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Molecular Cloning, Characterization and Overexpression of a Novel Cyclin from *Leishmania mexicana*

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Abstract: We are reporting here, the cloning and characterization of the first cyclin from *Leishmania mexicana*. We have identified a cyclin-like motif from the *L. major* genome sequencing project. A cyclin homologue was cloned and sequenced from *L. mexicana* genome and it showed 96.1% amino acid identity with the putative *L. major* cyclin. It has also sequence identity to mitotic cyclins from other organisms. Southern analysis showed that it is present as a single copy gene. *CYCa* has been over-expressed in *E. coli* as a histidine fusion and western blot has confirmed the immunoreactive property of the recombinant cyclin, which then used to reconstitute active recombinant *L. mexicana* CRK3. No phosphorylation of histone HI was detected by both wild type and mutated CRK3 on the activation assays suggesting that phosphorylation status and cyclin binding are important for reconstituting protein kinase activity. The results confirm that we have isolated a cyclin molecule from *L. mexicana* (LmCYCa) which may play an important role in the regulation of the parasite cell cycle.

Key words: *Leishmania mexicana*, cyclin, molecular characterization, cell cycle, cdc2-related kinase

INTRODUCTION

To date, there are 19 human cyclins; 26 cyclins been identified in the budding yeast; *S. cerevisiae* and 9 cyclins in the unicellular parasite *T. brucei* (Doerig *et al*., 2000; Van Hellemond *et al*., 2000; Hammarton *et al*., 2003). Studies on yeast have shown that *S. cerevisiae* cells are viable in the absence of any two, but not three of CLNs 1-3, suggesting that CLN function is redundant (Reed *et al*., 1992). Moreover, cyclins C, D and E can functionally substitute for the absence of CLNs 1-3 in yeast when overexpressed (Reed *et al*., 1992). Further more, the mitotic cyclins, when overexpressed, can substitute for the G1 cyclins in yeast. As a consequence, yeast complementation approach has been established as a tool for identification of novel cyclins from different organisms. Using this approach recently, a novel cyclin has been isolated from the parasite *Toxoplasma gondii* (Kvaal *et al*., 2002). The role that some of these cyclins play in the organism is well known, for example the cyclin B/Cdc2 complex acts as the primary promoter of mitosis. On the other hand, the role of others cyclins is not fully understood, even their CDK-partner has yet to be identified. With the advent of the most recent RNA interference technique (RNAi), the role of some trypanosomatid cyclins has been investigated (Hammarton *et al*., 2003). The activities of the Cdk/cyclin complexes are periodic throughout the cell cycle. This periodicity is achieved by regulation at many levels and is necessary for the proper coordination of cell cycle events (Gould, 2000).

The relative specificity of cyclin localization compared with that of Cdc2 has led to the suggestion that cyclins target Cdks to particular intracellular sites as substrates.

The first putative cyclin to be identified from trypanosome was *TbCYC1* (Affranchino *et al*., 1993). Later work on *TbCYC1* (Hua *et al*., 1997) showed that there is no fluctuation on its expression during the cell cycle. Another work on TbCYC1 (Hammarton *et al*., 2000) showed its inability to complement a budding yeast G1 cyclin mutant and to bind neither the fission yeast CDK binding protein $p13^{suc1}$ nor the leishmanial homologue $p12^{cks1}$. Moreover same workers have shown a 22 amino acid frameshift in the cyclin box of the previously published CYC1. Therefore it is concluded that TbCYC1 is not a mitotic cyclin. Two more cyclins have been isolated from *T. brucei* (Van Hellemond *et al*., 2000) CYC2

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and CYC3 both are identified by rescue of the *Saccharomyces cerevisiae* mutant DL1, which is deficient in CLN G1 cyclin function. In addition, the CYC3 is reported with a low level of sequence identity to mitotic B-type cyclins from a variety of organisms; since it contains a destruction box-like motif within its C-terminus. However, the CYC2 has sequence identity to the *Neurospora crassa* PREG1 and the *S. cerevisiae* PHO80 cyclin. More over, both CYC2 and CYC3 are present as single copy genes in the *T. brucei* genome and expressed in all life cycle stages (Van Hellemond *et al*., 2000). Further more, immuno-precipitation and yeast two-hybrid system have demonstrated the ability of CYC2 to interact with CRK3 from *T. brucei* (Van Hellemond *et al*., 2000).

The *in vivo* overexpression of the trypanosomatid cyclins in the live trypanosome has been investigated. For instance, the expression of the tagged-cyclin in the trypanosome has no effect on either the morphology or the growth as compared to the native over-expressed cyclin (Van Hellemond and Mottram, 2000). The degradation of mitotic B-type cyclins is dependent on the presence of the destruction box (King *et al*., 1996). The destruction box motif is required for the formation of cyclin-ubiquitin conjugates, which are subsequently targeted to and degraded by the proteasome (King *et al*., 1996).

The *L. major* genome sequences are now serving as a basis for examining and comparing specific functional regions of other *Leishmania* species. Exploring the sequences of the cosmid LM25. Contig98 (LmFlchr25) by blasting the *L. major* genome with mammalian cyclin sequences, using the VectorNTI software, has revealed the presence of two Open Reading Frames (ORFs). In addition, a blast search of the GenBank/EMBL database with (752 d2) CDS from *L. major* Contig LM.25 revealed many sequences with significant alignment to all genes that encode G2/mitotic-specific cyclins or G1/S-specific cyclins from different organisms. In the light of these results, we proposed that (752 d2) CDS is a leishmanial cyclin. Therefore, we set our goal to isolate and characterize this cyclin genetically and biochemically from *L. mexicana*. We also wish for to study the possible *in vitro* activation of recombinant *L. mexicana* CRK3 by this cyclin.

MATERIALS and METHODS

Study area: The study was carried out at the Wellcome Centre for Molecular Parasitology, Anderson College and the period of the study was from May 1999 to March 2002.

Cloning of *L. mexicana CYCa* coding sequence: Genomic DNA was prepared from *L. mexicana* as previously described (Ali *et al*., 2003). Two oligonucleotide primers for the PCR reaction were designed. One PCR primer OL813 includes a *Nde*I restriction site at 5′ end and a priming sequence (752-770 bp) of the LM24. Contig98, which is homologous to the 5′ end of the DNA corresponding to the N-terminus of the putative *L. major* cyclin motif that serves as the forward primer. The second primer OL814 contains an *Xho*I restriction site and a priming sequence (1657-1675 bp) of the LM24.Contig98 which is homologous to the 3′ end of the DNA corresponding to the C-terminus of the putative *L. major* cyclin motif that serves as the reverse primer. Twenty five cycles of PCR were performed with the following parameters: 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. The final extension reaction was increased to 7 min to ensure maximal full-length product. The PCR product of expected size (about 926 bp) was purified and subsequently sub-cloned into pCR-Script Vector (Stratagene) to give plasmid pGL612.

Sequence analysis of *LmmCYCa* gene: Two recombinant clones, one from each separate PCR reaction, were selected for sequencing. Plasmid isolations from *E. coli* were performed using Qiaprep columns (Qiagen) and sequencing was done using different primers by the dideoxy chain termination method, using an ABI automatic sequencer. The commercially available T_3 and T_7 sequencing primers were used together with other sequencing primers based on the *LmmCYCa* sequence. The obtained sequences were assembled and aligned using the Vector NTI software; the Contig Express and AlignX programs, respectively.

Over-expression of *L. mexicana* CYCa in *E. coli* cells: The cloned *L. mexicana CYCa* fragment was obtained from the construct pGL612. This fragment was then sub-cloned in frame with the histidine-tag in the pET21-a expression vector (Invitrogen) to generate the plasmid pGL630, in which the leishmanial cyclin ORF is tagged by a six-histidine residue from the C-terminus end. This was done in order to over-express the *L. mexicana* cyclin under the control of the T_7 promoter. The resulting pGL630 construct was used to transform the BL-21 (DE3) strain of the *E. coli*. Expression of the recombinant protein was induced as previously described, with some conditions having been changed. Induction was performed in LB medium containing ampicillin, by adding isopropyl β-D-thiogalactoside (IPTG; Sigma) at a final concentration of 4 mM to culture with an OD_{600} of 0.6,

followed by further 4 h of incubation at 30°C and then overnight at 19°C. The induced samples were analyzed by SDS-polyacrylamide gel electrophoresis and stained with 0.25% Coomassie Blue. Finally, expression of the *L. mexicana* cyclin in the bacterial cells was checked by western blot analysis using the Mono-clonal anti-$(his)_4$ anti-bodies (Qiagen).

Kinase assay of the *L. mexicana* CRK3his incubated with the *L. mexicana* CYCahis: The constructs CRK3his (pGL448), CRK3hisE178 (pGL665) and CYCahis (pGL630) were separately purified. CRK3his was purified by FPLC chromatography (BioCAD). While, CYCahis was partially purified by chromatography over Ni^{2+}-NTA-resin (Qiagen Purification kit). Purified CRK3 was combined with the purified CYChis (equal volumes), alternatively, both lysates were, incubated at 4°C for 4 h, purified on Ni-NTA agarose beads and then the eluates, together with the bound beads were subjected to activation assay. Phosphorylation reactions were performed in a total volume of 20 μL containing KAB. Six different recombinant CRK3his and recombinant CYCahis amounts were used that ranged from 1 to 10 μL for each reaction. Reactions were initiated by the addition of 1 μL (10 μCi) of [γ-^{32}P] ATP diluted in KAB buffer (1M MOPS (pH7.2), 500 mM EGTA, 1 M $MgCl_2$, 500 mM DTT) and incubated at 30°C for 20 min. Reactions were terminated by adding 20 μL Laemmli SDS-PAGE loading buffer (2x) and heating to 100°C for 5 min. Incorporation of [γ-^{32}P] ATP into the substrate histone H1 was then quantified by separation of samples on 12.5% Laemmli gels and exposing dried gels to autorad film.

Southern blot analysis of *L. mexicana CYCa*: Genomic DNA was prepared from 10×10^8 promastigotes. Cells were collected by centrifugation and incubated in 4% SDS at 65°C for 30 min in SE buffer (100 mM EDTA pH 8.0, 300 mM NaCl, 10 mM Tris HCl pH 7.5). A volume of 25 μL (20 mg mL^{-1}) proteinase K were added and incubated at 37°C for another 30 min. DNA was extracted using the phenol-chloroform extraction method. Five micrograms of genomic DNA was digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Nco*I, *Sal*I and *Xho*I (New England Biolabs). The digested samples were resolved on a 0.8% TAE agarose gel. After electrophoresis, DNA was denatured in 0.05 N NaOH, neutralized with 0.5 M Tris HCl, transferred onto Hybond-N nylon membrane (Amersham Life Science) by capillary action through a 20 x Standard Saline Citrate (SSC) solution and UV cross-linked using a UV cross-linker (Stratagene).

Before hybridization, nylon membrane was blocked during 4-5 h at 65°C in solution (Church Gilberts) (0.34 mM Na_2HPO_4, 0.16 mM $NaH_2PO_4.H_2O$, 7% (w/v) SDS, 1 mM EDTA (pH 8.0). Probe was radiolabeled with the random priming DNA labeling method (Stratagene), using [α-^{32}P]-dCTP (Amersham Pharmacia Biotech). Hybridization was carried out overnight at 65°C, in blocking solution containing 4 μCi mL^{-1} of denatured probe. After hybridization, membrane was washed twice with 2 x SSC, 0.1% SDS at 65°C and autoradiographed.

RESULTS

Cloning and isolation of a new cyclin gene from *L. mexicana*: A putative *L. major* cyclin was identified by blasting the *L. major* genome sequence with the mammalian cyclin sequences. Consequently, the cyclin homologue was amplified by PCR from the *L. mexicana* genomic DNA and the cloned PCR was sequenced. The *LmmCYCa* coding region is 50.7% GC, which is consistent with the average GC content of 56% found in *L. mexicana* genes (Swindle and Tait, 1996).

High identity was observed with the mitotic cyclins from a variety of organisms. Moreover, a single Open Reading Frame (ORF) was obtained that encoding a predicted protein of 309 amino acid with an estimated molecular mass of 35.28 kDa. The analysis of the novel cyclin protein sequence revealed that it has an isoelectric point (Ip) of 6.28. The amino acid composition is rich in hydrophobic residues that constitute 40%. It is suggested that Leucine might contributed largely to this hydrophobicity of the cyclin protein, since it presents as 11%.

BLAST searches (Altschul *et al*., 1997) with the *L. mexicana* CYCa identified cyclin family members budding yeast, fission yeast, *T. brucei*, *T. cruzi* and humans. To define conserved features of the so-far identified mitotic cyclins, amino acid sequences from 18 cyclins were aligned and 16 conserved amino acids were identified. *LmmCYCa* cyclin contains all of the 16 conserved amino acids (not shown) and 56 of the 109 semi-conserved amino acids (shown by dots in Fig. 1. Obviously the *Leishmania* contains a cyclin box, but it is not conserved enough with other species to be identifiable.

The sequence comparison has shown that the *L. mexicana* cyclin as well as the putative *L. major* cyclin has some features not present in other cyclins. For example, there is seven randomly distributed dipeptides of the phenylalanine/leucine residues (FL) in the cyclin-box region (Two in TbCYC6 and one in Hs cyclin A). In addition, this FL dipeptide is not only restricted to the cyclin-box region of TbCYC6, but it also present in the C-terminus region (Fig. 1). Given that both phenylalanine

Fig. 1: Sequence alignment of *L. mexicana* CYCa with those of the putative *L. major* CYC, *T. brucei* CYC6, human cyclins A and B. Multiple alignment of cyclin family sequences, performed by AlignX™ program from the Vector NTI package software. Shaded black and grey residues represent identities among these cyclins. Asterisks represent the serine phosphorylation sites in the human and *Xenopus* cyclin B1. Dashes represent gaps in sequences. Hs, *Homo sapiens*; Tb, *Trypanosoma brucei*; Lmaj, *Leishmania major*. GenBank™ accession numbers as follows: 1 NP001228; 2, XP043879. TbCYC6 (AJ496539.2). The cyclin-box (CDK-binding domain) is underlined. The FL dipeptides are shown in red

and Leucine are hydrophobic residues, therefore, the cyclin-box is the most hydrophobic part of the cyclin; this may suggest a special requirement for the binding of the PSTAIRE-box of the CRK.

The predicted amino acid sequence shares the highest similarity with the putative *L. major* cyclin (LmajCYC) with 98.1% similarities and 96.1% identities through the entire coding sequence. A homology has also been shown to the *T. brucei* CYC6 (Hammerton, unpublished) with 23% identities through the entire coding sequence and 31.7% in the region 47-225 amino acids. The later region is the cyclin-box, the domain that thought to be involved in the CDK binding (Pines, 1995). However, from the sequence of the newly identified leishmanial cyclin, the central region (Under-lined sequence in Fig. 1 seems to contain all parts that are highly conserved in the mitotic cyclins subfamily. In addition, the LmmCYCa has significant similarities with human cyclins A (40.1%) and B1 (35%) in the cyclin-box region. Moreover, LmmCYCa shows 43 of the conserved residues of the mitotic cyclins, most of them in the cyclin-box region and few in the C-terminus regions (Black- highlighted residues in Fig. 1.

Comparison of the LmmCYCa with human cyclins A and B1 together with the *T. brucei* CYC6 (Fig. 2), have revealed that no N-terminus extension is present in the leishmanial cyclin; this feature is also shared by the *T. brucei* CYC6. The human cyclins A and B1 also have 50-amino acid insertion near the N-terminus this is absent from the LmmCYCa. Interestingly, all of the phosphorylation sites that shown to be important for the nuclear translocation of the human as well as *Xenopus* (Li *et al*., 1997, Winters *et al*., 2001) cyclin B are present in this insertion. This observation raises the possibility that leishmanial cyclins might have a different mechanism that regulates their spatial as well as temporal existence during the cell cycle.

T. brucei CYC6 is the only trypanosomal cyclin out of the eight identified to date that shows significant homology to LmmCYCa, however, some differences were observed. For example there are two insertions in the cyclin-box domain. One insertion is short, while the second is Q-rich 60-amino acids region that forms of repeats of glutamine residues. In addition, a C-terminus extension is present in *T. brucei* CYC6 and absent from the *L. mexicana* CYCa.

The evolutionary rates of the yeast, trypanosome, *Leishmania* and human cyclin family members are expected to be different. Therefore, when compared with other cyclin superfamily proteins, using the Neighbor Joining method (NJ), *LmmCYCa* diverges within the mitotic cyclin family class A and B, providing a

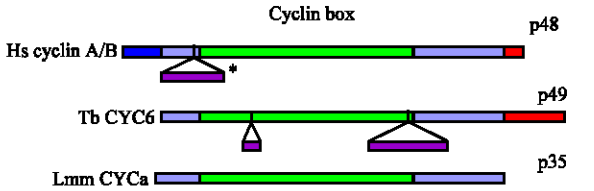

Fig. 2: A schematic representation showing the domains characteristic of the mitotic cyclins of trypanosomatids. The green colored region represents the cyclin-box. The blue is the N-terminus region, while the red colored is the C-terminus region. The insertions are illustrated in pink colour

reasonable degree of confidence for identifying *LmmCYCa* as a member of the mitotic cyclin family (Fig. 3). Interestingly, *LmmCYCa* apparently diverges before human B cyclins family branching into the B1 and B2 subfamilies.

Genomic organization of *L. mexicana CYCa*: To determine the copy number of *LmmCYCa* gene, southern blot analysis of *L. mexicana* genomic DNA was performed. A single hybridizing fragment under high stringency conditions was detected with digests using the enzymes *Bgl*II, *Eco*RI, *Hin*dIII, *Nco*I and *Xho*I (which do not cut inside the *LmmCYCa* coding region). A 6.0 kb *Bgl*II fragment, a 1.2 kb *Eco*RI fragment, a 1.8 kb *Hin*dIII, a 4.0 kb *Nco*I and a 2.7 kb *Xho*I fragment were hybridized to the labelled probe. However, two hybridized bands, a 3.5 and 1.2 kb fragments of *Sal*I were shown, consistent with the presence of *Sal*I site within the ORF of the *LmmCYCa*. No further hybridizing bands appeared after longer exposures with high stringently washed membranes. Taken together, this southern blot analysis revealed single bands, suggesting that *LmmCYCa* is present as a single copy gene in the *L. mexicana* genome (Fig. 4). This pattern resembles other characterized trypanosome cyclin genes, reinforcing the hypothesis of preferential organization of cyclins in trypanosomatid as single copy genes.

Over-expression and production of recombinant *L. mexicana* CYCa: Recombinant cyclin for preparative purification was obtained by large-scale cultivation of pGL630-containing cells. As shown in Fig. 5 all samples were resuspended in Laemmli buffer, boiled for 5 min and were then subjected to SDS-PAGE to estimate the size of the recombinant protein in the total cell protein extract. In the induced cells, protein band with a molecular mass of approx. 35 kDa was detected, which was consistent with the predicted size of his-tagged cyclin.

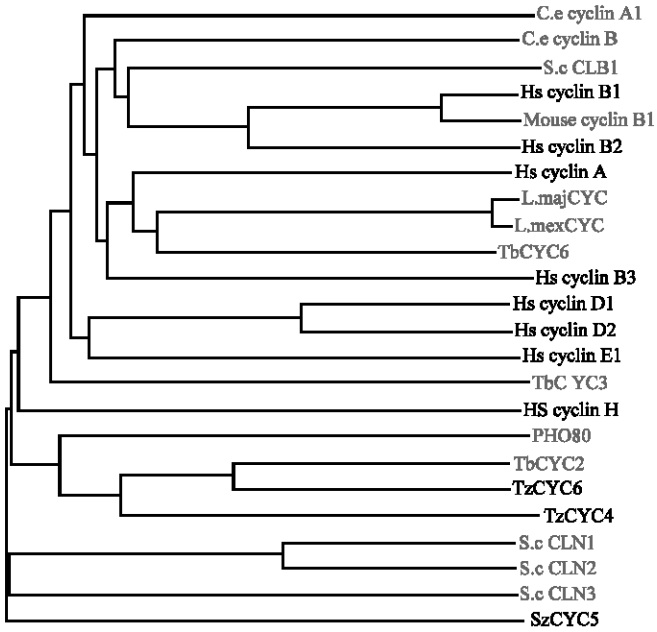

Fig. 3: Phylogenetic analysis of *L. mexicana* cyclin with *T. brucei*, yeast and human cyclins using AlignX™ program of the VectorNTI suite. Species abbreviations are as follows: Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*; Tb, *T. brucei*; Tz, *T. cruzi*, Lmm, *Leishmania mexicana*; Lmaj, *Leishmania major*; C.e, *Caenorhabditis.. elegans*. Accession numbers as follows: H.s cyclin A, (Accession No. NP001228), H.s cyclin B1, (Accession No. XP043879), H.s cyclin B2, (Accession No. AAD09309), H.s cyclin D1, (Accession No. XP029777), H.s cyclin D2, (Accession No. X68452), H.s cyclin E1, (Accession No. BC035498), H.s cyclin H, (Accession No. AF477979), C.e cyclin A1 (Accession No. P34638), C.e cyclin B, (Accession No.AAA84394), Mouse cyclin B1, (Accession No. XM193189) CLB1 (Accession No. M65069), CLN1 (Accession No. M33264), CLN2 (Accession No. M33265), CLN3 (Accession No. S14054), PHO80 (Accession No. X07464), TzCYC4, (Accession No. AF237588) TzCYC5, (Accession No. AF237589) TzCYC6 (Accession No. AF237587), TbCYC2 (Accession No. AJ242519), TbCYC3 (Accession No. AJ242520), TbCYC6 (Accession No. AJ496539.2)

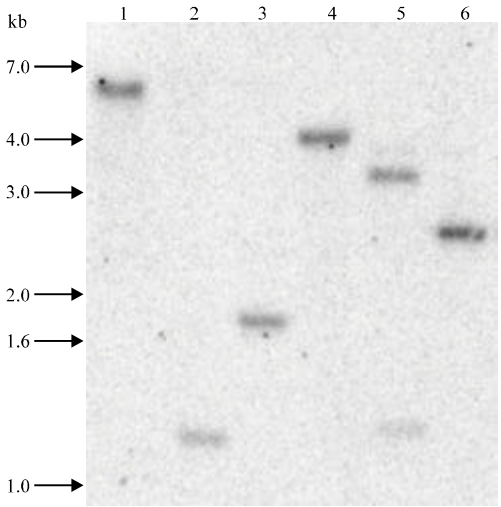

Fig. 4: Southern blot analysis of *L. mexicana* genomic DNA, probed with the *LmmCYCa* fragment under high stringency conditions. 5 μg of *L. mexicana* (MNYC/BZ/62/M379) genomic DNA was digested with *Bgl*II (lane 1), *Eco*RI (lane 2), *Hin*dIII (lane 3), *Nco*I (lane 4), *Sal*I (lane 5) and *Xho*I (lane 6), electrophoresed through a 0.8% agarose gel, blotted to a nylon membrane and probed with the 936 bp *Nde*I/*Xho*I fragment of pGL612

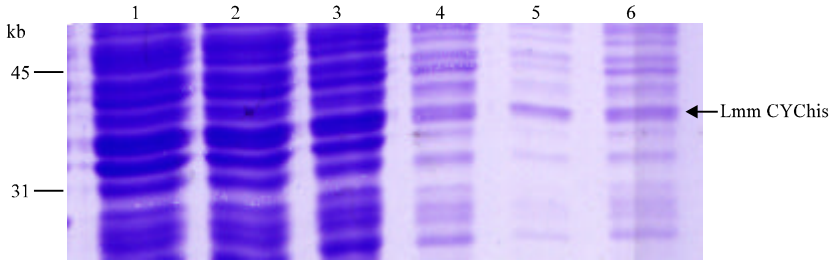

Fig. 5: SDS-PAGE Localization of the recombinant *L. mexicana* CYChis. Cells of the strain BL-21 (DE3) [pET-21a, pGL630], either non-induced (Lane 1 and 4) or IPTG-induced (Lanes 2, 3, 5 and 6) were extracted and subjected to SDS-PAGE (12.5%). Pellets preparations were loaded on lanes 1, 2 and 3, while supernatants preparations were loaded on lanes 4, 5 and 6. The molecular mass marker (bio-Rad) was indicated. The proteins in lanes 1-6 were stained with Coomassie Blue. The 35 kDa band of the *L. mexicana* histidine-tagged CYCa was detected on the supernatant preparation, upon induction overnight at 19°C with both 1 mM (lanes 2 and 5) and 5 mM (lanes 3 and 6) IPTG

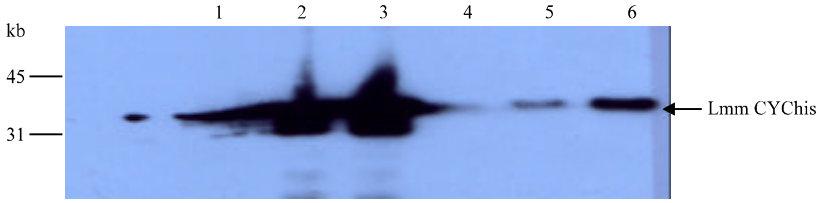

Fig. 6: Western blot analysis of expression of *L. mexicana* CYCahis in BL-21 (DE3) bacterial cells. Lysates of induced bacterial cells; pellet preparations (lanes 2 and 3), supernatant preparations (lanes 5 and 6) and non-induced BL-21 cells (lanes 1 and 4) were resolved by SDS-PAGE on a 12.5% gel: non-induced cells (lane 1), transformed cells induced at 19°C with 1 mM IPTG (lanes 2 and 5) and transformed cells induced at 19°C with 5 mM IPTG (lanes 3 and 6). All were probed with anti-$(His)_4$ anti-bodies. ECL detection was carried out after incubation with horseradish peroxidase-linked anti-rabbit IgG

In order to examine the type and nature of the newly identified *L. mexicana* cyclin in *E. coli* cell extract, we used a commercial available anti-$(his)_4$ anti-body that recognize the histidine-tagged protein with high specificity. No anti-body specific for CYC protein was available at that time. Previous studies showed that Trypanosomatid cyclins are present at low levels in any life cycle stage due to their turnover mechanism (Van Hellemond and Mottram, 2000; Van Hellemond *et al*., 2000). As shown in Fig. 6, the immunoblot using anti-$(his)_4$ anti-bodies detected a single protein band. In addition, the size of the detected signals is consistent with the size of the recombinant LmmCYCahis that was analysed by the SDS-PAGE. However, this signal was not detected in the non-induced cells

Reconstitution of the recombinant *L. mexicana* CRK3his kinase complex through incubation with recombinant *L. mexicana* cyclin: CYC-CRK kinase assay: Evidence is accumulating that cyclin-dependent kinase 2 (Cdk2) and its homolog Cdc2, requires cyclin binding and phosphorylation of Thr-160 for activation *in vivo* (Gu *et al*., 1992; Brown *et al*., 1999). However, less information about the mechanism of the activation process is available. Particularly, no information is available for the natural cyclin partner(s) of the *L. mexicana* CRK3 and of its mechanism of activation. In this study, novel bacterial expression and purification systems for LmmCRK3 (Ali *et al*., 2003) and LmmCYCa were developed that allow mechanistic studies of the activation process to be performed in the absence of cell extracts.

To elucidate the possibility that *L. mexicana* CYCa cyclin is an activator of a leishmanial CRK, we decided to test its interaction with the CRK3 protein kinase. From the finding that the *L. mexicana* CRK3 is active at the G2/M phase of the *Leishmania* cell cycle (Hassan *et al*., 2001) together with the presence of significant homology of LmmCYCa to mitotic cyclins, we decided to test the interaction of these two proteins.

To assay for the kinase activity of the LmmCRK3his, pure and partially pure recombinants LmmCRK3his and

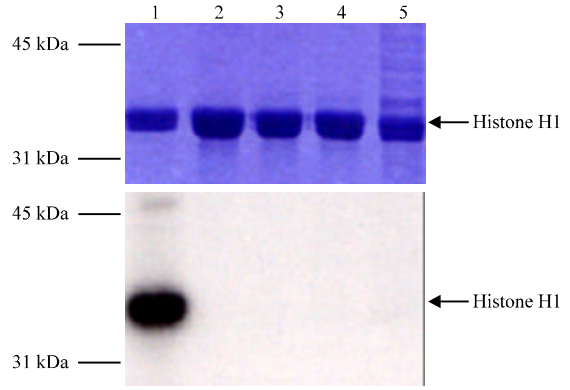

Fig. 7: Reconstitution of the recombinant *L. mexicana* CRK3his kinase activity through incubation with recombinant *L. mexicana* cyclin: Ni-NTA purified wild type LmmCRK3his (lane 2), mutated LmmCRK3hisE178 (lane 4), combinations of purified wild type LmmCRK3his and purified LmmCYCahis (lane 3), combinations of purified mutated LmmCRK3hisE178 and partially purified LmmCYCahis (lane 5), were assayed for kinase activity. Preparations were used each in a separate kinase assay containing 5 μL (lanes 2-5) of the recombinant protein, while 5 μL of Cdc2/cyclin B was used in the control reaction (lane 1), together with 50 μg histone H1, 10 μCi [γ-^{32}P] ATP (Amersham). Radiolabeled proteins were analyzed by SDS-PAGE (12.5% gel) (Top panel) and visualized by autoradiography (Bottom panel). The gel was exposed to X-ray film at room temperature for 3 days and quantified on phospho-imager (Typhoon)

LmmCYCahis, respectively were obtained by small-scale purification on Ni-NTA agarose beads and were incubated as shown in Fig. 7, only one signal was detected by p34Cdc2/cyclin B, but none of the other reactions.

DISCUSSION

A novel cyclin from *L. mexicana* was isolated its gene product is highly homologous to the mitotic cyclins, particularly, A-type ones, from several organisms, mainly in the cyclin-box region. In spite of the high homology to cyclin B-type from yeast and mammals, the absence of the destruction-box is clearly indicated. This observation suggests that LmmCYCa leishmanial cyclin may be directed for degradation by ubiquitination pathway different from that known for others cyclin A/B family. This is in consistence with the fact that trypanosomatids have mechanisms regulating their cell cycle differ from that of other organisms (Doerig *et al*., 2000; Hammarton *et al*., 2003; Banerjee *et al*., 2006)

L. mexicana cyclin is more similar in sequence to the mitotic cyclins A/B and CLBs than to the CLNs. Although of its similarity to mitotic cyclins, *L. mexicana* cyclin may act elsewhere in the cell cycle. Indeed, mere sequence similarity may not be enough to define the point of action of a cyclin in the cell cycle. This speculation is supported by previously finding (Hunter and Pines, 1991), when it showed that the *S. pombe cig1*$^{+}$ gene resembles the mitotic B-type cyclins in sequence but may act in G1. This confirmed later (Martín-Castellanos *et al*., 2000) as fission yeast cells lacking cig1 and cig2 had a cell cycle distribution similar to that of wild-type cells, with a short G1 and a long G2. The findings of the sequence homology and the phylogenetic branching, suggest that *LmmCYCa* is probably an ancestral mitotic cyclin family member, related to the mammalian class A or B as shown in (Fig. 3).

The Southern blot analysis showed that *LmmCYCa* is present as a single coy gene in the *L. mexicana* genome. There are several lines of evidence that most of the so far identified trypanosome cyclins are present as a single copy. *CYC2* and *CYC3* are present as single-copy genes in the genome of the African Trypanosome (Van Hellemond *et al*., 2000), *T. brucei CYC6* is also present as single copy (Hammarton *et al*., 2003), a similar genomic organization to that of the *LmmCYCa*, which can add more to the degree of similarity between these two cyclins in addition to their sequence homology. CYC6 has shown to have a direct role in the cell cycle of the African trypanosome (Hammarton *et al*., 2003), a similar role for the LmmCYCa could possibly be present in the parasite *Leishmania* in the light of structure/function relationship.

Cell cycle research in trypanosomatids has contributed to the successful approach of *in vitro* activation of CDKs. Transfection of mammalian COS-7 cells with TzCRK1 has proved that a trypanosome CRK protein can bind mammalian cyclins E, D3 and A (Gomez *et al*., 1998). Yeast two hybrid interaction studies and co-immune-precipitation experiments have demonstrated that CYC2 could bind to CRK3 in *T. brucei* (Van Hellemond *et al*., 2000). These findings have proved that similar complexes exist in the unicellular organism that could possibly be responsible for the regulation of the cell cycle in a similar way to multi-cellular organisms. In this study we tested the possibility that the newly identified CYCa is the activating partner for CRK3 i.e., an attempt to *in vitro* activate the bacterial produced CRK3his with the CYCahis was performed. Although, a very faint signal was observed but it is difficult to declare that the histone H1 was phosphorylated by an active CRK3his /CYCa

complex. However, as the phosphorylation status might be responsible for this inactivity, a mutated CRK3his was used in parallel, to check for this possibility. All conditions gave negative results. The cyclin has not been purified optimally and the whole experimental conditions need to be optimized before conclusions are drawn on the possibility of reconstituting CRK3his kinase activity. Our very recent study (Gomes *et al*., 2010) has shown that CRK3: CYCA complex is active in the absence of T-loop phosphorylation. A recent study in *L. donovani* (Banerjee *et al*., 2006) has shown that *Leishmania donovani* cyclin 1 (LdCyc1) forms a complex with cell cycle kinase subunit CRK3 (LdCRK3) and is possibly involved in S-phase-related activities.

Taken together, the high sequence and structural homology to the mammalian mitotic cyclins, these results raise the possibility that the newly identified leishmanial cyclin has a role in regulating the cell cycle in the *Leishmania* and itself may be regulated by a mechanism different from that of the unicellular yeast, but more similar to multicellular organisms, possibly via the ubiquitination pathway.

In conclusion, initial experiments have shown no activity for the recombinant histidine-tagged *L. mexicana* CRK3. This may be due to insufficient trials, thus more optimization is needed in order to draw a conclusion on whether the novel mitotic leishmanial cyclin (LmmCYCa) is the regulatory partner for the G2/M phase kinase (LmmCRK3) or not.

Finally, all these results indicate that *L. mexicana* might have a class of cyclins that control the activity of the CRK proteins and that a complex mechanism would exist in order to regulate the kinases involved in the cell cycle and the differentiation processes of the parasite.

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