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Recombinant *Leishmania mexicana* CRK3:CYCA has protein kinase activity in the absence of phosphorylation on the T-loop residue Thr178

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Abstract

The activity of cyclin dependent kinases (CDKs), which are key regulators of the eukaryotic cell cycle, is regulated through post-translational mechanisms, including binding of a cyclin and phosphorylation. Previously studies have shown that *Leishmania mexicana* CRK3 is an essential CDK that is a functional homologue of human CDK1. In this study, recombinant histidine tagged *L. mexicana* CRK3 and the 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. Protein kinase activity was observed in the absence of phosphorylation of the T-loop residue Thr178, but increased 5-fold upon phosphorylation by the CDK activating kinase Civ1 of *Saccharomyces cerevisiae*. Seven recombinant *L. major* CRKs (1, 2, 3, 4, 6, 7 and 8) were also expressed and purified, none of which were active as monomers. Moreover, only CRK3 was phosphorylated by Civ1. HA tagged CYCA expressed in *L. major* procyclic promastigotes was co-precipitated with CRK3 and exhibited histone H1 kinase activity. These data indicate that in *Leishmania* CYCA interacts with CRK3 to form an active protein kinase, confirm the conservation of the regulatory mechanisms that control CDK activity in other eukaryotes, but identifies biochemical differences to human CDK1.

1. Introduction

Despite recent advances in understanding of the cell biology of the protozoan parasite, *Leishmania*, its cell cycle remains relatively unexplored. Fundamentally, the parasite's cell cycle is the same as every other eukaryote's featuring growth, DNA replication, mitosis and cytokinesis. In addition, *Leishmania* must ensure the duplication and faithful segregation of their singular organelles: the nucleus, the kinetoplast, the flagellum and the Golgi apparatus. *Leishmania* possess orthologues of many of the protein kinases that have been shown to be key players in controlling the eukaryotic cell cycle, including cyclin-dependent kinases, Aurora and polo-like kinases [1-6], but direct evidence linking these orthologues to a role in *Leishmania* cell cycle control is limited [1,7]. In other eukaryotes, cyclin-dependent kinases (CDKs) act at the boundaries between different cell cycle stages, to prevent premature or

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inappropriate transition through key checkpoints. Their activity is tightly regulated through a variety of mechanisms including binding of a cyclin partner and phosphorylation [8]. Cyclin binding is further regulated at the transcriptional level, resulting in cyclical expression, and post-translationally through targeted destruction by the proteasome [9]. There are two main sites of phosphorylation on CDKs: close to the catalytic site, at Y15 and T14 (in human CDK1), and on the activation or T-loop, at T161 (in human CDK1). Phosphorylation of Y15 and T14 has an inhibitory effect [10,11] which can be reversed by dephosphorylation by the CDC25 phosphatase [12]. Phosphorylation at the T-loop residue is required for the full activity of CDK1, CDK2 [13] and CDK4 [14] resulting in a dramatic conformational change in the T-loop, creating the substrate binding site and orientating ATP correctly for phospho-transfer [15]. However, CDK6 activity *in vivo* appears to be independent of its T-loop phosphorylation status [16].

In mammalian cells, the kinase responsible for phosphorylating the T-loop threonine (CDK activating kinase or CAK) is itself a CDK (CDK7) that is found in a complex with cyclin H and an assembly factor, MAT1 [17]. In contrast, in budding yeast the CDK activating kinase consists of a single protein, known as CAK or Civ1 (CAK *in* vivo) [18]. Both can phosphorylate CDKs but they possess quite different substrate specificities: Civ1 is predominantly cytoplasmic [19] and preferentially phosphorylates CDK monomers, whilst CDK7/cyclin H/MAT1 favours CDK/cyclin complexes [20]. *In vitro*, CDK7/cyclin H (with or without MAT1) can phosphorylate CDK1, CDK2, CDK3, CDK4 and CDK6 [14,21,22]. However, although T-loop phosphorylation of CDK4 is required for activity [14], CDK7 may not be responsible for this phosphorylation *in vivo* [16], implying that there may be more than one human CAK enzyme. *Saccharomyces cerevisiae*, Civ1, can also phosphorylate and activate most mammalian CDKs *in vitro* [20,23,24], implying that the effect of the T-loop phosphorylation *in vitro* is independent of the activating enzyme. No CDK activating kinase has been identified in the *L. major* genome [5].

In comparison with *S. cerevisiae*, *Leishmania* possess a relatively expanded repertoire of 12 cdc2-related kinases [5], perhaps reflecting the relative complexity of the parasite's cell division cycle and the need to integrate that with the developmental life cycle, in which the parasite oscillates between proliferative and cell-cycle arrested forms. CRK3 is the best described of the leishmanial CDKs. It is highly conserved between different species of *Leishmania* (for example, there is only one amino acid difference between CRK3 of *L. mexicana*, *L. major* and *L. donovani*), complements a *Schizosaccharomyces pombe* cdc2 mutant [25] and functions at the G2/M boundary [7], suggesting it is a functional CDK1 homologue. CRK3 is predicted to be regulated by similar mechanisms to other CDKs, since it possesses a conserved cyclin-binding domain and the three regulatory phosphorylation sites (Aligned to T14, Y15 and T160 of human CDK1) [2]. Eleven cyclins have been identified in the *L. major* genome and these fall into 3 classes based on their sequence characteristics [4]; mitotic cyclins (CYCA, CYC3, CYC6 and CYC8), PHO80-like cyclins (CYC2, CYC4, CYC5, CYC7, CYC10, CYC11) and transcriptional cyclins (CYC9). All the cyclins are conserved with other trypanosomatids, such as *Trypanosoma brucei*, except CYCA, which appears to be specific to *Leishmania* species. To date the only CDK:cyclin pair identified in *Leishmania* is the *L. donovani* CRK3:CYC1 (the syntenic homologue of *L. major* CYCA) [26].

In the present work we successfully expressed, purified and reconstituted an active recombinant CRK3:CYCA protein kinase complex *in vitro*. Recombinant CRK3:CYCA has histone H1 protein kinase activity in the absence of phosphorylation on the T-loop threonine, a feature that distinguishes it from mammalian CDK1. Phosphorylation of the T-loop threonine by *S. cerevisiae* Civ1, however, is associated with a 5-fold increased kinase activity. Thus these results confirm that the activity of the leishmanial CDK, CRK3, is

regulated in a similar fashion to other eukaryotic CDKs, but that CRK3:CYCA has some differences from human CDK1 .

2. Materials and methods

2.1 Parasites

L. major (MHOM/JL/80/Friedlin) and promastigotes were grown in modified Eagle's medium with 10% (v/v) heat-inactivated foetal calf serum (designated complete HOMEM medium) at 25°C [27].

2.2 Cloning Leishmania CRKs and CYCA

N-terminally histidine tagged *L. mexicana* CRK3 was expressed from plasmid pGL751, which was constructed as follows: *CRK3* was PCR amplified using primers OL225 and OL894 (Table 1), which added *Nde*1 and *Xho*1 sites onto the 5′ and 3′ ends of the ORF respectively. The PCR product was cloned into *Nde*1/*Xho*1 digested pET28a to create pGL751. To make a non-tagged version, CRK3 was excised from pGL751 using *Nde*I/*Bam*H1 and cloned into pET21a generating pGL1072. *L. mexicana* CYCA was amplified from genomic DNA with oligonucleotides primers OL813 and OL814 which added *Nde*1 and *Xho*1 sites onto the 5′ and 3′ end of the ORF respectively. This was cloned into *Nde*1/*Xho*1 digested pET21a, to give plasmid pGL630, which encodes CYCA with a C-terminal six histidine tag.

To generate histidine tagged *L. major* CRK3, PCR amplification of LmjF36.0550 was performed using *L. major* genomic DNA, oligonucleotides OL1787 and OL1788 and Invitrogen Thermozyme polymerase. The PCR product was subcloned into pET15b, which was pre-digested with *Bam*HI and *Nde*I, generating pGL1340. *L. major* CRK1 (LmjF21.1080; pGL1338), CRK2 (LmjF05.0550; pGL1339), CRK4 (LmjF16.0990; pGL1616), CRK6 (LmjF27.0560; pGL1341, CRK7 (LmjF26.0040; pGL1349), CRK8 (LmjF11.0110; pGL1342) in combination with the oligonucleotides shown in Table 1 were similarly PCR amplified and cloned into pET15b.

To create HA epitope tagged *L. mexicana* CYCA, the gene was amplified with oligonucleotides incorporating the HA tag at the N or C-terminus (OL1937 and OL1938 and OL1935 and OL1936 respectively) and cloned into the *Sma*I/*Bgl*II site of pXG [28].

To generate CRK3^{T178E}his site directed mutagenesis was performed using manufacturers instructions (QuikChange kit, Stratagene) on plasmid pGL751 using oligonucleotide primers OL877 and OL878, resulting in plasmid pGL1071.

2.3 Protein purification and kinase assays

L. mexicana CRK3his was expressed in BL21 (DE3) pLysS *Escherichia coli* cells (Stratagene), inducing with 100μM IPTG at 20°C overnight, and purified as described previously [2]. For *L. mexicana* CYCA, BL21 (DE3) pLysS *E. coli* cells were transformed with plasmid pGL630. Cells were induced for protein expression at 19°C over night using 5mM IPTG and CYCAhis was purified as described for CRK3his. Plasmids expressing *L. major* CRK1-CRK8 were transformed into BL21 (DE3) pLysS *E. coli* cells and induced with 1mM IPTG at 19 °C over night. All the CRKs produced soluble protein, but expression levels varied from low (CRK6 and CRK8) to high (CRK1, CRK2, CRK3 and CRK7). *S. cerevisiae* Civ1-GST was purified as described previously [24]. The expression and purification of CRK3:CYC6 will be described elsewhere (Walker et al., manuscript in preparation).

Protein kinase assays were performed as described previously [2]. Recombinant protein kinase was incubated in 50 mM MOPS pH 7.2, 20 mM $MgCl_2$, 10 mM EGTA, 2 mM DTT, 4 μM ATP, plus 1 μCi γ-P^{32}ATP (3000Ci/mmol) and 2.5 μg histone H1 per reaction. Reactions were incubated at 30 °C for 30 min. Final volume of each reaction was 20 μl and at the end of the 30 min incubation 20 μl of two times Laemmli protein loading buffer was added to stop the reaction, samples then were incubated at 100 °C for 5 min and loaded on 12% acrylamide gel. The gel was dried and exposed to KODAK sensitive film overnight. Protein kinase activity was quantified by scanning the dried gels on a Typhoon phosphor imager (GE Healthcare).

To assess the interaction of *L. mexicana* CRK3 with CYCAhis *in vitro,* BL21 DE3 *E. coli* cells were transformed with plasmid pGL630 to express CYCAhis. Cell lysate was incubated with 200 μl of Ni-NTA agarose (Qiagen) bead slurry for 5 min at room temperature and centrifuged for 5 min at 2100 g. This column of Ni-NTA + CYCAhis was washed 2 times with PBS 7.4 and incubated with a soluble bacteria lysate containing non tagged CRK3 for 30 min, mixing at room temperature to permit the binding of the two proteins. The beads were then centrifuged at 1000 g for 5 min. The column was washed 2 times with PBS 7.4 and eluted in 100 μl fractions with phosphate buffer consisting of 100 mM NaPi 7.4, 10 mM NaCl and 0.5 M imidazole (pH 8.0). 10 μl of each elution fraction was mixed with 10 μl Laemmli protein loading buffer and the total volume of 20 μl was loaded on a 12% SDS-PAGE gel. The proteins on the gel were transferred to a PVDF membrane and a western blot was performed using α-CRK3 antibodies [7] diluted 1:2000.

2.4 Immunoprecipitation

L. major were transformed with plasmids pGL1388 and pGL1389 using the method of Robinson and Beverley [29]. Transformants were selected in the presence of 50μg ml^{-1} G418. These cell lines were grown to mid log phase and 50ml of culture was harvested at 1000 g for 10 min at 4°C. The cell pellet was then washed twice in cold PBS and resuspended in 1ml of IP lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Nonidet P40) containing protease inhibitors (100 μg ml^{-1} leupeptin, 500 μg ml^{-1} Pefabloc, 5 μg ml^{-1} pepstatin, 1mM 1-10, phenanthroline, 1mM EDTA and 1mM EGTA). To this lysis suspension, 50 μl of HA affinity purification matrix (Roche) was added and an overnight incubation at 4 °C with agitation was done. The matrix was then washed 3 times with 1ml of lysis buffer and resuspended in 50 μl of lysis buffer. 10 μl was loaded on an SDS-PAGE gel, which was used either for Western blotting or silver staining. 5 μl of matrix was used in a kinase assay using histone H1 as a substrate. For western blots to detect HA tagged proteins, monoclonal mouse HRP conjugated antibody (Roche) was used diluted at 1 in 500.

3. Results

3.1 *Leishmania* CYCA binds and activates CRK3 in vitro

Leishmania mexicana CRK3 and CYCA were histidine tagged, expressed and purified from *Escherichia coli* (Fig. 1A, lanes 1 and 2 respectively). A construct expressing CRK3 without a histidine tag was also generated. To investigate the interaction of CRK3 and CYCA, an *in vitro* binding assay was carried out whereby CYCAhis was bound onto a Ni-NTA column and then incubated with an *E. coli* cell lysate containing non tagged CRK3. After washing to remove non-specifically bound proteins, CYCAhis was eluted from the column and the presence of co-eluting CRK3 in the eluant was assessed by Western blotting with an anti-CRK3 antibody [2]. CRK3 was found to bind immobilised CYCAhis (Fig. 1B, lane 1) but not control beads (lane 2), showing that *L. mexicana* CRK3 can interact with CYCA *in vitro*. Recombinant monomeric CRK3his had negligible histone H1 protein kinase activity (Fig. 1C, lane 1), but when increasing concentrations of CYCAhis were pre-incubated with a

fixed concentration of CRK3his, escalating histone H1 kinase activity was detected (lanes 2-7). No histone H1 kinase activity was detected with cyclin alone (lane 8). Optimal CRK3his:CYCAhis protein kinase activity was detected when CRK3 and CYCA were mixed in an approximate 1:1 molar ratio (lane 6).

Phosphorylation of the canonical Thr residue in the T-loop of CDKs is essential for maximal activity in yeast, plants and mammals [30]. Substitution of a Thr residue with a negatively charged Glu can mimic phosphorylation of the Thr [31] and when applied to the T-loop residue in the *Plasmodium* CDK, PfPK5, resulted in a 5 to 10-fold activation [32]. To test if this was also the case for CRK3, site directed mutagenesis was carried out on the conserved T-loopThr residue (Thr 178) of CRK3his to produce $CRK3^{T178E}$his. Affinity purified $CRK3^{T178E}$his (Figure 1D) lacked histone H1 kinase activity both in the absence (Figure 1F, lane 1) and presence of CYCA (Figure 1E, lane 2). The results show that CYCAhis is able to activate CRK3his (Figure 1E, lane 1) but not $CRK3^{T178E}$his (lane 2), indicating that the mutation abolishes histone H1 kinase activity. CRK3 is also activated by the cyclin CYC6 to produce a kinase with histone H1 kinase activity (Walker et al., manuscript in preparation; Figure 1F, lane 3). $CRK3^{T178E}$his, however, is not activated by CYC6 (Figure 1F, lane 2), showing that T178 is essential for CRK3 protein kinase activity with two different cyclin partners.

L. mexicana CRK3his affinity purified from the parasite has been shown to have histone H1 kinase activity and to be inhibited by a variety of CDK inhibitors [2,33]. Although it is not known how many cyclins bind and activate CRK3 or the Thr178 phosphorylation status of CRK3 *in vivo*, the CRK3 purified from *L. mexicana* promastigotes could be compared to the recombinant purified CRK3his:CYCAhis by comparing their inhibition with two well established CDK inhibitors, flavopiridol [34] and indirubin-3′-monoxime [35]. IC_{50} values of 102 nM for flavopiridol and 3.1 μM for indirubin-3′-monoxime with CRK3his:CYCAhis (Figure S1) were similar to the IC_{50} values of 100 nM [7] and 1.35 μM [33] respectively for CRK3his affinity purified from *L. mexicana*. The variation in IC_{50} between recombinant CRK3 and that purified from the parasite might be due to the presence of a complex mixture in the parasite-derived enzyme preparation. Monomeric CRK3, CRK3:CYCA, CRK3:CYC6 or potentially other CRK3:cyclin complexes might be present, possibly each with different inhibition profiles.

The genome of *Leishmania major* contains over 170 protein kinase genes [5], but it has not been possible to identify using bioinformatics analysis which of these genes might encode a functional *Leishmania* CDK activating kinase (CAK). For this reason we tested if the GST-tagged *S. cerevisiae* CAK (Civ1-GST) [18], expressed and purified from *E. coli* (Fig 2A, lower panel), would phosphorylate CRK3 on Thr178. The yeast Civ1-GST was able to phosphorylate recombinant CRK3his in a dose dependent manner (Fig. 2A, lanes 2-6, upper panel). Civ1-GST did not auto-phosphorylate (lane 7) or phosphorylate $CRK3^{T178E}$his (lane 8) indicating that Thr178 in CRK3 was the most likely site of phosphorylation. In order to assess whether the phosphorylation of CRK3his Thr178 would increase its protein kinase activity, a time course was carried out where CYCAhis and CRK3his were incubated in the presence and absence of Civ1-GST and histone H1 kinase activity assessed at various time intervals (Figure 2B). A 5-fold increase in phosphorylated histone H1 was observed after Thr178 phosphorylation by Civ1-GST (compare histone H1 signal in lanes 5 and 10). Civ1-GST does not phosphorylate histone H1 significantly (Figure 2C, compare lane 1, in the absence of Civ1, with lanes 2 and 3 in the presence of 0.3mg and 1.8mg Civ1 respectively).

The natural substrate for Civ1 in *Saccharomyces cerevisiae* is CDC28 [18]. The fact that *Leishmania* CRK3 can be phosphorylated by Civ1 indicates that the phosphorylation site is conserved between these two species (see Figure 3A) and implies that this phosphorylation

may play a role in regulating CRK3 activity, as it does for CDC28 [18]. *L. major* has 12 CRKs and 10 of these have a conserved T-loop Thr or Ser residue [4]. To assess if other CRKs could be phosphorylated by Civ1-GST, *L. major* CRKs 1-8 (Fig 3B) were cloned into pET15b and expressed and purified from *E. coli* (Figure 3B). CRK5 was not included as it has been reclassified as a MOK-family MAP kinase and is unlikely to be cyclin-dependent [4]. *L. major* CRKs were chosen as the *L. mexicana* genome was unavailable for analysis at the time and the CRK family in that species was unknown. Only *L. major* CRK3his was found to be phosphorylated by Civ1-GST (Figure 3B). The purified monomeric CRKs were tested for histone H1 kinase activity, but none were active (data not shown). These data show that yeast Civ1-GST has specificity for CRK3, the *Leishmania* CRK with the highest homology to Civ1's natural substrate, CDC28 (Figure 3A), and that *Leishmania* CRKs are not active histone H1 kinases, when expressed as soluble monomeric proteins. This does not, however, preclude their activation by a cognate cyclin partner(s), yet to be identified or activity as monomers towards other substrates.

3.2 An active CRK3:CYCA complex in *L. major*

CYCA was amplified with a C- or N-terminal HA tag and cloned into an episomal expression vector pXG to generate pGL1388 (N-terminal HA tag; HA-CYCA), and pGL1389 (C-terminal HA tag; CYCA-HA). *L. major* promastigotes were transfected with each plasmid and cell lines resistant to G418 isolated (designated *L. major* [pXG-CYCA-HA], for C-terminal tag, and *L. major* [pXG-HA-CYCA], for the N-terminal tag). The expression of both CYCA-HA (Figure 4A, lane 1) and HA-CYCA (lane 2) was detected in procyclic promastigote cell lysates at the predicted size of 35 kDa, while no HA-tagged protein was detected in wild type cells (lane 3).

An immuno-precipitation (IP) of *L. major* and *L. major* [pXG-HA-CYCA] was performed using a column of conjugated anti-HA antibody (Figure 4B). The proteins immunoprecipitated from cell lysates were separated by SDS-PAGE and stained with silver stain. A protein corresponding to the expected size of HA tagged CYCA was immuno-precipitated from *L. major* [pXG-HA-CYCA] (Figure 4B upper panel, lane 1), but not wild type *L. major* (lane 2). CRK3 was detected with a CRK3-specific antibody in immuno-precipitates of *L. major* [pXG-HA-CYCA] (Figure 4B, lower panel, lane 1) but not of wild type *L. major* (lane 2), confirming that CRK3 interacts with CYCA in procyclic promastigotes. The precipitated material was assayed for histone H1 kinase activity. Activity was detected in immuno-precipitates from *L. major* [pXG-HA-CYCA] (Figure 4C, lane 1), but not from wild type *L. major* (lane 2). These data show that CYCA interacts with CRK3 *in vivo* and forms an active histone H1 kinase.

4. Discussion

The work presented here is the first to describe the production of a defined active recombinant CRK3 kinase complex and demonstrates that, although the leishmanial CDK shares some regulatory features with mammalian and yeast CDKs, there are also some important differences. In this study, soluble CRK3 was expressed in bacteria, purified and found to possess negligible histone H1 kinase activity. A putative cyclin, CYCA [4], was identified in *L. mexicana* and also expressed in bacteria. The purified CYCA protein was found to bind and activate CRK3 *in vitro* in a dose-dependent manner, with optimal kinase activity occurring when the molar ratio of kinase to cyclin was 1:1. The syntenic homologue of CYCA in *L. donovani*, LdCYC1, has previously been shown to bind LdCRK3 *in vivo* but could not activate bacterially expressed LdCRK3 *in vitro* [26], possibly as a result of the recombinant protein(s) being mis-folded and therefore inactive.

Previously, active CRK3 enzyme was purified from leishmanial lysates [33], but the complex was uncharacterised in terms of the cyclin partner(s) and the phosphorylation status of the kinase subunit. The ability to re-constitute active kinase complex entirely from bacterially expressed protein ensures that the enzyme preparation is clearly defined, consistent and reproducible. The accurate biochemical characterisation of this complex may help to further elucidate the role(s) of CRK3 in *Leishmania*. Indeed it has enabled us to scrutinise the role of phosphorylation of the T-loop Thr-178 in the regulation of recombinant CRK3 protein kinase activity.

Phosphorylation of the T-loop Thr in CDK1, CDK2 and CDK4 is required for full activation [13,14] and is associated with a dramatic increase in protein kinase activity [36]. This increased activity is explained by the conformational change elicited by phosphorylation, which creates the substrate binding site and orientates ATP for phospho-transfer [15,37]. Mutation of a Thr residue to Asp or Glu is thought to mimic phosphorylation at this site. In cAMP-dependent kinase, phosphorylation of a Thr in the catalytic subunit is essential for the formation of the hetero-tetrameric complex. Mutation of this Thr to either Asp or Glu mimics the presence of the phospho-threonine and allows the association of the subunits. This effect is specific for the acidic amino acids; mutation to any other residue abolishes complex formation [30]. There is some evidence that this approach can be used to mimic T-loop phosphorylation in CDKs. Mutation of the T-loop residue in *Schizosaccharomyces pombe* cdc2 (CDK1) to Glu results in a phenotype *in vivo* that is consistent with constitutive activation of this CDK; deregulated mitosis and premature cytokinesis [38]. Replacement of the T-loop Thr with Glu in the *Plasmodium falciparum* CDK, PfPK5, results in a 5-10-fold increase in kinase activity [31,32]. Mutation of the T-loop residue of CRK3 to Glu ($CRK3^{T178E}$), however, did not activate the enzyme; instead it abolished protein kinase activity in the presence of CYCA. Although this was unexpected, it is consistent with what is observed for *Saccharomyces cerevisiae* CDC28; Mutation of the T-loop Thr to Glu inhibits both kinase activity and biological function, although second suppressor site mutations can generate T169E mutants with partially recovered biological activity [39]. On its own, Glu cannot fully complement for the phospho-threonine in CDC28. Moreover, mutation of the T-loop Thr abolishes catalytic activity in CDK1 and CDK2: in CDK1 mutation to Val abolishes cyclin-binding and kinase activity [38] and in CDK2 mutation to Ala abolishes activity of bacterially-expressed protein [40]. *Leishmania* CRK3 has an additional Thr residue (T176) close to the T-loop T178, which might also be a site of phosphorylation (Figure 3A). T176 is conserved in human CDK1 and CDK2, but not in *S. cerevisiae* CDC28. To our knowledge, this residue has not been identified as a site of phosphorylation in CDK proteins of other eukaryotes, but it could potentially be an additional site of regulation for T-loop function in *Leishmania*. Since this approach to mimic T-loop phosphorylation was unsuccessful and because the leishmanial CAK has not yet been identified, we further explored the requirement for CRK3 to be phosphorylated on its T-loop using the *S. cerevisiae* monomeric CAK, Civ1 [18].

The natural substrate for Civ1 is CDC28 but Civ1 can also phosphorylate and activate most mammalian CDKs *in vitro* [20,24]. Civ1 could phosphorylate wild type CRK3 *in vitro* but not $CRK3^{T178E}$, indicating that T178 is likely to be the phosphorylation site in CRK3, as predicted. Pre-incubation of the CRK3:CYCA complex with Civ1 resulted in phosphorylation of the kinase subunit and a 5-fold increase in its histone H1 kinase activity. Compared to the 80-100-fold increase observed for CDK1 and CDK2 [36], this is a fairly modest stimulation of activity. Possible reasons for this include: In the experimental conditions used, Civ1 may not be able to fully phosphorylate CRK3 because (a) the conditions are sub-optimal; the conditions used were those optimised for the subsequent phosphorylation of histone H1 by the CRK3 complex, (b) the sequence of the T-loop is only partially conserved (Fig 3A, CRK3 72% identical to CDC28) and CRK3 is an inefficient

substrate for Civ1 or (c) because Civ1 prefers CDK monomer as its substrate and may not have been able to efficiently phosphorylate the CRK3:CYCA complex. Indeed, it is known that Civ1 phosphorylates monomer CDK2 much more efficiently than CDK2/cyclin A complexes [20] and the intensity of the phosphorylated CRK3 (CRK3 monomer in Fig 2A) appears greater than when Civ1 was pre-incubated with CRK3:CYCA complex (Fig 2B). Future experiments will test the relative efficiency of CRK3 phosphorylation and activation when CRK3 is pre-incubated with Civ1 and then allowed to associate with CYCA.

However, the modest increase in CRK3 kinase activity upon phosphorylation by Civ1 may simply reflect the fact that T-loop phosphorylation is less important in the regulation of CRK3 activity than it is for CDK1, CDK2 and CDK4. Not all protein kinases are activated through phosphorylation of their T-loop; those that are include CDKs, MAPKs and cAPK. Immediately adjacent to the conserved aspartate residue within their catalytic domain, these protein kinases invariably have an arginine residue (RD kinases). Whilst all protein kinases that are activated by phosphorylation of their T-loop possess this RD motif, the reciprocal is not true; not all RD kinases require T-loop phosphorylation for activation. CRK3 does possess this RD motif but it may fall into the latter category, along with CDK5 and CDK6, whose activity appears to be independent of their T-loop phosphorylation status [16]. Protein kinases that do not utilise T-loop phosphorylation can adopt an active conformation without this post-translational modification [41]. CRK3 appears to lie somewhere between these two extremes: it is active in the absence of T-loop phosphorylation but its activity is further stimulated upon phosphorylation of its T-loop, albeit to a much lesser extent than observed with CDK1, CDK2 and CDK4.

In some cases, T-loop phosphorylation is required for CDK/cyclin complex formation: T-loop phosphorylation is a pre-requisite for CDK1/cyclin B complex formation *in vivo* but CDK2 can form complexes with cyclins in the absence of T-loop phosphorylation [13]. CRK3 appears to be more like CDK2 in this regard since CRK3 can form active complexes with CYCA in the absence of phosphorylation of T178. However, based upon current results, it cannot be ruled out that phosphorylation of CRK3 before incubation with CYCA would increase the efficiency of complex formation and the observed kinase activity.

In a recent analysis of the phosphoproteome of bloodstream form *T. brucei*, CRK3 was found to be phosphorylated on T33 and Y34, sites that correspond to human CDK1 T14 and Y15 [42,43]. In humans phosphorylation of Y15 by the wee1 kinase is a negative regulator of protein kinase activity [44] and the presence of wee1 in both the trypanosome and *Leishmania* genomes would suggest that CRK3 is regulated by a similar mechanism. In contrast, no phosphorylation was detected on T-loop threonine residue of *T. brucei* CRK3 [42] and no CAK-like protein kinases have been identified in either the trypanosome or *Leishmania* genomes [4,5]. Whilst the lack of detection of a T178 CRK3 phosphopeptide does not rule out its presence in the cell, it is possible that the trypanosomatids have evolved alternative mechanisms to positively regulate CRK3 activity.

In contrast to the phosphorylation and activation of CRK3 by Civ1, none of the other leishmanial CRKs could be phosphorylated by Civ1 *in vitro*. This may simply reflect the fact that the sequence similarity across the T-loop between these CRKs and the natural Civ1 substrate is lower than for CRK3. However, none of these CRKs displayed any histone H1 kinase activity as monomers either. As the CRKs are likely to be cyclin-dependent [4,5], these are likely to have to bind their cognate cyclin partners and possibly also be phosphorylated by the leishmanial CAK before they can form an active kinase. Future work will strive to identify the cyclin partners for the remaining leishmanial CRKs.

In summary, this work reports that the leishmanial CDK, CRK3, can associate with and be activated by the cyclin, CYCA; that the T-loop Thr-178 residue is essential for kinase activity in vitro and that phosphorylation of T178 by the yeast CAK, Civ1, can further increase kinase activity, in an analogous fashion to mammalian CDKs, albeit to a much lesser degree than mammalian CDKs. These results demonstrate that the way in which CDK activity is controlled in other eukaryotes is conserved in *Leishmania* but that there may be significant differences in the relative importance of the different regulatory mechanisms in the parasite.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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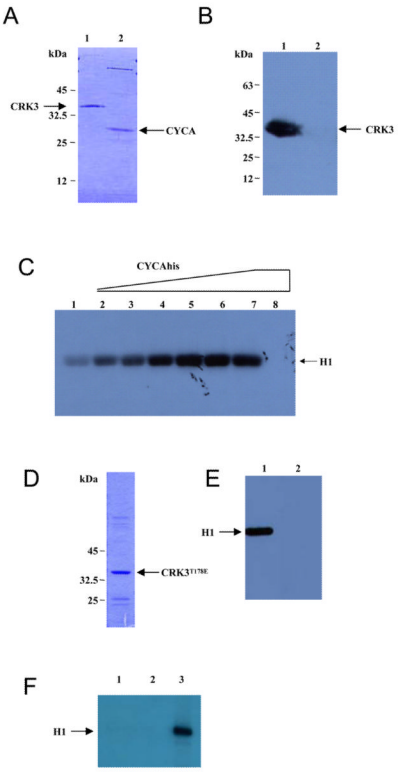

Figure 1. ***L. mexicana*** **CRK3:CYCA**

(A) SDS PAGE of CRK3his (lane 1) and CYCAhis (lane 2) purified from *E. coli* and stained with Coomassie blue R-250. (B) CYCAhis binds CRK3. Ni-NTA beads, with (lane 1) or without (lane 2) bound CYCAhis, were incubated with *E. coli* lysates expressing CRK3, washed, eluted and the eluted protein subjected to Western blot analysis with α-CRK3 antibody. (C) Activation of CRK3:CYCA. Phosphorylation of histone H1 by *L. mexicana* CRK3:CYCA was performed by mixing increasing quantities of CYCAhis (0 μg-3 μg in 0.5 μg increments from lanes 1-7) to a fixed amount of CRK3his (4 μg, lanes 1-7) in an *in vitro* kinase assay buffer containing 2.5 μg of histone H1 per reaction and γ-P^{32}-ATP. Lane 8 contains 3 μg CYCAhis only. Phosphorylated histone H1 was detected following SDS-PAGE and autoradiography. (D) SDS PAGE of $CRK3^{T178E}$his purified from *E. coli* and stained with Coomassie blue R-250. (E) $CRK3^{T178E}$his kinase assay with CYCA. 4 ug of CRK3his (lane 1) or $CRK3^{T178E}$his (lane 2) was incubated with 3 μg of CYCAhis and histone H1 kinase activity assessed as in panel C. H1; histone H1. (F) $CRK3^{T178E}$his kinase assay with CYC6. 3.5 μg of $CRK3^{T178E}$his (lanes 1 and 2) or CRK3his (lane 3) was incubated with (lanes 2 and 3) or without (lane 1) 3 μg of CYC6his and histone H1 kinase activity assessed as in panel C.

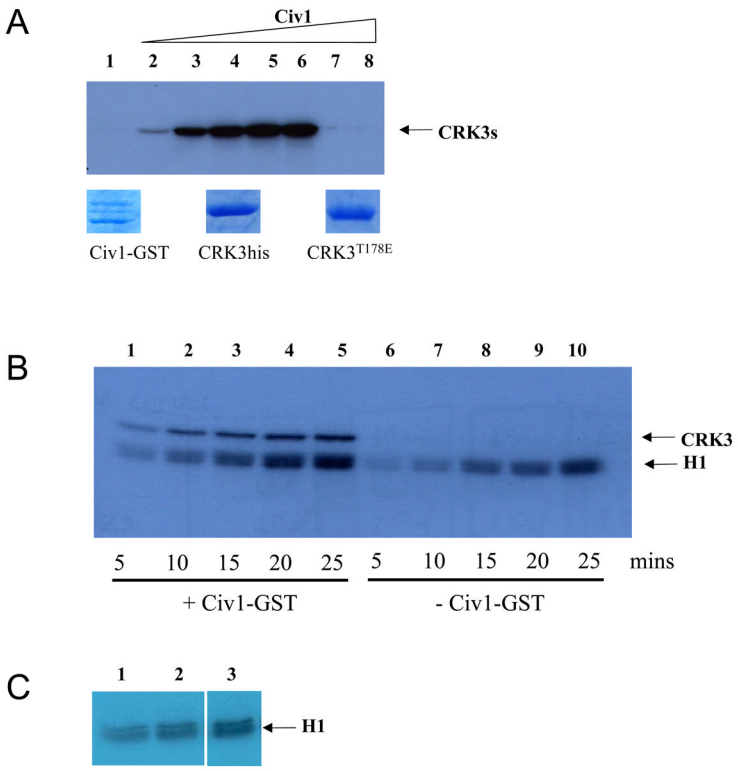

Figure 2. Phosphorylation of CRK3 with a CDK-activating kinase

(A) Upper panel. Phosphorylation of CRK3his or CRK3^{T178E}his by *S. cerevisiae* Civ1-GST. CRK3his (3 μg, lanes 1-7) or CRK3^{T178E}his (3 μg, lane 8) were incubated with increasing concentrations of Civ1-GST (lanes 1, 0 μg, lanes 2-6, 0.5μg increasing in 0.5μg increments, lanes 7 and 8, 2.5μg) for 30 mins in the presence of γ-P^{32}-ATP. Phosphorylated CRK3his or CRK3^{T178E}his was detected following SDS-PAGE and autoradiography. Lower panel. Coomassie blue R-250 stained protein used in the assay. (B) Histone H1 kinase assay. CRK3:CYCA complex (4 μg) was incubated with 0.5 μg Civ1-GST (lanes 1-5) or control buffer (lanes 6-10) for 15 mins prior to addition of histone H1 substrate. Samples were taken at 5, 10, 15 and 20 mins after addition of histone H1 and analysed by SDS-PAGE and autoradiography. (C) Histone H1 is not a Civ1-GST substrate. Histone H1 was incubated in the presence of Civ1-GST (0ug, lane 1; 0.3 μg in lane 2 and 1.8 μg in lane 3) for 30 mins in the presence of γ-P^{32}-ATP. Phosphorylated histone H1 was detected following SDS-PAGE and autoradiography.

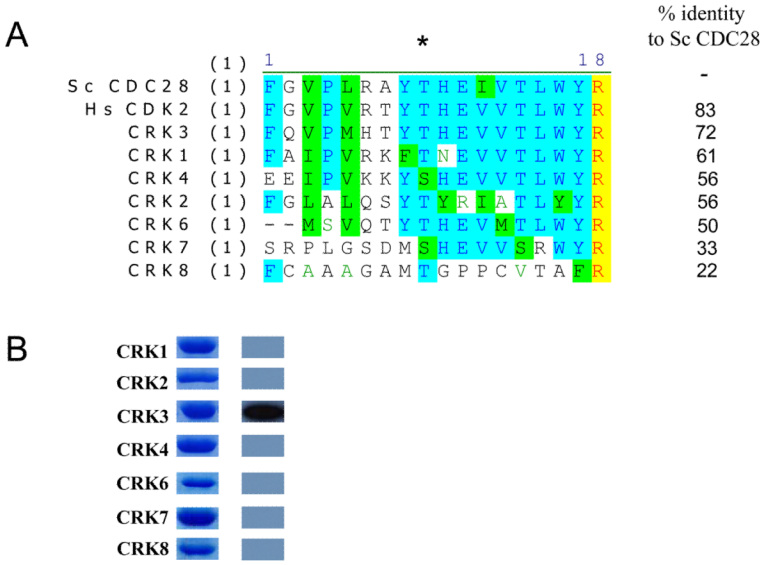

Figure 3. Phosphorylation of Leishmania CRKs with Civ1

(A) Sequence alignment of *L. major* CRK1-4, 6-8, *S. cerevisiae* CDC28, the natural substrate for Civ1, and human CDK2. The T-loop residue is indicated (*) (B) Phosphorylation of *L. major* CRKs by Civ1-GST. Left panel: purified recombinant histidine-tagged CRK proteins. Right panel: Phosphorylation by Civ1-GST.

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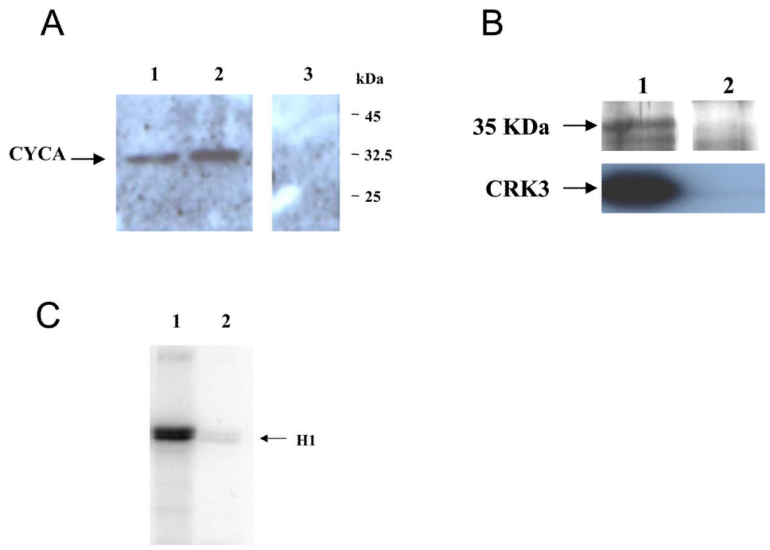

Figure 4. CRK3:CYCA in *L. major*

(A) Western blot of *L. major* promastigote cell lysates probed with anti-HA antibody. Lane 1: *L. major* [pXG-CYCA-HA], Lane 2: *L. major* [pXG-HA-CYCA], Lane 3, wild type *L. major* (B) Upper panel: Silver stained SDS-PAGE gel of protein eluted from anti-HA antibody affinity column. Lower panel. Western blot with anti-CRK3 antibody. Lane 1: *L. major* [pXG-HA-CYCA] Lane 2: wild type *L. major*. (C) histone H1 kinase assay with immuno-precipitated HA-CYCA Lane 1: *L. major* [pXG-HA-CYCA], Lane 2: wild type *L. major*. H1; histone H1

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Table 1

Gene	Destination vector	Primer	Primer sequence 5′ to 3′
***L. mexicana* CRK3**	pET28a	OL225	GAATTCCATATGTCTTCGTTTGGCCGTGTGA
***L. mexicana* CRK3**	pET28a	OL894	CTCGAGCTACCAACGAAGGTCGCTGA
***L. mexicana* CRK3**	Mutagenesis primer	OL877	CCCATGCACACCTAC**GAG**CACGAGGTGGTT ACG
***L. mexicana* CRK3**	Mutagenesis primer	OL878	CGTAACCACCTCGTG**CTC**GTAGGTGTGCAT GGG
***L. mexicana* CYCA**	pET21a	OL813	CATATGGCGGTCCCACTGCGAATG
***L. mexicana* CYCA**	pET21a	OL814	CTCGAGCGCAGAAGTTGAAATGAA
***L. major* CRK1**	pET15b	OL1783	CCATATGACCAGCCGGTACGAGCGGCA GGAGAAGATC
***L. major* CRK1**	pET15b	OL1784	CGGATCCCTAAAACTGGAGGCTAAAGTACG GGTG
***L. major* CRK2**	pET15b	OL1785	CCATATGCGGAGCAGCGGCCCCACCCCAGC GC
***L. major* CRK2**	pET15b	OL1786	CGGATCCTTACGACTGCTGCTGCTGCTGCTG CTG
***L. major* CRK3**	pET15b	OL1787	CCATATGTCTTCGTTTGGCCGTGTTACCGCCC
***L. major* CRK3**	pET15b	OL1788	CGGATCCCTACCAGCGAAGGTCACTGAACC ACGGG
***L. major* CRK4**	pET15b	OL1789	CCATATGTCGACGGCGGGTCGGTACAAGCA CG
***L. major* CRK4**	pET15b	OL1790	CGGATCCTCATAGCAAGTGGCAGGCCTCCA TCGTC
***L. major* CRK6**	pET15b	OL1791	CCATATGTCCGCGTCAGTGAACGACTTGGA TG
***L. major* CRK6**	pET15b	OL1792	CGGATCCCTACGCATCCTTCATAAAGGGGT GTTCC
***L. major* CRK7**	pET15b	OL1793	CCATATGGACAAGTACGCGTTGGGGCCG GTTATC
***L. major* CRK7**	pET15b	OL1794	CGGATCCTCATGCACGCAGCAAGGTATCTG AGAG
***L. major* CRK8**	pET15b	OL1795	CCATATGGGAGGGGAACTGGATAACCAGAAC
***L. major* CRK8**	pET15b	OL1796	CGGATCCTCAATGCTCCAGCTCCTTCCGCTT GACC

Gene	Destination vector	Primer	Primer sequence 5′ to 3′
L. mexicana **CYCA**	pXG	OL1935	CCC CGG GAT GGC GGT CCC ACT GCG AAT GAG GA
L. mexicana **CYCA**	pXG	OL1936	TGG ATC CTC AGG CAT AGT CCG GGA CGT CGT AGG GGT
L. mexicana **CYCA**	pXG	OL1937	CCC CGG GAT GTA CCC CTA CGA CGT CCC GGA CTA TGC
L. mexicana **CYCA**	pXG	OL1938	GTG GAT CCT CAC GCA GAA GTT GAA ATG A AA GG

Restriction endonuclease sites are underlined

Mutagenesis sites are in **bold**

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