

Full Length Research Paper

Isolation and characterization of a cdc2-related protein kinase 3 (CRK3) from a Sudanese strain of *Leishmania donovani*

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Cyclin-dependent kinases; (cdc2) are key regulators of the eukaryotic cell cycle. A number of cdc2-related kinase (CRK) genes have been isolated from trypanosomatids. The present study was designed to identify and analyze (CRK3) from a Sudanese strain of *Leishmania donovani*, the causative agent for Kala-azar. CRK3. Sequence analysis showed that *L. donovani* CRK3 encodes a protein of 311 amino acids with 99.7%; 99.4% and 49.4% identity with *L. mexicana* CRK3; *L. major* CRK3 and human HsCDC2, respectively. Genetic analysis by southern blot hybridisation of the genomic DNA has demonstrated that *LdCRK3* gene is not tandemly repeated and is present as a single copy in the *L. donovani* genome. Phylogenetic analysis showed that all of the three leishmanial CRK3s proteins exist with the trypanosome CRK3s in the same clade. CRK3 has potential as drug target, so the scientific data presented in this paper could be useful to explore the possibility of designing a drug that can be used in a low-cost manner to treat all forms of leishmaniasis based on the fact that CRK3 is highly conserved in *Leishmania* spp.

Keywords: *Leishmania donovani*, CRK3, protein kinase, molecular cloning, Sudan.

INTRODUCTION

Leishmania are parasitic protozoa that have a complex life cycle, during which the parasite shuttles between rapidly dividing stages (promastigotes and amastigotes) and the cell cycle arrested metacyclic stage. *Leishmania donovani* is the prototype microorganism responsible for visceral leishmaniasis (VL) in tropical and sub-tropical regions of the world including the Sudan (Zijlstra and el-Hassan, 2001). Genetic studies have shown the relationship between various genes of both *L. donovani* (causative agent of VL) and *L. major* (causative agent of cutaneous leishmaniasis [CL]). For instance, analysis of

sequence variations in the cytochrome oxidase II gene in an assortment of *Leishmania* isolates has shown that the Sudanese isolates of *L. donovani* possessed the most ancestral sequences and were of a single haplotype that significantly resembled the sequence of *L. major* (Ibrahim and Barker, 2001).

There is an inherent link between the control of the parasite's life cycle and cell cycle regulation. Cyclin-dependent kinases (CDKs), exemplified by cdc2 regulate progression through the eukaryotic cell cycle (Lee and Nurse, 1987). They are also involved in the regulation of other processes such as gene expression and phosphate metabolism. Two control points of the cell cycle are thought to be regulated by the CDKs, the START and the G2/M transition (Pines, 1995). The latter is highly regulated by the activity of the cdc2 gene. A large number of homologues have been identified from differ-

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Table 1. Oligonucleotides primers used in this study.

Primer	Length	Sequence 5'-3'	Comments
OL322 (Forward)	25	GGCCATGGCTTCGTTTGGCCGTGTG	Pc PCR Cloning of <i>Lmmcrk3</i>
OL323 (Reverse)	27	GCGGATCCCTACCAACGAAGGTCGCTG	

ent eukaryotes, including the unicellular trypanosomatids, which were designated cdc2-related kinases rather than CDKs at that time because none of them had been shown to bind cyclins (Mottram, 1994). The first CRK/cyclin complex identified in *T. brucei* was the CRK3/CYC2 complex (Van Hellemond *et al.*, 2000). Most recent study has shown that a recombinant *L. mexicana* CRK3 and cyclin CYCA were combined *in vitro* to produce an active histone H1 kinase that was inhibited by the CDK inhibitors, flavopiridol and indirubin-3'-monoxime (Gomes *et al.*, 2010).

CRK3 homologues have been described in the trypanosomatids *Leishmania mexicana*, LmCRK3 (Grant *et al.*, 1998), *Leishmania major*, LmajCRK3 (Wang *et al.*, 1998), *Trypanosoma brucei*, TbCRK3 (Mottram and Smith, 1995) and *Trypanosoma cruzi*, TzCRK3 (Hassan *et al.*, 2001). CRK3 proteins have been tested for their ability to complement different *Schizosaccharomyces pombe* cdc2 and / or *Saccharomyces cerevisiae* CDC28 temperature sensitive mutants, while, *L. mexicana* CRK3 failed to complement cdc28^{ts} mutants (Grant *et al.*, 1998). Only *L. major* CRK3 was able to restore Cdc2p / CDC28 activity (Wang *et al.*, 1998). Taken together, these results suggest that, at least for trypanosomatids and yeast, cross-species complementation may not be a powerful method to establish functional homologies between proteins. Gene disruption experiments in *L. mexicana* indicated that LmmCRK3 was essential to the promastigote form, and the histidine tagged CRK3 has shown high activity towards the substrate histone H1 that was mainly observed at the G2/M, suggesting a possible role in controlling the cell cycle (Hassan *et al.*, 2001).

The aims of this study: firstly, to isolate and characterize the full-length *CRK3* gene from *L. donovani*, using PCR cloning, library screening, automated-sequencing techniques. Secondly, characterization of the encoded CRK3 protein, analysis of the structure and comparison with cdc2 homologs from other organisms, mainly, *Leishmania* spp. And the human by using computer-based sequence analysis programs.

MATERIALS AND METHODS

Isolation of the positive λ phage clones LdC1 & LdC2

The probe that was used in this screen was derived from a PCR product that was produced from *L. donovani* (MHOM/SD/63/ittered) genomic DNA using

oligonucleotides mainly based on the sequence that flank the START and the STOP codons of the *L. mexicana* *CRK3* gene (Grant., 1998). The PCR-amplified cloned fragment of *L. donovani* *CRK3* was labelled by random priming to an approximate specific activity of 50 μ Ci, and was used as a probe to screen a λ Zap Cdna library (9×10^7 pfu / μ l) synthesized from the promastigote stage of the *L. donovani* (Christensen *et al.*, 2000). Two positives λ clones were isolated after two rounds of screening, were confirmed by PCR analysis and found to contain the *L. donovani* *CRK3* gene (LdC1 & LdC2) (Ali *et al.*, 2003).

Excision of the LdC1 and LdC2 phagemids:

The positive clones (LdC1 & LdC2) were excised with helper phage to generate subclones in pBlue Script SK (-) phagemid vector. The Rapid Excision kit (Stratagene) was used for this purpose. Briefly, a volume of 200 μ l of XL-1 Blue was co-infected with 250 μ l of the cored plaque and 1 μ l (10^{10} pfu/ml) of the ExAssist helper phage and were incubated as usual at 37°C. The excised phagemid was tittered by adding (1-3) μ l to 200 μ l XL0LR strain of *E. coli* and were then plated onto LB-tetracycline (25 μ g/ml) agar plates and incubated overnight at 37°C.

Purification of λ DNA:

The bacteriophage DNA of the positive clones was purified using Qiagen Miniprep kit (Qiagen). Briefly, a volume of 3 ml LB broth containing the appropriate antibiotics, were inoculated with a single colony and then incubated overnight at 37°C in a 200 rpm shaker. The suspension, lysis, neutralization, washing, and elution were all performed as described by the manufacturer. The DNA was precipitated and quantified as previously described (Ali *et al.*, 2003).

Sequencing of the isolated LdC2 clone:

Sequencing internal primers (Table 1) were designed based on sequences obtained by using T₃ (forward) and T₇ (reverse) primers. The purified LdC2 clone was used as a template in those sequencing reactions. The gene was identified as a cdc2-related kinase by computer analysis using the AlignX software, part of Vector NTI Suite V 6.0.

Southern Blot Analysis

Six micrograms of *L. donovani* strain (1s) (MHOM/SD/63/Khartoum) genomic DNA was digested

with restriction endonucleases *EcoRI*, *HindIII*, *BglII*, *BamHI*, *Sall* and *NotI*, electrophoresed through a 0.8% agarose gel, and blotted onto Hybond-N membrane (Amersham Pharmacia, UK). The blot was hybridized with a 2 Kb ³²P random-primed *HindIII* fragment of *L. mexicana crk3* (Containing *Lmmcrk3* ORF, Accession number AJ001275) at 65°C overnight. Washes were for 20 min at 65°C with 2x SSC / 0.1% SDS and then twice for 15 min with 0.1 x SSC / 0.1% SDS. Finally, the membrane was autoradiographed.

RESULTS

Isolation of *L. donovani* CRK3

A 900 bp DNA fragment was produced from the genomic DNA of the *L. donovani*, using a set of oligonucleotide primers (OL322 and OL323) that have been used successfully to isolate the *L. mexicana CRK3* gene. Sequencing of the LdC2 λ clone has shown a nearly full-length *L. donovani CRK3*, as it contains a complete 3' UTR end (data not shown). However, the 5' UTR end is incomplete as it lacks the spliced leader (SL). In addition, the sequence was found to contain a single ORF, translation of which, revealed the presence of an encoded sequence of 311 amino acids. Further analysis of the ORF revealed that, as with other leishmanial genes, the *L. donovani CRK3*-encoding sequence preferentially preserved a fairly high G or C codon at the third base of the codon (wobble position). Approximately 51% of the codon used contained either a G or a C residue at the third position. The full sequence of the LdC2 λ clone, which contains *LdCRK3* (*L. donovani* cdc2-related kinase 3), has been deposited in the EMBL (Accession number AJ426472).

Characterization of *L. donovani* CRK3

The predicted protein encoded by the *L. donovani CRK3* gene shows the greatest degree of sequence identity to the cdc2 family of serine/threonine protein kinases when compared with protein sequence databases. The alignment of *L. donovani* CRK3 with *L. mexicana* CRK3 (LmCRK3), *L. major* CRK3 (LmajCRK3) and human cdc2 (HsCDC2) are shown in Figure 1. The alanine residue at position 7 (Ala-7) was replaced by a valine residue (Val-7) in both of the *L. mexicana* CRK3 and *L. major* CRK3, while the conserved alanine residue at position 217 (Ala-217) in both of the *L. donovani* CRK3 and *L. mexicana* CRK3 was replaced by threonine residue (Thr-217) in the *L. major* CRK3. The three leishmanial CRK3s each have an unusual 19-amino acid N-terminal extension, when compared with human cdc2, which is highly conserved in sequence. However, all the three leishmanial CRK3s have the domains and residues characteristic of the

serine/threonine protein kinase family, mainly those which are important for the regulation of cdc2 activity. This comprised equivalent residues to human CDC2 at Thr-14 (T14) and Tyr-15 (Y15) in the ATP-binding domain, and T161 (Figure 1). The most highly conserved regions between HsCDC2 and *L. donovani* CRK3 include a 10-amino acid block GEGTYGVVYK, in the ATP-binding region (Figure 1), that is thought to be identical in all functional CDC2/CDK kinases. However, the *L. donovani* CRK3 has a single substitution, where the lysine residue (K) is replaced by arginine residue (R), interestingly, all the three leishmanial CRK3s have the same substitution. As both amino acids are basic, this may suggest that no significant differences exist between these kinases based on the ATP-binding domain. The 16-aa sequences EGVPTAIREISLLKE known as the 'PSTAIR' box is a highly conserved domain thought to be important for the binding of cyclins. In this 'PSTAIR' box six substitutions occurred; in the first one isoleucine (I) for valine (V), glutamine (Q) for serine (S) in the second substitution, leucine (L) for the isoleucine (I) in the third substitution, valine (V) for isoleucine (I) in the fourth substitution, isoleucine (I) for leucine (L) in the fifth substitution, and glutamine (Q) for (K) in the sixth substitution. Two of these substitutions give 'PQTALR' motif instead of 'PSTAIR'. However, this feature is common in all trypanosomatid CRK3. LdCRK3 also contains sequence features that distinguish it from the functional CDC2s. The region near the COOH terminus is of low homology (subdomain X). *L. donovani* CRK3 has a GDSEIGQ motif in place of the highly conserved GDSEIDQ box (subdomain IX) that plays major role in the control of the phosphorylation at the conserved 161-Thr residue.

Sequence identity (%) between the *L. donovani* CRK3, cdc2-related kinases (CRKs) from other trypanosomatids, budding yeast CDC28, and human CDC2 has been investigated (Table 2). *L. donovani* CRK3 shares over 99% identity with both *L. mexicana* CRK3 and *L. major* CRK3, over 78% identity with *T. brucei* CRK3 and *T. cruzi* CRK3, and over 49% with both human CDC2 and *Saccharomyces cerevisiae* CDC28. In contrast to *L. mexicana* CRK3, *L. mexicana* CRK1 has only 42% identity with the *L. donovani* CRK3, which indicates the degree of divergence between the two CRKs, and may also suggest that they play two different roles in the parasite *Leishmania*. The lowest identity is shown by the *L. mexicana* CRK4, to which *L. donovani* CRK3 has less than 23% identity.

Phylogenetic analysis

As illustrated in Figure 2, phylogenetic analysis using AlignX program of the *L. donovani* CRK3 with other cdc2-related kinases show that all three leishmanial CRK3s proteins are grouped with the trypanosome CRK3s in one

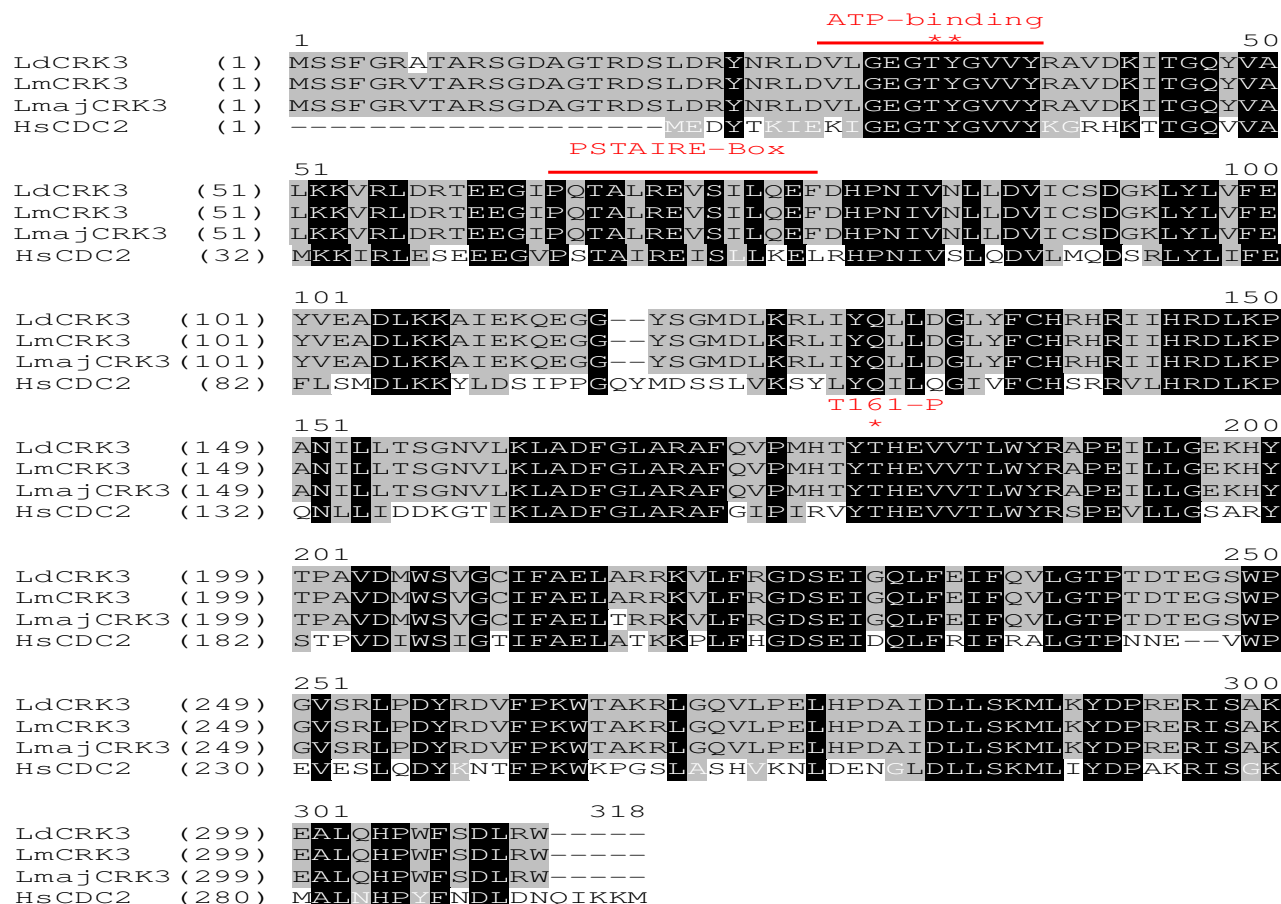


Figure 1. Comparison of the Amino Acid Sequence of *L. donovani* CRK3 (LdCRK3) with *L. mexicana* CRK3 (LmCRK3), *L. major* CRK3 (LmajCRK3) and Human CDC2 (HsCDC2).

Sequences were aligned using AlignX™ software part of the VectorNTI package. Dashes indicate gaps made to maximize alignments. Tyrosine and Threonine residues (*) shown to be phosphorylated *in vivo* in vertebrate CDC2s are indicated. The black shading represents sequences those are identical in human and the parasite *Leishmania*, grey shading represents sequences those are identical in all *Leishmania* cdc2-related kinase 3 (CRK3). The ATP-binding domain and the PSTAIRE-Box are illustrated. The numbers between brackets show the start of the amino acid sequence of every protein in each line, while the other numbers show the start and the end of the amino acid sequence in each line based on the *Leishmania* CRK3s proteins.

Table 2. Sequence identity (%) between *L. donovani* CRK3, Trypanosomatid cdc2-related kinases (CRKs), Budding yeast CDC28, and Human CDC2.

% Identity	LdCRK3	HsCDC2	ScCDC28
LdCRK3	-	49.4	50.2
LmajCRK3	99.4	49.1	50.3
LmmCRK3	99.7	49.4	50.2
LmmCRK1	42.5	51.5	50.3
LmmCRK4	22.7	28.8	26.8
TbCRK3	78.1	49.4	47.9
TzCRK3	77.8	48.7	47.9

Reference: HsCDC2 (Accession # X05360), *L. mexicana* CRK3 (Accession # AJ001275), *L. mexicana* CRK1 (Accession # X60385), *L. mexicana* CRK4 (Accession # AJ293288), *L. major* CRK3 (Accession # AF073381), *L. donovani* CRK3 (Accession # AJ426472), ScCDC28 (Accession # X00257), *T. brucei* CRK3 (Accession # X74617), *T. cruzi* CRK3 (Accession # U69958).

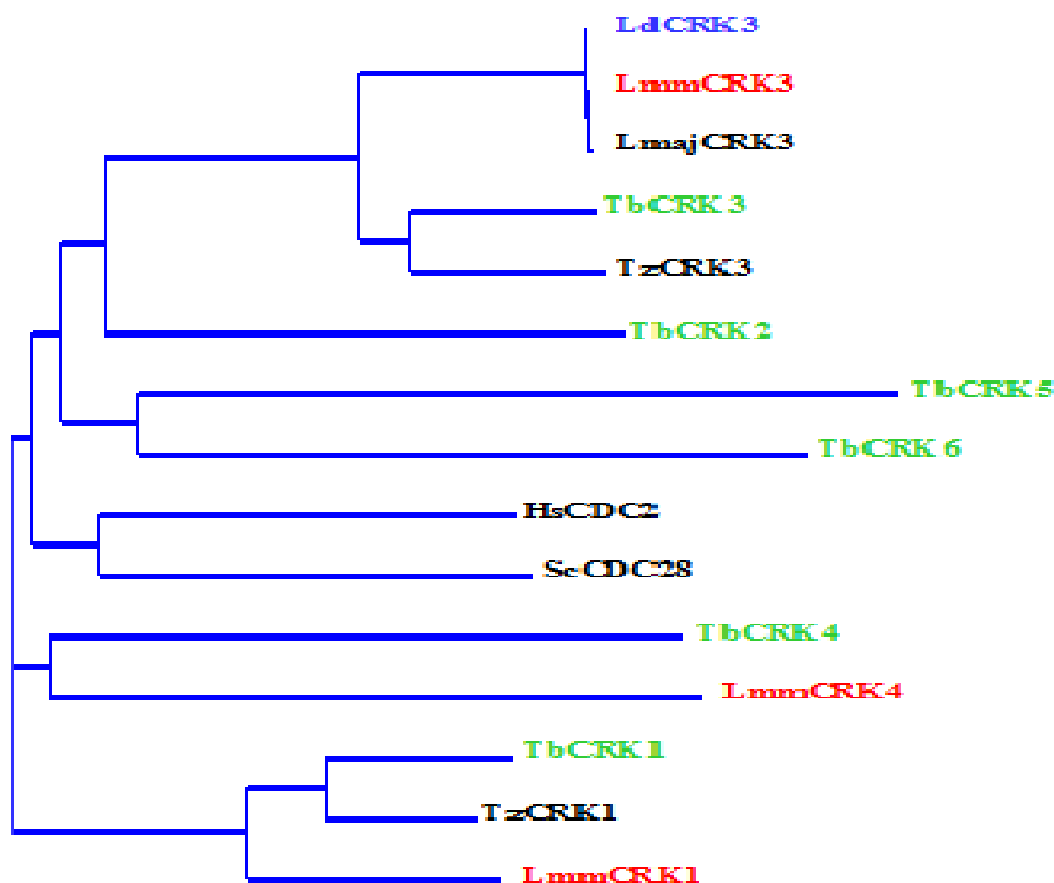


Figure 2. Phylogenetic analysis of *L. donovani* CRK3 with other cdc2-related kinases using AlignX™ program of the VectorNTI suite.

Catalytic domains were used in this analysis. Species abbreviations are as follows: Hs, human; Sc, *Saccharomyces cerevisiae*; Tb, *T. brucei*; Tz, *T. cruzi*; Lmm, *Leishmania mexicana*; Lmaj, *Leishmania major*; Ld, *Leishmania donovani*. Reference: LmmCRK1 (Accession # X60385), LmmCRK3 (Accession # AJ001275), LmmCRK4 (AJ293288), LmajCRK3 (Accession # AF073381) TbCRK1 (Accession # X64314), TbCRK2 (Accession # X74598), TbCRK3 (Accession # X74617), TbCRK4 (AJ413200), TbCRK5 (Similar to Accession # NP_036103; *Mus musculus* MAPK, and Accession # NP_055041; human MOK protein kinase), TbCRK6 (Similar to Accession # P54666; *T. brucei* Cell Division Control Protein 2 homolog 3).

subfamily. However, they are more divergent from the latter that includes both TbCRK3 and TzCRK3, which is in consistence with the fact that a minor evolutionary difference does exist between *Leishmania* and *Trypanosoma*. Moreover, there is almost no divergence among the three leishmanial CRK3s, although LdCRK3 has a single and two amino acids difference from LmCRK3 and LmajCRK3, respectively. This observation is also inconsistent with the fact that they are taxonomically from different complex, which give strong support that CRK3 must be an essential kinase for *Leishmania* parasite. In spite of the fact that the evolutionary rates of the yeast, *Leishmania*, *Trypanosoma*, and human CDK family members were expected to be different, the CRK3 subfamily falls in the same group with both human CDC2 and budding yeast

CDC28.

Analysis of the structure

Analysis of the predicted amino acid sequence of the *L. donovani* CRK3 has revealed a protein of 311 amino acids, which has an estimated molecular mass of 35.5 kDa. The isoelectric point (pI) is 6.8. The composition of the amino acids has revealed that hydrophobic residues present as 36.98%. *L. donovani* CRK3 also is rich in leucine residues (12.54%), which may contribute largely to the hydrophobicity of the protein. Using the on-line software (http://bioinformatics.weizmann.ac.il/hydrobin/plot_hydroph.pl) the hydropathicity of the *L. donovani* CRK3 was compared with the human CDC2 (Figure 3).

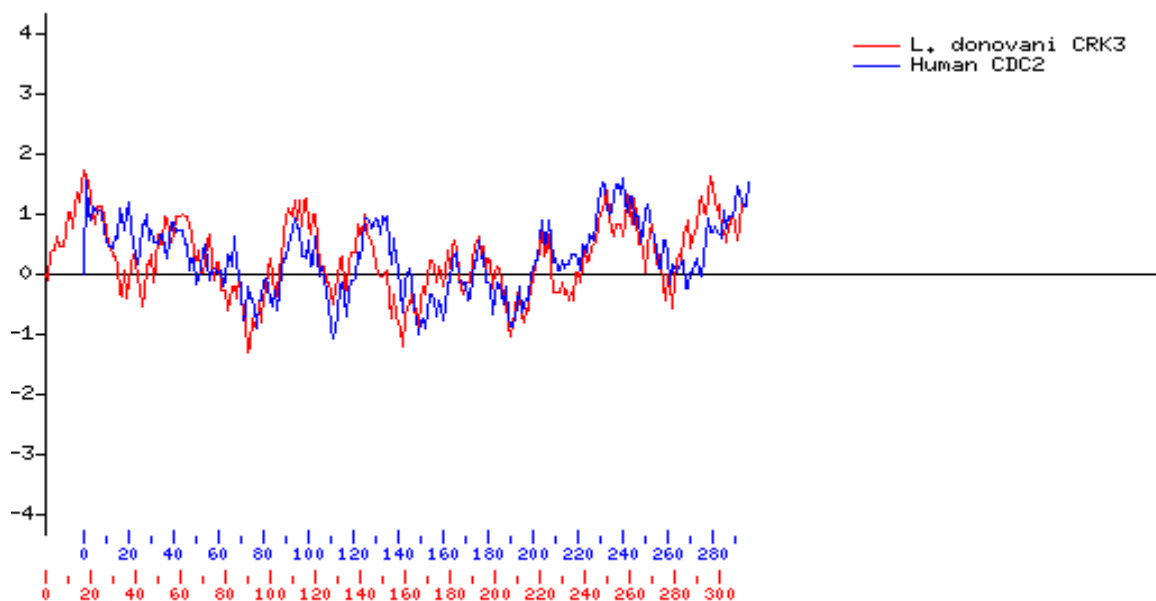


Figure 3. Comparison of the Hydropathic profile of *L. donovani* CRK3 & Human CDC2.

Using the on-line software, protein hydropathicity/hydrophobicity search and comparison server, the hydropathicity of the *L. donovani* CRK3 was compared with that of the human CDC2. Kyte Doolittle calculation method was used, in which A= -1.8; C= -2.5; D, E, N, & Q= 3.5; F= -2.8; G= 0.4; H= 3.2; I= -4.5; K=3.9; L= -3.8; M= -1.9; P= 1.6; R= 4.5; S=0.8; T= 0.7; V= -4.2; W= 0.9; & Y= 1.3. Unlike, human CDC2, *L. donovani* CRK3 has a hydrophobic N-terminal extension.

L. donovani CRK3 has a 19 aa extension on the N-terminus, this analysis shows that it is hydrophobic. However, the two plots have more or less same hydropathicity/hydrophobicity pattern, particularly, in the region 180-200 aa (human CDC2) and 200-220 aa (*L. donovani* CRK3).

3D - Modelling

The availability of crystallographic models for members of the kinase family permits the use of structural information, along with primary sequence alignment, to model the structure of homologous enzymes. The overall architecture of the catalytic core of the LdCKR3 is very similar to that of human CDK2, with a small and large lobe, in spite the fact that the overall amino acid identity is only 43%. In fact, the two kinases come from different branches of the kinase superfamily phylogenetic tree.

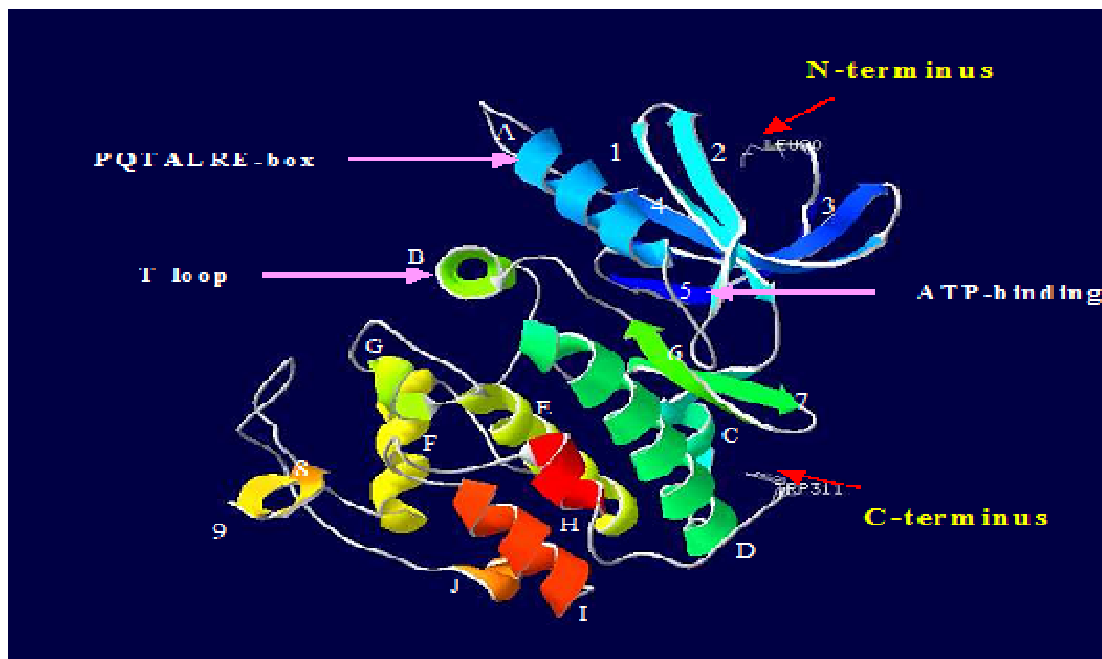
As shown in Figure 4 the molecule is illustrated in forms of β -sheet shown as arrows and α -helix shown as ribbons. All the α -helices are shown in letters (A-J), while the β -sheets are shown in numbers (1-9). The structure of *L. donovani* CRK3 is that of a protein kinase catalytic core, consisting of an N-terminal domain formed principally from seven β -sheets and four α -helices, and a C-terminal domain formed principally from six α -helices and two β -sheets. The kinase consists of two lobes with a deep cleft between them, which may be represent the

active site of the enzyme-the region in which most of the conserved residues lie (ATP-binding domain, PSTAIRE-box, and the T-loop). The larger amino-terminal lobe is composed of a seven-stranded antiparallel β -sheet. In contrast, the smaller carboxy-terminal lobe is largely α -helical, with two β -strands forming part of the cleft between the lobes. The two phosphorylation sites that correspond to the human CDC2 T-14 and Y-15 sites are located in the ATP-binding motif that is indicated by the green arrow within β 5 that lies near the cleft formed by the N-terminal and C-terminal domains. The C-terminal domain is colored blue, with the activatory phosphorylation site that corresponds to Thr-160 in the human CDC2, highlighted in red in the T-loop which is illustrated in yellow, and lie near the α B.

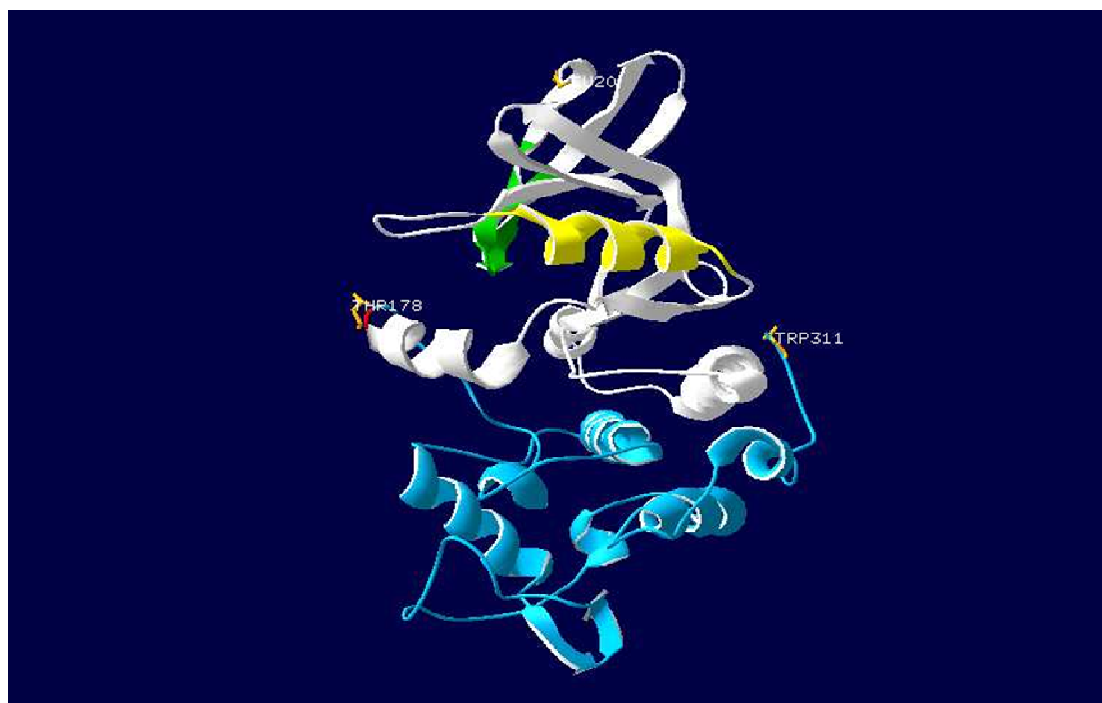
From this model it is clear that the T-loop is protruding in a similar way to that described for the inactive form of all CDK protein kinases. Upon phosphorylation, this loop will bend in a way to give the chance for the binding of the cyclin partner, and thus bring the kinase to the active status.

Genomic organization of the *L. donovani* CRK3

Southern blot analysis detected a single hybridising band under high stringency conditions in five out of six restriction digests (Figure 5, lanes 1-4, & 6). The *Sa*II restriction digest (lane 5) gave one strong hybridising



(A)



(B)

Figure 4. Molecular modelling of *L. donovani* CRK3.

The three-dimensional homology modelling was performed using the Swiss-Pdb Viewer v3.6 b2.

The modelling was done using human cyclin-dependent kinase 2 (CDK2) complexed with the inhibitor staurosporine as a template (accession code 1AQ1, Guex *et al.*, 1999²¹; and Guex and Peitsch, 1997²²). (A) Using the successive colouring automatically, while [B] manual colouring, and the molecule is rotated to show the important domains.

In (B), the N-terminal domain is principally white, with the exception of the 19 aa that shown by leucine 20 (colored yellow). The PQTALRE (corresponding to PSTAIRE in CDK2), is shown by the yellow helix and the ATP-binding motif is illustrated in green arrow within $\beta 5$. The C-terminal domain is colored blue with the activation site (Thr-178) highlighted in red.

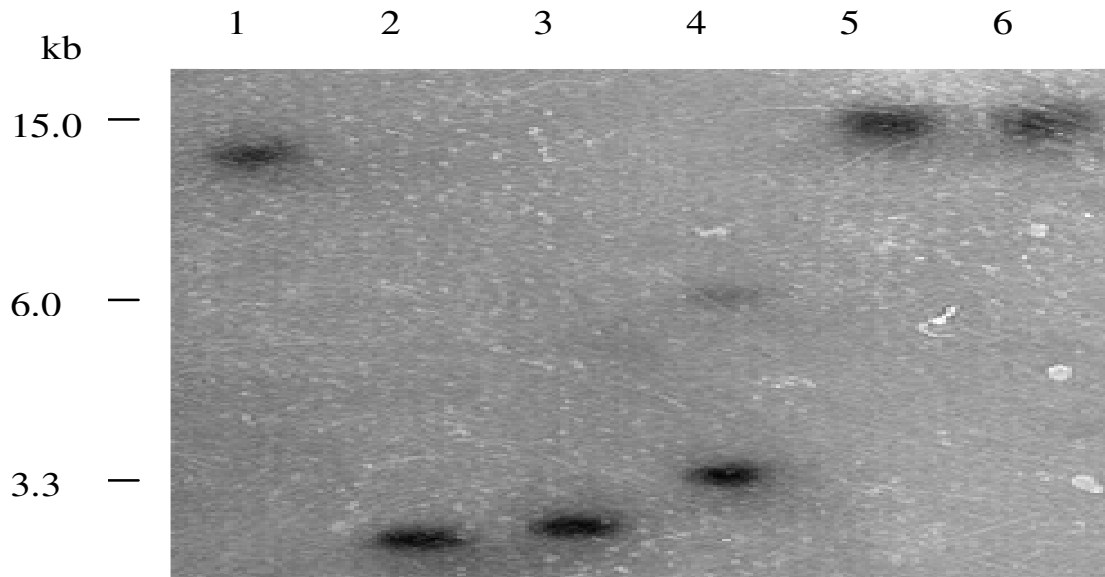


Figure 5. Southern blot analysis of *L. donovani* genomic DNA, probed with the *Hind*III fragment of pGL89 under high stringency conditions.

6 µg of *L. donovani* strain (1s) (MHOM/SD/63/Khartoum) genomic DNA was digested with *Eco*RI (lane 1), *Hind*III (lane 2), *Bgl*II (lane 3), *Sal*I (lane 4), *Bam*HI (lane 5), and *Not*I (lane 6), electrophoresed through a 0.8% agarose gel, blotted to a nylon membrane and probed with the 2 kb *Hind*III fragment of pGL89. Arrowheads indicate DNA markers in Kb.

fragment of approximately 3.3 kb and another weakly hybridising fragment of approximately 6.0 kb. This result is consistent with the physical map created for the putative *L. donovani crk3* that showed an internal *Sal*I site within the *crk3* (data not shown). Together these results indicate that *L. donovani crk3* is present as a single copy gene in the *L. donovani* genome.

DISCUSSION

Full-length open reading frame (ORF) for the *L. donovani CRK3* was obtained by screening a promastigote cDNA library. Genomic southern analysis demonstrated that the *LdCRK3* is present as a single copy in the *L. donovani* genome, which is consistent with the genomic organization of the all so-far identified trypanosomatid CRKs.

Sequence comparison shows that all the trypanosomatid cdc2-related kinases (CRKs) have more or less the same degree of identity to either the human CDC2 or the budding yeast CDC28. This observation is consistent with what is expected from the divergence in the evolution between the different eukaryotes; trypanosome parasite; yeast and human. Taken together, these results suggest that the CRK3 homologue is almost completely conserved and consequently is essential in all the trypanosomatids, including *T. brucei*, *T. cruzi*, *L. mexicana*, *L. major* (Beverley *et al.*, 1986), and *L. donovani*. However, the sequence data does not

allow us to conclude that *L. donovani CRK3* is essential, as there could be highly conserved non-essential proteins. Rather it is likely to be essential because it has been shown to be so in *L. mexicana*. Moreover, *L. donovani CRK3* is more similar to *L. mexicana CRK3* than any other *L. mexicana* proteins such as *L. mexicana tubulin* (Accession # AF345947) or *L. major actin-related protein* (Accession # AL445944) (data not shown), which may indicate a similar function of both kinases in the two *Leishmania* species, possibly in the cell cycle control.

Interestingly, threonine and tyrosine residues known to be important in CDC2 regulation by phosphorylation are also conserved in *L. donovani CRK3* (Thr-33 & Tyr-34). Therefore, the presence of these conserved residues in *L. donovani CRK3* indicates that the *Leishmania* protein kinase may be under similar post-translational control as CDC2-related kinases from other organisms.

The putative protein encoded by *L. donovani CRK3* has an N-terminal extension, which consists of 19-aa and in this it is similar to other trypanosomatid CRK3 protein kinases. The role of this N-terminal extension is poorly understood, but plays no role in the regulation of the kinase activity.

Phylogenetic analysis suggests that the CRK3s class of protein kinases are good candidates to be the functional homologue of *cdc2*, as they fall in the same phylogenetic group with both the human CDC2 and the budding yeast CDC28. However, it is not possible to use sequence identity to infer function. All CRKs are as similar to human CDC2 as to each other and biochemical

or genetic data is required to determine a function.

T. brucei has an unusually high number of CRK genes for a unicellular organism and this may indicate that the cell cycle regulatory mechanisms are so complicated that several molecules are required to co-ordinate the cell cycle steps during the complex life cycle. Early studies identified only three CRKs genes from *Leishmania* spp. as compared to *Trypanosoma*. However, all these three leishmanial CRKs have shown high structural and/or functional homology to the corresponding Trypanosome CRKs. Most recently five more Leishmanial CRKs (2, 4, 6, 7 and 8) were investigated in a search for their cyclin-partner and biological activity (Gomes *et al.*, 2010). A similar large numbers of CRKs, could possibly be present in *Leishmania*, thus more genes are waiting to be identified in order to understand the whole picture of the *Leishmania* cell cycle. For example a novel cyclin was identified from *L. mexicana* (Ali *et al.*, 2010) and was found to have ability to bind and activate *L. mexicana* CRK3 (Gomes *et al.*, 2010). In a search for new drug against Leishmaniasis the active CRK3:CYCA complex was tried against known CDK inhibitors (Gomes *et al.*, 2010) and inhibitors against Leishmania CRK3-CYC6 complex was identified most recently (Cleghorn *et al.*, 2011).

Investigation of expression of *L. donovani* CRK3 gene is beyond the scope of this study, but we would expect that, the *LdCRK3* to be also an essential gene, as it has the sequence characteristic of a cdc2-related kinase, including many of the domains and motifs thought to be important for CDK regulation. Moreover, *L. donovani* CRK3 is likely to be essential as it is 99% identical to *L. mexicana* CRK3, which has been shown experimentally to be essential for cell cycle regulation (Hassan *et al.*, 2001).

The argument that the *L. donovani* CRK3 gene is a *L. donovani* homologue of cdc2-related kinase subfamily rests on the sequence homology data and not on the functional data. In the present study, it is difficult to predict a function for *L. donovani* CRK3 based on the sequence data alone, in stead we need to carryout functional experiments to confirm whether it is the functional cdc2 homologue or not. One possible approach is to try genetically to manipulate the gene, using the gene knockout experiments or the most recent techniques; the RNA interference (RNAi) / inducible RNA/double-stranded RNA (Ngô *et al.*, 1998); (Wang *et al.*, 2000); (LaCount *et al.*, 2000), in order to assign a specific function to the gene in the *Leishmania* parasite. This technique has been established for the African *Trypanosoma*, but not yet for *Leishmania*, though ongoing research is focusing on testing RNAi in *Leishmania* sp.

Knowing the temporal and spatial expression of the protein (CRK3) and its activity would help to test synthetic inhibitors on that protein and thus help to produce specific drug or anti-leishmanial agent. However, several

pharmacological studies on anti-leishmania agents have been hampered by the insensitivity of *Leishmania* to commonly used drugs. Therefore, the existence of treatment failure highlighted the need to conduct studies to investigate an appropriate anti-leishmanial agent. This study have given the strong evidence that the CRK3 class of *Leishmania* species is conserved and hence, have contributed to the current research on the identification of the parasitic cdc2-related protein kinases as drug targets from several protozoa; including *Leishmania*, African *Trypanosoma*, and *Plasmodium falciparum*.

Therefore, the possibility of designing a drug that can be used in a costless manner to treat all forms of leishmaniasis could be extrapolated.

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