at very low levels, probably due to its rapid turnover. As the over-expressed *Tc*CYC2-HA affects the cell cycle, it is possible that the endogenous *Tc*CYC2 is found in few sub-cellular organelles and/or at very short periods throughout the cycle, only when, or where, it is required to perform a particular process, such as activate/deactivate CDKs and other proteins (Hochegger et al., 2008). In other organisms, cyclins are proteins which their expression is tightly regulated by several mechanisms (Bloom and Cross, 2007; Tarn and Lai, 2011). In some cancer cells, uncontrolled proliferation is correlated with failure on mechanisms that control cyclin expression or degradation, resulting in high levels of cyclin activity (Akli et al., 2007; Minella et al., 2005; Musgrove et al., 2011). In *T. brucei*, down regulation of CYC2 by RNAi (which has 52% of identity with *Tc*Cyc2 from *T. cruzi*), arrests the parasite at the G1 phase of the cell cycle (Hammarton et al., 2004; Li and Wang, 2003). In addition, double RNAi experiments in which both, CRK (CRK1, CRK2 or CRK3) and CYC2 (named by these authors Cyclin E1) were depleted, allowed to have an idea of the multiples roles of this protein in *T. brucei* cell cycle. In this regard, the RNAi for CYC2 + CRK1 results in a more pronounced growth arrest at G1 than the RNAi for each of them separately. Surprisingly, the double knock out for CYC2 + CRK3 caused an arrest in the G2/M boundary of the cell cycle, in a more efficient manner than RNAi for CRK3 only. The authors proposed then that CYC2 has different roles in the cell cycle control and could form different complexes with CRKs, promoting the passage through G1/S and G2/M transitions (Gourguechon et al., 2007). In this regard and taking into account our results, the cyclin *Tc*CYC2 would play a role in the cell cycle of the parasite, not restricted to G1/S boundary. We are now engaged in the study of mechanisms that produce the phenotype observed in this work as a result of the over-expression of *Tc*CYC2 in *T. cruzi*.

Acknowledgments

We thank to Dr. León Bouvier and Dr. Jeremy Mottram for vectors, Dr. Isabel Santori for help in complementation yeast assay and Dr. Alejandro Schijman for typifying the *T. cruzi* strains. MP thanks to Dr. Marcela Ferella for language assistance and the AMSUD-PASTEUR Training Programm for Young Investigators. This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Agencia Nacional de Promoción científica y Tecnológica (ANPCyT). MP and MTTI are members of the Research Career of CONICET.

References

Akli, S., Van Pelt, C.S., Bui, T., Multani, A.S., Chang, S., Johnson, D., Tucker, S., Keyomarsi, K., 2007. Overexpression of the low molecular weight cyclin E in transgenic mice induces metastatic mammary carcinomas through the disruption of the ARF-p53 pathway. Cancer Res. 67, 7212–7222.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Atwood, J.A., Weatherly, D.B., Minning, T.A., Bundy, B., Cavola, C., Opperdoes, F.R., Orlando, R., Tarleton, R.L., 2005. The *Trypanosoma cruzi* proteome. Science 309, 473–476.

Ayub, M.J., Atwood, J., Nuccio, A., Tarleton, R., Levin, M.J., 2009. Proteomic analysis of the *Trypanosoma cruzi* ribosomal proteins. Biochem. Biophys. Res. Commun. 382, 30–34.

Banerjee, S., Sen, A., Das, P., Saha, P., 2006. *Leishmania donovani* cyclin 1 (LdCyc1) forms a complex with cell cycle kinase subunit CRK3 (LdCRK3) and is possibly involved in S-phase-related activities. FEMS Microbiol. Lett. 256, 75–82.

Bendris, N., Lemmers, B., Blanchard, J.M., Arsic, N., 2011. Cyclin A2 mutagenesis analysis: a new insight into CDK activation and cellular localization requirements. PLoS One 6, e22879. http://dx.doi.org/10.1371/journal.pone.0022879.

Berglund, O., Sjöberg, B.M., 1979. Effect of hydroxyurea on T4 ribonucleotide reductase. J. Biol. Chem. 254, 253–254.

Bloom, J., Cross, F.R., 2007. Multiple levels of cyclin specificity in cell-cycle control. Nat. Rev. Mol. Cell Biol. 8, 149–160.

Body, A., Mackiewicz, P., Milanowski, R., 2010. Did Trypanosomatid parasites contain a eukaryotic alga derived plastid in their evolutionary past? J. Parasitol. 96, 465–475.

Burgos, J.M., Diez, M., Vigliano, C., Bisio, M., Risso, M., Duffy, T., Cura, C., Brusses, B., Favaloro, L., Leguizamon, M.S., Lucero, R.H., Laguens, R., Levin, M.J., Favaloro, R., Schijman, A.G., 2010. Molecular identification of *Trypanosoma cruzi* discrete typing units in end-stage chronic Chagas heart disease and reactivation after heart transplantation. Clin. Infect. Dis. 51, 485–495.

Chowdhury, A.R., Zhao, Z., Englund, P.T., 2008. Effect of hydroxyurea on procyclic *Trypanosoma brucei*: an unconventional mechanism for achieving synchronous growth. Eukaryot. Cell 7, 425–428.

Cordero, E.M., Nakayasu, E.S., Gentil, L.G., Yoshida, N., Almeida, I.C., da Silveira, J.F., 2009. Proteomic analysis of detergent-solubilized membrane proteins from insect-developmental forms of *Trypanosoma cruzi*. J. Proteome Res. 8, 3642–3652.

Coudreuse, D., Nurse, P., 2010. Driving the cell cycle with a minimal CDK control network. Nature 468, 1074–1079.

Datar, S.A., Jacobs, H.W., de la Cruz, A.F.A., Lehner, C.F., Edgar, B.A., 2000. The Drosophila cyclin D–Cdk4 complex promotes cellular growth. EMBO J. 19, 4543–4554.

Day, I.S., Reddy, A.S.N., Golovkin, M., 1996. Isolation of a new mitotic-like cyclin from *Arabidopsis*: complementation of a yeast cyclin mutant with a plant cyclin. Plant Mol. Biol. 30, 565–575.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.M., Gascuel, O., 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. 36, 465–469.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.
Faradji, F., Bloyer, S., Dardalhon-Cumenal, D., Randsholt, N.B., Peronnet, F., 2011. *Drosophila melanogaster* cyclin G coordinates cell growth and cell proliferation. Cell Cycle 10, 805–818.
Fenn, K., Matthews, K.R., 2007. The cell biology of *Trypanosoma brucei* differentiation. Curr. Opin. Microbiol. 10, 539–546.
Ferella, M., Nilsson, D., Darban, H., Rodrigues, C., Bontempi, E.J., Docampo, R., Andersson, B., 2008. Proteomics in *Trypanosoma cruzi* – localization of novel proteins to various organelles. Proteomics 8, 2735–2749.
Forsythe, G.R., McCulloch, R., Hammarton, T.C., 2009. Hydroxyurea-induced synchronisation of bloodstream stage *Trypanosoma brucei*. Mol. Biochem. Parasitol. 164, 131–136.
Franzen, O., Ochaya, S., Sherwood, E., Lewis, M.D., Llewellyn, M.S., Miles, M.A., Andersson, B., 2011. Shotgun sequencing analysis of *Trypanosoma cruzi* I Sylvio X10/1 and comparison with *T. cruzi* VI CL Brener. PLoS Negl. Trop. Dis. 5, e984.
Galanti, N., Dvorak, J.A., Grenet, J., McDaniel, J.P., 1994. Hydroxyurea-induced synchrony of DNA replication in the Kinetoplastida. Exp. Cell Res. 214, 225–230.
Gillis, L.D., Leidal, A.M., Hill, R., Lee, P.W.K., 2009. P21Cip1/WAF1 mediates cyclin B1 degradation in response to DNA damage. Cell Cycle 8, 253–256.
Gomes, F.C., Ali, N.O.M., Brown, E., Walker, R.G., Grant, K.M., Mottram, J.C., 2010. Recombinant *Leishmania mexicana* CRK3:CYCA has protein kinase activity in the absence of phosphorylation on the T-loop residue Thr178. Mol. Biochem. Parasitol. 171, 89–96.
Gómez, E.B., Kornblihtt, A.R., Tellez-Iñión, M.T., 1998. Cloning of a cdc2-related protein kinase from *Trypanosoma cruzi* that interacts with mammalian cyclins. Mol. Biochem. Parasitol. 91, 337–351.
Gómez, E.B., Santori, M.I., Laria, S., Engel, J.C., Swindle, J., Eisen, H., Szankasi, P., Tellez-Iñión, M.T., 2001. Characterization of the *Trypanosoma cruzi* Cdc2p-related protein kinase 1 and identification of three novel associating cyclins. Mol. Biochem. Parasitol. 113, 97–108.
Gourguechon, S., Wang, C.C., 2009. CRK9 contributes to regulation of mitosis and cytokinesis in the procyclic form of *Trypanosoma brucei*. BMC Cell Biol. 10, 68–81.
Gourguechon, S., Savich, J.M., Wang, C.C., 2007. The multiple roles of cyclin E1 in controlling cell cycle progression and cellular morphology of *Trypanosoma brucei*. J. Mol. Biol. 368, 939–950.
Guo, N., Faller, D.V., Vaziri, C., 2000. A novel DNA damage checkpoint involving post-transcriptional regulation of cyclin A expression. J. Biol. Chem. 275, 1715–1722.
Hammarton, T.C., 2007. Cell cycle regulation in *Trypanosoma brucei*. Mol. Biochem. Parasitol. 153, 1–8.
Hammarton, T.C., Clark, J., Douglas, F., Boshart, M., Mottram, J.C., 2003. Stage-specific differences in cell cycle control in *Trypanosoma brucei* revealed by RNA interference of a mitotic cyclin. J. Biol. Chem. 278, 22877–22886.
Hammarton, T.C., Engstler, M., Mottram, J.C., 2004. The *Trypanosoma brucei* cyclin, CYC2, is required for cell cycle progression through G1 phase and for maintenance of procyclic form cell morphology. J. Biol. Chem. 279, 24757–24764.
Hammarton, T.C., Monnerat, S., Mottram, J.C., 2007. Cytokinesis in trypanosomatids. Curr. Opin. Microbiol. 10, 520–527.
Han, W., Rhee, J.S., Maximov, A., Lin, W., Hammer, R.E., Rosenmund, C., Sudhof, T.C., 2005. C-terminal ECFP fusion impairs synaptotagmin 1 function. J. Biol. Chem. 280, 5089–5100.
Hochegger, H., Takeda, S., Hunt, T., 2008. Cyclin-dependent kinases and cell-cycle transitions: does one fit all? Nat. Rev. Mol. Cell Biol. 9, 910–916.
Huang, K., Ferrin-O'Connell, I., Zhang, W., Leonard, G.A., O'Shea, E.K., Quiocho Florante, A., 2007. Structure of the Pho85–Pho80 CDK-cyclin complex of the phosphate-responsive signal transduction pathway. Mol. Cell 28, 614–623.
Jetton, N., Rothberg, K.G., Hubbard, J.G., Wise, J., Li, Y., Ball, H.L., Ruben, L., 2009. The cell cycle as a therapeutic target against *Trypanosoma brucei*: hesperadin inhibits aurora kinase-1 and blocks mitotic progression in bloodstream forms. Mol. Microbiol. 72, 442–458.
Kaldis, P., 1999. The cdk-activating kinase (CAK): from yeast to mammals. Cell. Mol. Life Sci. 55, 284–296.
Kang, S., Metzenberg, R.L., 1993. Insertional mutagenesis in *Neurospora crassa*: cloning and molecular analysis of the preg (+) gene controlling the activity of the transcriptional activator NUC-1. Genetics 133, 193–202.
Kikuchi, S.A., Sodre, C.L., Kalume, D.E., Elias, C.G.R., Santos, A.L.S., de Nazare Soeiro, M., Meuser, M., Chapeaurouge, A., Perales, J., Fernandes, O., 2010. Proteomic analysis of two *Trypanosoma cruzi* zymodeme 3 strains. Exp. Parasitol. 126, 540–551.
Kumar, P., Wang, C.C., 2006. Dissociation of cytokinesis initiation from mitotic control in an eukaryote. Eukaryot. Cell 5, 92–102.
Lew, D.J., Duli, V., Reed, S.I., 1991. Isolation of three novel human cyclins by rescue of G1 cyclin (cln) function in yeast. Cell 66, 1197–1206.
Li, Z., Wang, C.C., 2003. A PHO80-like cyclin and a B-type cyclin control the cell cycle of the procyclic form of *Trypanosoma brucei*. J. Biol. Chem. 278, 20652–20658.
Lillico, S., Field, M.C., Blundell, P., Coombs, G.H., Mottram, J.C., 2003. Essential roles for GPI-anchored proteins in African trypanosomes revealed using mutants deficient in GPI8. Mol. Biol. Cell 14, 1182–1194.
Mendenhall, M.D., Hodge, A.E., 1998. Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. 62, 1191–1243.
Miller, M.E., Cross, F.R., 2001. Mechanisms controlling subcellular localization of the G1 cyclins Cln2p and Cln3p in budding yeast. Mol. Cell. Biol. 21, 6292–6311.
Minella, A.C., Welcker, M., Clurman, B.E., 2005. Ras activity regulates cyclin E degradation by the Fbw7 pathway. Proc. Natl. Acad. Sci. USA 102, 9649–9654.
Mumberg, D., Muller, R., Funk, M., 1994. Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res. 22, 5767–5768.
Musgrove, E.A., Caldon, C.E., Barraclough, J., Stone, A., Sutherland, R.L., 2011. Cyclin D as a therapeutic target in cancer. Nat. Rev. Cancer 11, 558–572.
Mutomba, M.C., Wang, C.C., 1996. Effects of aphidicolin and hydroxyurea on the cell cycle and differentiation of *Trypanosoma brucei* bloodstream forms. Mol. Biochem. Parasitol. 80, 89–102.
Ngo, H., Tschudi, C., Gull, K., Ullu, E., 1998. Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. Proc. Natl. Acad. Sci. USA 95, 14687–14692.
Ossenbuhl, F., Inaba-Sulpice, M., Meurer, J., Soll, J., Eichacker, L.A., 2006. The *Synechocystis* sp. PCC 6803 oxa1 homolog is essential for membrane integration of reaction center precursor protein pD1. Plant Cell 18, 2236–2246.
Paba, J., Ricart, C.A.O., Fontes, W., Santana, J.M., Teixeira, A.R.L., Marchese, J., Williamson, B., Hunt, T., Karger, B.L., Sousa, M.V., 2004a. Proteomic analysis of *Trypanosoma cruzi* developmental stages using isotope-coded affinity tag reagents. J. Proteome Res. 3, 517–524.
Paba, J., Santana, J.M., Teixeira, A.R.L., Fontes, W., Sousa, M.V., Ricart, C.A.O., 2004b. Proteomic analysis of the human pathogen *Trypanosoma cruzi*. Proteomics 4, 1052–1059.
Parry, D.H., Hickson, G.R.X., ÓFarrell, P.H., 2003. Cyclin B destruction triggers changes in kinetochore behavior essential for successful anaphase. Curr. Biol. 13, 647–653.
Pines, J., Hunter, T., 1991. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. J. Cell Biol. 115, 1–17.
Ploubidou, A., Robinson, D.R., Docherty, R.C., Ogbadoyi, E.O., Gull, K., 1999. Evidence for novel cell cycle checkpoints in trypanosomes: kinetoplast segregation and cytokinesis in the absence of mitosis. J. Cell Sci. 112, 4641–4650.
Pontano, L.L., Diehl, J.A., 2009. DNA damage-dependent cyclin D1 proteolysis: GSK3B holds the smoking gun. Cell Cycle 8, 824–827.
Sakano, K., Oikawa, S., Hasegawa, K., Kawanishi, S., 2001. Hydroxyurea induces site-specific DNA damage via formation of hydrogen peroxide and nitric oxide. Cancer Sci. 92, 1166–1174.
Sant'Anna, C., Nakayasu, E.S., Pereira, M.G., Lourenço, D., de Souza, W., Almeida, I.C., Cunha-e-Silva, N.L., 2009. Subcellular proteomics of *Trypanosoma cruzi* reservosomes. Proteomics 9, 1782–1794.
Santori, M.I., Laría, S., Gómez, E.B., Espinosa, I., Galanti, N., Téllez-Iñón, M.T., 2002. Evidence for CRK3 participation in the cell division cycle of *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 121, 225–232.
Simpson, A.G.B., Stevens, J.R., Lukes, J., 2006. The evolution and diversity of kinetoplastid flagellates. Trends Parasitol. 22, 168–174.
Sodré, C., Chapeaurouge, A., Kalume, D., de Mendonca Lima, L., Perales, J., Fernandes, O., 2009. Proteomic map of *Trypanosoma cruzi* CL Brener: the reference strain of the genome project. Arch. Microbiol. 191, 177–184.
Tarn, W.Y., Lai, M.C., 2011. Translational control of cyclins. Cell Division 6, 5. http://dx.doi.org/10.1186/1747-1028-6-5.
Thomas, C.L., Maule, A.J., 2000. Limitations on the use of fused green fluorescent protein to investigate structure–function relationships for the cauliflower mosaic virus movement protein. J. Gen. Virol. 81, 1851–1855.
Tu, X., Wang, C.C., 2004. The involvement of two cdc2-related kinases (CRKs) in *Trypanosoma brucei* cell cycle regulation and the distinctive stage-specific phenotypes caused by CRK3 depletion. J. Biol. Chem. 279, 20519–20528.
Tu, X., Kumar, P., Li, Z., Wang, C.C., 2006. An aurora kinase homologue is involved in regulating both mitosis and cytokinesis in *Trypanosoma brucei*. J. Biol. Chem. 281, 9677–9687.
Tyler, K.M., Engman, D.M., 2001. The life cycle of *Trypanosoma cruzi* revisited. Int. J. Parasitol. 31, 472–481.
Ulrich, P.N., Jimenez, V., Park, M., Martins, V.P., Atwood, J., Moles, K., Collins, D., Rohloff, P., Tarleton, R., Moreno, S.N.J., Orlando, R., Docampo, R., 2011. Identification of contractile vacuole proteins in *Trypanosoma cruzi*. PLoS One 6, e18013.
van Hellemond, J.J., Neuville, P., Schwarz, R.T., Matthews, K.R., Mottram, J.C., 2000. Isolation of *Trypanosoma brucei* CYC2 andCYC3 cyclin genes by rescue of a yeast G1cyclin mutant. J. Biol. Chem. 275, 8315–8323.
Vazquez, M.P., Levin, M.J., 1999. Functional analysis of the intergenic regions of TcPB gene loci allowed the construction of an improved *Trypanosoma cruzi* expression vector. Gene 239, 217–225.
Verweij, F.J., van Eijndhoven, M.A.J., Hopmans, E.S., Vendrig, T., Wurdinger, T., Cahir-McFarland, E., Kieff, E., Geerts, D., van der Kant, R., Neefjes, J., Middeldorp, J.M., Pegtel, D.M., 2011. LMP1 association with CD63 in endosomes and secretion via exosomes limits constitutive NF-kB activation. EMBO J. 30, 2115–2129.
Wang, G., Kong, H., Sun, Y., Zhang, X., Zhang, W., Altman, N., de Pamphilis, C.W., Ma, H., 2004. Genome-wide analysis of the cyclin family in *Arabidopsis* and comparative phylogenetic analysis of plant cyclin-like proteins. Plant Physiol. 135, 1084–1099.
Weingartner, M., Pelayo, H.R., Binarova, P., Zwerger, K., Melikant, B., de la Torre, C., Heberle-Bors, E., Bogre, L., 2003. A plant cyclin B2 is degraded early in mitosis and its ectopic expression shortens G2-phase and alleviates the DNA-damage checkpoint. J. Cell Sci. 116, 487–498.
Zingales, B., Andrade, S.G., Briones, M.R.S., Campbell, D.A., Chiari, E., Fernandes, O., Guhl, F., Lages-Silva, E., Macedo, A.M., Machado, C.R., Miles, M.A., Romanha, A.J., Sturm, N.R., Tibayrenc, M., Schijman, A.G., 2009. A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. Mem. Inst. Oswaldo Cruz 104, 1051–1054.