ELSEVIER

Molecular & Biochemical Parasitology 121 (2002) 225–232

MOLECULAR & BIOCHEMICAL PARASITOLOGY

www.parasitology-online.com

Evidence for CRK3 participation in the cell division cycle of *Trypanosoma cruzi*

María I. Santori [a], Sebastián Laría [a], Eliana B. Gómez [a,1], Ingrid Espinosa [b], Norbel Galanti [b], María T. Téllez-Iñón [a,*]

[a] *Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI, CONICET) and Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (UBA), Vuelta de Obligado 2490 (1428) Buenos Aires, Argentina*

[b] *Programa de Desarrollo de Biología Molecular y Celular, Instituto de Ciencias Biomédicas (ICBM), Facultad de Medicina, Universidad de Chile, Santiago, Chile*

Received 7 November 2001; accepted in revised form 17 February 2002

Abstract

Trypanosoma cruzi CRK3 gene encodes a Cdc2p related protein kinase (CRK). To establish if it has a role in the regulation of the parasite cell cycle we studied CRK3 expression and activity throughout three life cycle stages. CRK3 from epimastigote soluble extracts interacted with p13[suc1]-beads. Endogenous CRK3 phosphorylated histone H1 and this activity was inhibited by specific CDK inhibitors: Olomoucine, Flavopiridol and Roscovitine. Flavopiridol partially inhibited the growth of *T. cruzi* epimastigotes at 50 nM, the lowest concentration used, but even with the highest (5 μM), cell growth was not completely arrested. CRK3 from Flavopiridol-inhibited epimastigote extracts exhibited a dose dependent inhibition of histone H1 phosphorylation. *T. cruzi* p13[suc1]-binding CRK displayed the same inhibition profile. This suggests that CRK3 is the enzyme responsible for the majority of the kinase activity associated with p13[suc1]. CRK3 activity of hydroxyurea (HU) synchronized epimastigotes peaked in G2/M boundary while the kinase activity associated to p13[suc1]-beads increased at the same time point but remained high until late G2/M. In addition, CRK3 expression was constant during the cell cycle. This is a common pattern of CDK activity regulation. Taken together, these results support the idea that CRK3 is involved in control of the cell cycle in *T. cruzi*. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Trypanosoma cruzi*; Cyclin-dependent kinase; Cell cycle control; Flavopiridol; Hydroxyurea synchronization

1. Introduction

The parasitic protozoan *Trypanosoma cruzi* belongs to the Trypanosomatidae family and is responsible for the clinically important Chagas' disease. Trypanosomes have a complex life cycle that alternates between insect and mammalian hosts with different developmental stages. The rapidly dividing forms such as amastigotes, in the vertebrate host cells, and epimastigotes, in the vector gut, maintain the infection. The non-dividing trypomastigote form, which is probably arrested in G1/G0 phase of the cell cycle, can be found in the vertebrate bloodstream or in the vector hindgut and is preadapted for survival when the parasite passes from one organism to the other [1,2]. The coordination between replication and segregation of the nucleus, kinetoplast and flagellum is another important aspect of *T. cruzi* and other kinetoplastids cell cycle [3]. This parasite undergoes a complex series of morphogenetic changes suggesting that there is an integral link between control of the cell cycle and cell differentiation. Trypanosomes have gained complexity in cell cycle control and it is likely that special molecular mechanisms have evolved to meet special requirements of the parasite.

Abbreviations: CDK, cyclin dependent kinase; CRK, Cdc2p-related kinase; CKI, CDK inhibitor; FP, flavopiridol; HU, hydroxyurea.

* Corresponding author. Tel.: +54-11-4783-2871; fax: +54-11-4786-8578.

E-mail address: mtellez@dna.uba.ar (M.T. Téllez-Iñón).

[1] Present Address: Molecular and Cell Biology Laboratory, The Salk Institute, 10010 N. Torrey Pines Rd, La Jolla, CA 92037, USA.

0166-6851/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0166-6851(02)00039-7

Cyclin dependent kinases (CDKs), such as *Schizosaccharomyces pombe* Cdc2p and *Saccharomyces cerevisiae* CDC28 are essential regulators of the cell cycle and are highly conserved, ubiquitous proteins throughout eukaryotes [4]. Control of cell cycle progression in both budding and fission yeast, is associated with the activation of a single CDK, CDC28 and Cdc2p, respectively. In higher eukaryotes, the regulation of the cell cycle is under the control of many CDKs. CDK levels tend to remain constant during the cell cycle, and their activities are controlled mainly post-translationally by different mechanisms: association of the kinase subunit with the positive regulatory cyclin partner, phosphorylation of conserved residues and binding of CDK inhibitor peptides [5,6]. Over the last years considerable effort has been made to identify compounds that specifically inhibit CDKs (CKIs) to be used as anti-cancer agents. A number of chemical compounds have been identified and many of them appear to inhibit kinase activity by competitive binding to the ATP binding pocket. Some CKIs have remarkable selectivity and can differentiate the human CDK family into two subfamilies: (1) CDK1, CDK2 and CDK5, and (2) CDK4 and CDK6. Three chemical inhibitors, Olomoucine, Roscovitine and Flavopiridol have shown selectivity for CDK1 and CDK2 proteins [7,8]. Flavopiridol (FP), a semi-synthetic flavone, is a potent growth inhibitor of a number of tumor cell lines [9,10]; it blocks mammalian cells in either G1 or G2 [11] and it can also affect S-phase progression in *Plasmodium falciparum* [12]. In addition, it was found that Flavopiridol significantly inhibited glycogen phosphorylase a and b from rabbit skeletal muscle [13].

Investigations into the molecules that regulate the cell cycle of trypanosomatids have led to the identification of many proteins belonging to the Cdc2p related kinase (CRK) family. In *T. cruzi* we have isolated two CRK genes: *TzCRK1* and *TzCRK3* [14,15]. CRK1 homologues have been found in the trypanosomatids *Leishmania mexicana* [16], *Trypanosoma brucei* [17] and *Trypanosoma congolense* (acc.# Z30312). Gene disruption experiments in *L. mexicana* indicated that CRK1 was essential to the promastigote form [18]. CRK1 proteins have been tested for their ability to complement Cdc2p/CDC28 mutations in yeast, but under the assayed conditions none of them could rescue the deficient phenotype [14,16]. *T. cruzi* CRK3 predicted aminoacid sequence [14] has 82, 78 and 77% identity with *T. brucei* [17], *L. mexicana* [19] and *L. major* [20] CRK3, respectively. Genetic manipulation showed that CRK3 is essential to *L. mexicana* promastigotes [21]. There have been reports indicating that CRK3 could be the CDK1 functional homologue in Leishmania [19–22].

In this study we report the characterization of *T. cruzi* CRK3 and show evidence indicating that this enzyme could be the cdk1 homologue in *T. cruzi*.

2. Materials and methods

2.1. Cellular cultures and protein preparations

T. cruzi epimastigotes from Tul 2 strain were cultured as described [23]. Metacyclic trypomatigotes were obtained by axenic culture under differentiating conditions. Amastigotes were obtained from Vero cell cultures as described in [24].

Parasite protein extracts were prepared by resuspending the parasite pellets in SK buffer with proteinase inhibitors (0.25 M sucrose, 5 mM KCl, 0.5 mM *N*-Tosyl-L-lysine chloromethyl ketone, 1 mM benzamidine, 1 mM phenylmethyl-sulphonyl fluoride, 25 U ml^{-1} aprotinine, 10 μg ml^{-1} leupeptin, 2 μg ml^{-1} trypsine 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 in [23]. Protein concentration in every fraction was assessed by Bradford method.

2.2. Raising antiserum against CRK3 protein

To raise a CRK3 antiserum (anti-TzCRK3) the complete ORF of *tzcrk3* was inserted into the pRSET-A (Invitrogen) expression vector in order to generate recombinant CRK3 with a six histidine tag at the N-terminus (His-CRK3). His-CRK3 was expressed in *E. coli* and the purified recombinant protein was used to immunize rabbits. The recombinant protein (His-TzCRK3) was Ni^{2+}-agarose affinity purified from induced culture lysates. One mg of purified fusion protein was mixed with complete Freund's adjuvant, injected into a rabbit which was boosted again 1 month later with 1 mg of purified fusion protein mixed with complete Freund's adjuvant. The rabbit was bled 1 month after the second injection and the purified IgGs were used for Western blot analysis and immunoprecipitation assays. IgGs were purified as described previously in Gómez et al. [15].

2.3. Immunoprecipitations, $p13^{suc1}$-agarose co-precipitations and kinase assays

One hundred micrograms of parasite soluble fractions were preclarified with protein A-agarose (Gibco-BRL) and incubated with anti-CRK3 or anti-CRK1 purified IgGs or with $p13^{suc1}$-agarose beads (Calbiochem). The $p13^{suc1}$-agarose complexes and protein A-agarose immunocomplexes were washed four times with phosphate-buffered saline (PBS) and incubated with kinase 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, 5 μCi

[γ-^{32}P]ATP (3000 Ci mmol^{-1}, NEN) and 0.1 mg ml^{-1} histone H1, Calbiochem), or resuspended in 1× Laemmli's buffer for Western blot analysis. The CRK1 kinase buffer contained 50 μM ATP while CRK3 and p13^{suc1} kinase buffer contained 25 μM ATP. Reactions were performed at 30 °C for 30 min in a total volume of 40 μl and stopped with 5× Laemmli's buffer. Samples were analyzed by 12% SDS-PAGE, stained with Coomassie Blue R-250 or electrotransferred to Hybond C (Amersham) membranes and exposed to autorradiographic films. Signal was scanned with a phosphorimager Storm 820 (Amersham Pharmacia Biotech) and quantitated with ImageQuant software.

2.4. Inhibition assays

Immunoprecipitated CRK3 was incubated with different CDK inhibitors: Flavopiridol, Olomoucine and Roscovitine (Calbiochem). The inhibitors Olomoucine and Roscovitine were dissolved in DMSO as indicated by the manufacturer. Flavopiridol was resuspended to 10 mM in 70% ethanol while serial dilutions were performed in water. Increasing concentrations up to 1 mM for Roscovitine and Olomoucine, and 0.5 mM for Flavopiridol, were tested. Enzyme without the addition of inhibitors was used as positive controls to compare the inhibition effect. Kinase assays were performed according to Section 2.3.

2.5. Northern blot analysis

Total RNA from the different life cycle stages of *T. cruzi* (epimastigote, trypomastigote, and amastigote) was obtained using TRIzol reagent (Gibco BRL) according to the manufacturer's instructions. Total RNA was Northern blotted according to [14]. The BamHI/NotI fragment from the pGEX4T-3/tzcrk3 plasmid, corresponding to the full length gene was used as probe. The probe was radiolabeled with [α-^{32}P]dCTP (10^9 cpm pmol^{-1}, NEN) using Prime-a-Gene Labeling System (Promega). Signal was scanned with a phosphorimager Storm 820 (Amersham Pharmacia Biotech) and quantitated with ImageQuant software.

2.6. Western blot analysis

Nitrocellulose membranes were blocked with 5% powdered milk in Tris-buffered saline containing 0.05% Tween 20 (TBST-M), and incubated for 2 h with anti-TzCRK3 purified IgGs, diluted 1:800 in TBST-M. Blots were washed with TBST and incubated with 1:5000 dilution of anti-rabbit IgG alkaline phosphatase-conjugated (Gibco-BRL). Bands were visualized using BCPIP/NBT as substrate for alkaline phosphatase.

2.7. Synchronized cultures

Epimastigotes from Tulahuen strain were synchronized according to [25]. Briefly, epimastigotes were grown at 28 °C in supplemented Diamond medium, 2.5% fetal bovine serum (FBS). Cells were transferred to fresh medium containing 10% FBS and 20 mM hydroxyurea (HU) for 24 h, since it has been reported that doubling time is about 22 h. Following HU incubation, cells were washed two times in PBS and resuspended in fresh medium containing 10% FBS. Samples were taken at the indicated time points. Cell counts were performed using a Neubauer chamber. DNA synthesis was estimated by measuring [^{3}H] Thymidine incorporation. About 1×10^6 cells ml^{-1} were incubated in 10 μCi of [^{3}H] Thymidine (60 Ci mmol^{-1}, NEN) in 1 ml PBS at 28 °C for 1 h with constant shaking. The incorporation was stopped by washing the cells twice in cold PBS. The remainder of the assay procedure was as described in [26].

3. Results

3.1. Stage specific Northern blot analysis of the CRK3

Total RNA isolated from three life cycle stages of *T. cruzi* was Northern blotted using full length *CRK3* as hybridization probe (Fig. 1). As previously reported for the epimastigote form [14], *CRK3* mRNA was detected as a band ranging between 1.3 and 1.4 kb. An approximate 2-fold increase in *CRK3* mRNA levels was detected in amastigotes in three independent experiments (Fig. 1). The same Northern blot was rehybridized with a ribosomal probe and the loaded RNA was normalized. There are at least two transcripts for the *crk3* gene, with a difference of 21 nt in their 5′ UTR [14], but it was not possible to assess whether the

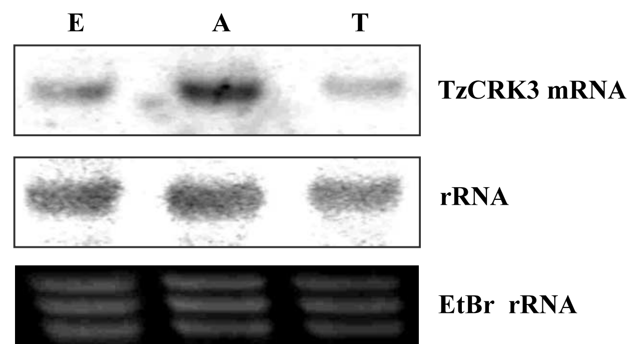

Fig. 1. Northern blot analysis of CRK3 in three stages of the parasite. Fifteen μg total RNA from each form were used; ethidium bromide staining of rRNA and rRNA labeled probe were used as loading controls. Results are representative of three independent experiments. Signal was scanned with a phosphorimager Storm 820 and quantitated with ImageQuant software.

increment of *CRK3* mRNA in amastigotes included one or both of the transcripts.

3.2. CRK3 expression throughout the life cycle of T. cruzi

Western blot analysis was performed with affinity purified His-CRK3 and cell extracts from epimastigote, amastigote and trypomastigote stages of *T. cruzi* (Fig. 2). Purified IgGs from the CRK3 antiserum detected a 37 kDa band in the recombinant protein lane. A protein of the predicted molecular weight, 35 kDa, was detected in the epimastigote soluble (S_{20}) and nuclear-enriched (P_{20}) fractions. Both in soluble and nuclear-enriched fractions of amastigote and trypomastigote extracts, the antiserum revealed at least two proteins of higher molecular weight.

The specificity of the binding was assessed by depleting the antibody with recombinant His-CRK3 (Fig. 2, lane 1). The preimmune serum was used as negative control (not shown).

3.3. CRK3 interacts with yeast p13^{suc1}

Although a small proportion of *S. pombe* Cdc2p kinase is associated in vivo with the constitutively present p13^{suc1}, in vitro these two proteins show very high affinity. Based on this property p13^{suc1} coupled to agarose beads has been used to isolate CDKs from a wide variety of organisms [27–29]. The interaction between yeast p13^{suc1} and CRK3 was analyzed. Epimastigote soluble fractions were incubated with p13^{suc1}-agarose beads and the precipitated proteins were analyzed by Western blot using anti-CRK3 antibodies. Fig. 3 shows that CRK3 coprecipitates with p13^{suc1} beads. Interestingly, CRK3 antibodies failed to recognize the CRK3 protein in the p13^{suc1}-depleted supernatant (Sn). These results suggest that the protein kinase associated to p13^{suc1}-agarose in *T. cruzi* epimastigote extracts described in Gómez et al. [15] is CRK3.

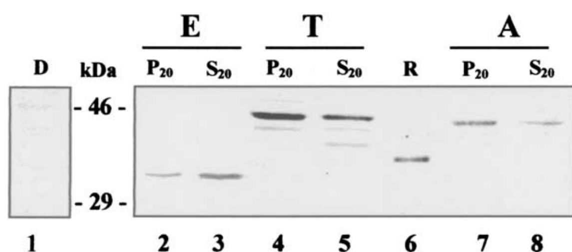

Fig. 2. Western blot analysis. Purified recombinant His-CRK3 (lane 6) and soluble (S_{20}) or nuclear-enriched (P_{20}) extracts from three life cycle stages of *T. cruzi*, epimastigote (lanes 2 and 3), trypomastigote (lanes 4 and 5) or amastigote (lanes 7 and 8) were analyzed as described in Section 2. Equivalent amounts of each cell extract were loaded to the gel. R: recombinant His-TzCRK3. Lane 1: epimastigote whole cell extract, developed with CRK3 antibody pre-incubated with recombinant His-CRK3.

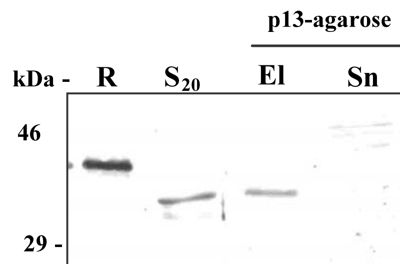

Fig. 3. Endogenous CRK3 co-precipitates with p13^{suc1}. Epimastigote soluble extracts were incubated with p13^{suc1} beads. Co-precipitated proteins were eluted with 1 × loading buffer, analyzed by SDS-PAGE and western blotted as described in Section 2. R: His-TzCRK3, S_{20}: soluble protein extract, El: proteins eluted from the p13^{suc1}-agarose beads, Sn: proteins in the supernatant after p13^{suc1} co-precipitation.

3.4. Endogenous CRK3 activity is sensitive to CDK inhibitors in vitro

In order to investigate the kinase activity of CRK3, the recombinant protein was affinity purified and used in histone H1 phosphorylation assays. His-CRK3 did not have kinase activity under the conditions used (data not shown). This may be due to an incorrect folding of the protein in *E. coli* or to the lack of positively regulator proteins.

The ability of the anti-CRK3 antiserum to immunoprecipitate a soluble CDK-like kinase activity was tested. Purified anti-CRK3 antibodies were used to precipitate CRK3 from epimastigote soluble extracts. Immunocomplexes were incubated with different cold ATP concentrations. Anti-CRK3 antibodies immunoprecipitated a kinase activity that was able to phosphorylate histone H1 (Fig. 4A). CRK3 endogenous activity was assessed in three life cycle stages of the parasite. Immunoprecipitation followed by kinase assays were performed with epimastigote, amastigote and trypomastigote soluble extracts (Fig. 4B). CRK3 kinase activity in epimastigote and trypomastigote showed an approximate 3-fold increase compared with amastigote stage. These results agree with the low levels of CRK3 protein in the soluble extracts of amastigotes (see Fig. 2).

CKIs have been developed to specifically block cell cycle progression. In *T. cruzi* a p13^{suc1}-associated kinase activity was described [15] that is inhibited by CKIs. In the present study the effect of the three inhibitors on CRK3 kinase activity was tested. As shown in Fig. 4C, histone H1 phosphorylation was inhibited by the CKIs Olomoucine (Olom), Roscovitine (Rosco) and Flavopiridol (FP) indicating that, at least in vitro, CRK3 shares biochemical properties with other proteins of the CDK family. The pattern of inhibition observed in vitro for the immunoprecipitated CRK3 is similar to the p13^{suc1}-associated CDK1-related kinase activity reported by Gómez et al. [15]. This was the first evidence in *T. cruzi*

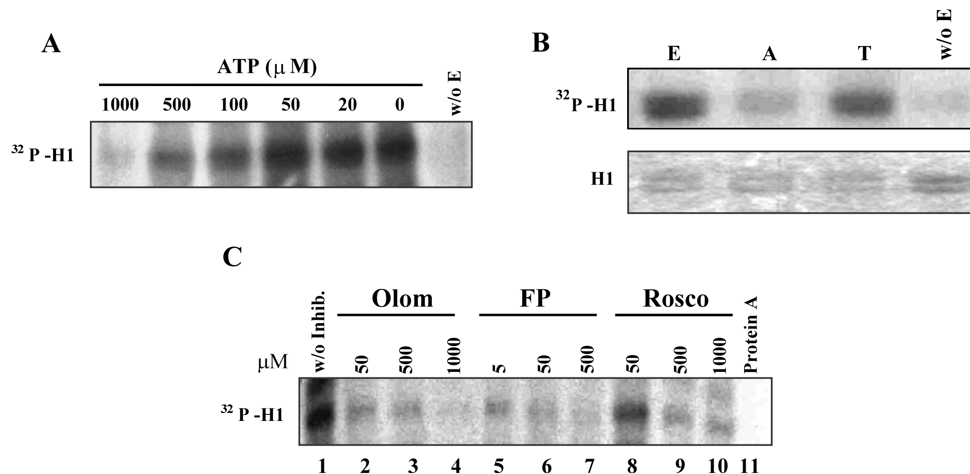

Fig. 4. Endogenous TzCRK3 activity. Soluble protein extracts from epimastigotes were preclarified with protein A-agarose, immunoprecipitated and assayed for H1 kinase activity as described in Section 2. (A) Increasing cold ATP (µM) concentrations were used. 50 µM ATP reaction mix without enzyme (w/o E) was used as histone H1 basal level of ATP incorporation. (B) Kinase activity levels are different in epimastigote, amastigote and trypomastigote forms of *T. cruzi*. Signal was detected with Storm 820 scanner and quantitated with ImageQuant software. (C) CRK3 H1 kinase activity from epimastigotes is inhibited by CKIs (lanes 2–10). Protein A-agarose precipitation of the preclarified extract is shown in lane 11. FP (Flavopiridol), Olom (Olomoucine) and Rosco (Roscovitine). Results are representative of three independent experiments.

of a CRK with similar properties to CDK1 and CDK2 proteins.

3.5. *FP inhibits the growth of T. cruzi epimastigotes*

The effect of FP on *T. cruzi* epimastigote cultures was tested. Parasites were seeded at a density of 0.75×10^6 cells ml^{-1} in the presence of increasing concentrations of the inhibitor ranging from 0 to 5 µM. Samples were taken at 24 h intervals, cell number was determined and mean values were plotted. FP inhibited the growth of epimastigotes in a dose dependent manner. Although the growth of the culture was significantly inhibited it was not completely arrested even at the highest FP tested concentration. Concentrations higher than 5 µM caused cell death. Results presented in Fig. 5A suggest that the cell cycle of epimastigotes is mainly controlled by one or more CDKs that are sensitive to FP.

3.6. *Epimastigote CRK3 kinase activity is inhibited by FP*

Given that CRK3 is inhibited in vitro by FP and that the CKI inhibits cell growth, the activity of CRK3 in those cultures was assessed. CRK3 from soluble epimastigote extracts grown for 48 h with different concentrations of FP was immunoprecipitated and assayed for histone H1 kinase activity. The inhibition profile of CRK3 and the CRK co-precipitated with p13^{suc1} are shown in Fig. 5B. Both enzymatic activities display the same dose-dependent inhibition pattern. Accurate IC_{50} values were impossible to calculate since

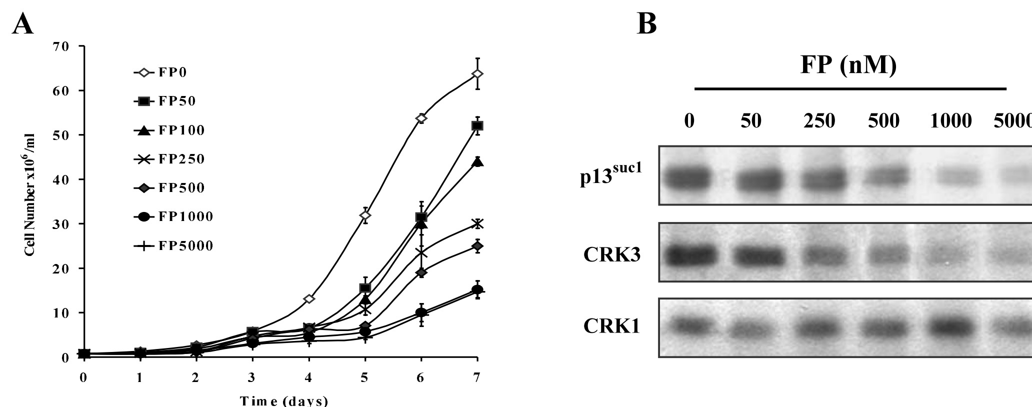

Fig. 5. Flavopiridol prevents the growth of *T. cruzi* epimastigotes, and inhibits endogenous CRK3 and p13^{suc1}-binding activities. (A) Epimastigotes were seeded at a density of 0.75×10^6 cells ml^{-1} and incubated in the presence of FP. Cell density was determined every 24 h. The mean result of quadruplicated values is plotted and S.D. is indicated. (B) Inhibition profile of CRK3, CRK1 and p13^{suc1} binding activities. Immunoprecipitated CRK1 activity was used as positive control. Each condition was assayed in duplicate. Results shown are representative of three independent experiments.

Flavopiridol inhibition is reversible and it is likely that PBS washes off some of the drug. Therefore, apparent IC_{50} values were calculated. CRK3-FP apparent IC_{50} was about 146±14 and 174±19 nM for the p13^{suc1}-associated CRK. Experiments were repeated three times and no significant difference was observed. Since endogenous CRK1 was reported not to be inhibited by FP [15], it was immunoprecipitated from the treated culture and used as a positive control (Fig. 5B).

3.7. *CRK3 expression and activity in HU-synchronized T. cruzi epimastigotes*

CDK activity is controlled post-translationally. CRK1 and CRK3 are both *T. cruzi* putative cell cycle controllers and they are expressed constitutively during epimastigote cell cycle (this work and Gómez EB, unpublished results). In order to study the regulation of CRKs activity throughout *T. cruzi* cell cycle, epimastigotes were HU synchronized. H-U growth arrest is the only approach shown to date to efficiently synchronize *T. cruzi* cells [25,30], thus CRK3 expression during the epimastigote cell cycle was investigated. After removal of the HU, cells were collected at different time points over a 24 h period. Samples were used to isolate total RNA and to obtain protein extracts. As shown in Fig. 6A, upon release from HU, the G1 population synchronously entered into S phase. Peak DNA synthesis occurred at approximately 8 h. By 14 h post-HU release, the incorporation of [^{3}H] Thymidine into DNA decreased as the population synchronously entered G2/M phase; by 24 h synchrony had decayed and the population could not be distinguished from an untreated, asynchronous, exponential-phase culture.

Fig. 6B shows that the majority of the epimastigotes CRK3 activity peaks at the G2/M boundary. Interestingly, CRK1 endogenous activity showed a different pattern, it was high in G1/S and during DNA synthesis, decreasing at the beginning of G2/M. These results suggest that CRK1 and CRK3 are involved in the regulation of different phases of *T. cruzi* cell cycle.

p13^{suc1}-associated kinase activity peaked at the same time as CRK3, remaining active until late G2/M (Fig. 6B). The report of a CDK-like activity that associates with p13^{suc1} suggests that there is at least one protein homologous to the yeast Cdc2p kinase. This result supports the idea that CRK3 is the CRK interacting with p13^{suc1}. Nevertheless, by the methods employed in this study, the presence of another unidentified CRK in the p13^{suc1}-precipitates along with CRK3 can not be ruled out.

Northern and Western blot analyses of CRK3 showed that the gene is expressed at constant levels throughout (Fig. 6C and D).

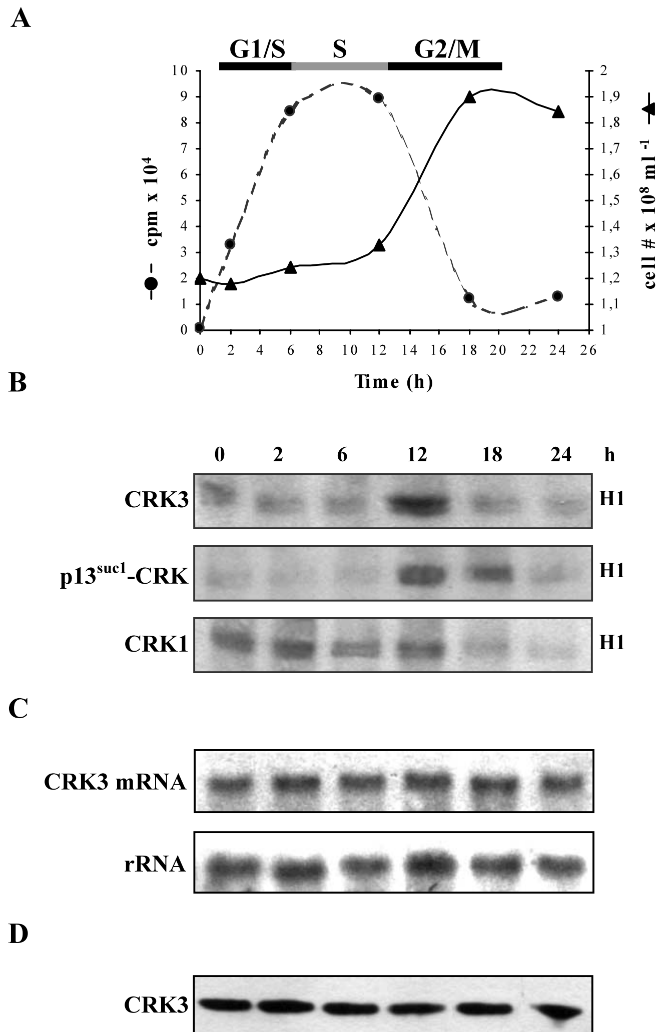

Fig. 6. p13^{suc1}-binding CRK, CRK1 and CRK3 expression and activities during *T. cruzi* epimastigote cell cycle. Cells were synchronized with 20 mM HU as described in Section 2. (A) [^{3}H] Thymidine incorporation and cell number of synchronized culture. (B) CRK kinase activities in HU synchronized parasites. (C) Northern blot analysis of *CRK3* expression during epimastigote cell cycle, rRNA hybridization was used as loading control. (D) Western blot of CRK3 during epimastigote cell cycle. Soluble cell extracts were processed as described in Section 2.

4. Discussion

In this work we report the characterization of *Trypanosoma cruzi* CRK3 and show evidence indicating that this protein kinase could be the CDK1 homologue in this parasite.

Western blot analysis using CRK3 antiserum revealed in epimastigote extracts a band of predicted molecular weight. Since the depleted CRK3 antiserum did not detect any signal we can confirm the specificity of the antibodies. Amastigote and trypomastigote stages showed bands of higher molecular weight (ca. 46 kDa). These bands could be either unidentified CRK proteins sharing epitopes with CRK3 or CRK3 itself, in

a modified state that is resistant to SDS and β-mercaptoethanol. Other works have shown high molecular weight proteins cross-reacting with cell cycle control-related proteins, even in presence of SDS and β-mercaptoethanol [27,22].

Substrates for in vivo CRK phosphorylation in Trypanosomatids have not been described so far. Histone H1 has been widely used as in vitro substrate in CDK and CRK studies [6]. Even though CRK3 from dividing and non dividing forms phosphorylated histone H1, it can be assumed that this enzyme may have different substrate specificities in each stage.

We previously demonstrated that in epimastigotes there is a $p13^{suc1}$-binding CRK, which is not CRK1, that is inhibited by CKIs [15]. Flavopiridol, Olomoucine and Roscovitine inhibited CRK3 activity in epimastigotes. However, Olomuocine appears to inhibit CRK3 more than Roscovitine, which is different from the relative potencies towards Cdc2p [7]. We show here that CRK3 binds to $p13^{suc1}$ in epimastigote soluble extracts. The inhibition pattern of the epimastigote CRK3 endogenous activity by the CKIs mentioned above and the observation of the CRK3 and $p13^{suc1}$ interaction, indicate that this kinase is a reliable candidate to be the *T. cruzi* $p13^{suc1}$-binding CRK. Nevertheless it should be noted that the oligomerization state of the Suc proteins has important implications for function [31–33] and, as in the case of *L. mexicana* [22], in *T. cruzi* the $p13^{suc1}$-binding activity could be composed by more than one kinase.

Since the immunoprecipitated CRK3 activity was inhibited by FP and Olomoucine, the effect of these compounds on the growth of *T. cruzi* epimastigotes was tested. It was previously reported [21] that FP reversibly arrested *L. mexicana* cultures in the G2/M phase of the cell cycle. Olomoucine had no effect on the growth rate of *T. cruzi* epimastigotes. FP had a significant effect on the cell division cycle of the parasite but it did not completely block the growth of the parasites. This observation suggests that the main CDKs involved in *T. cruzi* cell cycle are sensitive to FP. However, at least another protein, not sensitive to the CKI and with overlapping properties, is able to partially rescue the arrested cells. Another explanation to the incomplete block of the parasites growth by FP could be that not enough of the drug, at the concentrations used, managed to enter the cell compartment where CRK3 is to cause complete inhibition of the kinase and complete inhibition of growth. Endogenous CRK3 activity was inhibited in FP treated parasites. The dose dependent profile of this inhibition was the same as the observed for the kinase activity associated to $p13^{suc1}$-agarose beads and the apparent IC_{50} values had no significant differences. These results support the idea that CRK3 could be one of the CDKs interacting with the $p13^{suc1}$ homologous in *T. cruzi* and thus responsible for the majority of the protein kinase activity associated to $p13^{suc1}$ in vitro.

Although hydroxyurea (HU) is not suitable for synchronizing *T. brucei* cells since it was reported to inhibit the parasite cell division due to its cytotoxicity [34,35], it was successfully employed in *T. cruzi* epimastigotes [25,30]. After removal of the HU, cells arrested at the G1/S phase boundary and proceeded synchronously through the cell cycle (Fig. 6A). CRK3 activity peaked at the G2/M boundary, 12 h after HU release, the expected timing of activity for a G2/M cyclin-dependent kinase. Interestingly, this regulation pattern is also observed in higher eukaryotes CDK1 proteins. This suggests that there could be more than one active CRK at G2/M. CRK1 kinase activity was high during G1 and S phases of epimastigotes cell cycle. These results suggest that CRK1 may be controlling G1/S transition and could be the kinase responsible for preventing the re-initiation of DNA replication during S-phase.

The results obtained in this work allow us to postulate that CRK3 shares functional homology with CDK1 and that it has a role in the control of the G2/M transition in *T. cruzi*.

Acknowledgements

We are indebted to Dr. Berta Franke de Cazzullo from the National University of General San Martín, for providing *T. cruzi* amastigotes and trypomastigotes cultures. This work was supported by grants from the National Research Council (CONICET), the World Health Organization Special Program for Research and Training in Tropical Diseases, Ministerio de Salud and the University of Buenos Aires (UBA). M.I. Santori is a Doctoral Fellow of UBA; S. Laría is a Doctoral Fellow of Ministerio de Salud; M.T. Téllez-Iñón is Career Investigator of CONICET, Argentina.

References

[1] De Souza W. Cell biology of *Trypanosoma cruzi*. Int Rev Cytol 1984;86:197–283.

[2] Tyler KM, Engman DM. The life cycle of *Trypanosoma cruzi* revisited. Int J Parasitol 2001;31:472–81.

[3] Turner CMR. Cell cycle co-ordination in Trypanosomes. Parasitol Today 1992;8:3–4.

[4] Morgan DO. Principles of CDK regulation. Nature 1995;374:131–4.

[5] Pines J. Cyclins and cyclin-dependent kinases: a biochemical view. Biochem J 1995;308:697–711.

[6] Pines J, Hunter T. Cyclin-dependent kinases: an embarrassment of riches? In: Hutchison C, Glover DN, editors. Cell cycle control. Oxford: IRL Press, 1995:144–76.

[7] Meijer L. Chemical inhibitors of cyclin-dependent kinases. Trends Cell Biol 1996;3:393–7.

[8] Meijer L, Kim SH. Chemical inhibitors of cyclin-dependent kinases. Methods Enzymol 1997;283:113–28.
[9] Czech J, Hoffman D, Naik R, Sedlacek HH. Antitumoral activity of flavone L86 8275. Int J Oncol 1995;6:31–6.
[10] Carlson BA, Dubay MM, Sausville EA, Brizuela L, Worland PJ. Flavopiridol induces G1 arrest with inhibition of cyclin-dependent kinase CDK2 and CDK4 in human breast carcinoma cells. Cancer Res 1996;56:2973–8.
[11] Worland PJ, Kaur G, Stetler-Stevenson M, Sebers S, Sartor O, Sausville EA. Alteration of the phosphorylation state of $p34^{Cdc2p}$ kinase by the flavone L86-8275 in breast carcinoma cells. Correlation with decreased H1 kinase activity. Biochem Pharmacol 1993;46:1831–40.
[12] Graeser R, Wernli B, Franklin RM, Kappes B. *Plasmodium falciparum* protein kinase 5 and the malarial nuclear division cycles. Mol Biol Parasitol 1996;82:37–49.
[13] Oikonomakos N, Schnier J, Zographos S, Skamnaki V, Tsitsanou K, Johnson L. Flavopiridol inhibits glycogen phosphorylase by binding at the inhibitor site. J Biol Chem 2000;275:34566–73.
[14] Gómez E, Kornblihtt AR, Téllez-Iñón MT. Cloning of a Cdc2p-related protein kinase from the parasite *Trypanosoma cruzi* that interacts with mammalian cyclins. Mol Biol Parasitol 1998;91:337–51.
[15] Gómez EB, Santori MI, Laria S, Engel JC, Swindle J, Eisen H, Szankasi P, Téllez-Iñón MT. Characterization of the *Trypanosoma cruzi* Cdc2p-related protein kinase 1 and identification of three novel associating cyclins. Mol Biochem Parasitol 2001;113:97–108.
[16] Mottram JC, Kinnaird JH, Shiels B, Tait A, Barry D. A novel Cdc2p-related protein kinase from *Leishmania mexicana* LmmCRK1, is post-translationally regulated during the life cycle. J Biol Chem 1993;268:21044–52.
[17] Mottram JC, Smith G. A family of trypanosome Cdc2p-related protein kinases. Gene 1995;162:147–52.
[18] Mottram J, McCready P, Brown K, Grant K. Gene disruption indicate an essential function for the LmmCRK1 Cdc2p-related kinase of *Leishmania mexicana*. Mol Microbiol 1996;22:573–82.
[19] Grant KM, Hassan P, Anderson JS, Mottram JC. The crk3 gene of *Leishmania mexicana* encodes a stage-regulated Cdc2p-related histone H1 kinase that associates with $p12^{cks1}$. J Biol Chem 1998;24:10153–9.
[20] Wang Y, Dimitrov K, Garrity L, Sazer S, Beverley S. Stage-specific activity of the *Leishmania major* CRK3 kinase and functional rescue of a *Schizosaccharomyces pombe* Cdc2p mutant. Mol Biochem Parasitol 1998;96:139–50.
[21] Hassan P, Fergusson D, Grant KM, Mottram JC. The CRK3 protein kinase is essential for cell cycle progression of *Leishmania mexicana*. Mol Biochem Parasitol 2001;113:189–98.
[22] Mottram JC, Grant KM. Leishmania mexicana $p12^{cks1}$, a functional homologue of fission yeast $p13^{suc1}$, associates with a stage-regulated histone H1 kinase. Biochem J 1996;316:833–9.
[23] Gómez ML, Erijman L, Araujo S, Torres HN, Téllez-Inón MT. Protein kinase C in *Trypanosoma cruzi* epimastigote forms: partial purification and characterization. Mol Biochem Parasitol 1989;36:101–8.
[24] Andrews N, Colli W. Adhesion and interiorization of *Trypanosoma cruzi* in mammalian cells. J Protozool 1982;29:264–9.
[25] Galanti N, Dvorak JA, Grenet J, McDaniel JP. Hydroxyurea-induced synchrony of DNA replication in kinetoplastida. Exp Cell Res 1994;214:225–30.
[26] Rojas MV, Galanti N. Relationship between DNA methylation and cell proliferation in *Trypanosoma cruzi*. FEBS Lett 1991;295:31–4.
[27] Brizuela L, Draetta G, Beach D. p13Suc1p acts in the fission yeast cell division cycle as a component of the $p34^{Cdc2p}$ protein kinase. EMBO J 1987;6:3507–14.
[28] Tang L, Pelech L, Berger JD. A Cdc2p-like kinase associated with commitment to division in *Paramecium tetraurelia*. J Euk Microbiol 1994;41:381–7.
[29] Takahashi M, Amin N, Grant P, Pant HC. p13Suc1 associates with a Cdc2p-like kinase in a multimeric cytoskeletal complex in squid axoplasm. J Neurosci 1995;15:6222–9.
[30] Recinos RF, Kirchhoff LV, Donelson JE. Cell cycle expression of histone genes in *Trypanosoma cruzi*. Mol Biochem Parasitol 2001;113:215–22.
[31] Birck C, Raynaud-Messina B, Samama JP. Oligomerization state in solution of the cell cycle regulators p13suc1 from the fission yeast and p9cksphy from the myxomycete Physarum, two members of the cks family. FEBS Lett 1995;363:145–50.
[32] Parge HE, Arvai AS, Murtari DJ, Reed SI, Tainer JA. Human CksHs2 atomic structure: a role for its hexameric assembly in cell cycle control. Science 1993;262:387–95.
[33] Arvai AS, Bourne Y, Hickey MJ, Tainer JA. Crystal structure of the human cell cycle protein CksHs1: single domain fold with similarity to kinase N-lobe domain. J Mol Biol 1995;249:835–42.
[34] Mutomba MC, Wang CC. Effect of aphidicolin and hydroxyurea on the cell cycle and differentiation of *Trypanosoma brucei* bloodstream form. Mol Biochem Parasitol 1996;80:89–102.
[35] Brun R. Hydroxyurea: effect on growth, structure, and [^{3}H]thymidine uptake of *Trypanosoma brucei* procyclic culture forms. J Protozool 1980;27:122–8.