*Manuscript
Click here to view linked References

Leishmania mexicana cyclin-dependent kinase complex CRK3:CYCA is active in the absence of T-loop phosphorylation

Felipe C. Gomes[1], Nahla Osman M. Ali [1,*], Elaine Brown[1], Roderick G. Walker[1], Karen M. Grant[2] and Jeremy C. Mottram[1,§]

[1]Wellcome Centre for Molecular Parasitology and Division of Infection & Immunity, Faculty of Biomedical and Life Sciences, University of Glasgow, 120 University Place, Glasgow, G12 8TA, UK

[2]School of Health & Medicine, Division of Medicine, Faraday Building, Lancaster University, Lancaster, LA1 4YA

*Current address: Department of Parasitology, Faculty of Veterinary Medicine, Shambat Campus P. O Box 32, University of Khartoum, Sudan

§ Author for correspondence: (email: j.mottram@bio.gla.ac.uk)

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 activated by CYCA and 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 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.

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 20ºC overnight, and purified as described previously [2]. For *L. mexicana* CYCA, BL21 (DE3) pLYS *E. coli* cells were transformed with plasmid pGL630. Cells were induced for protein expression at 19^{o}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) pLYS *E. coli* cells and induced with 1mM IPTG at 19^{o}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].

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

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 2100g. This column of Ni-NTA + CYCAhis was washed 2 x 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 1000g for 5 min. The column was washed 2X with PBS 7.4 and eluted in 100 µl fractions with phosphate buffer consisting of 100mM NaPi 7.4, 10mM NaCl and 0.5M imidazole (pH 8.0). 10 µl of each elution fraction was mixed with 10 µl Laemmli protein loading buffer 2 x 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 1000g for 10 min at 4^{o}C. The cell pellet was then washed twice in cold PBS and resuspended in 1ml of IP lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 0.1% Nonidet P40) containing protease inhibitors. To this lysis suspension, 50 µl of HA affinity purification matrix (Roche) was added and an overnight incubation at 4^{o}C with agitation was done. The matrix was then washed 3X 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 threonine residue in the T-loop of CDKs can be mimicked by substitution of the Thr residue with a negatively charged Glu residue [30]. To test if this was also the case for CRK3, site directed mutagenesis was carried

out on the conserved T-loop threonine residue (Thr 178) of CRK3his to produce CRK3^{T178E}his. Affinity purified CRK3^{T178E}his (Figure 1A, lane 3) lacked histone H1 kinase activity both in the absence (data not shown) and presence of CYCA (Figure 1D, lane 2). The results show that CYCAhis is able to activate CRK3his (Figure 1D, lane 1) but not CRK3^{T178E}his (lane 2), indicating that the mutation abolishes histone H1 kinase activity.

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 [2,31]. Although it is not known how many cyclins bind and activate CRK3 or the Thr178 phosphorylation status of CRK3 *in vivo*, the IC_{50} of CRK3 purified from *L. mexicana* promastigotes could be compared to the recombinant purified CRK3his:CYCAhis with two well established CDK inhibitors, flavopiridol [32] and indirubin-3′-monoxime [33]. IC_{50} values of 25 nM for flavopiridol and 33 nM for indirubin-3'-monoxime with CRK3his:CYCAhis were somewhat different from the IC50 values of 100nM [7] and 1.35μM [31] respectively for CRK3his affinity purified from *L. mexicana*.

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) confirming that

Thr178 in CRK3 was the 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 (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).

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 2C) 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 2C) were cloned into pET15b and expressed and purified from *E. coli* (Figure 2D). 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 2D). The purified monomeric CRKs were tested for histone H1 kinase activity, but only CRK3 showed any activity (monomeric CRK3 displays a low level of H1 activity in the absence of cyclin, see lane 1, Fig 1C; data not shown for other CRKs). 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 2C), and that other *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.

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 3A, 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 3B). The proteins immunoprecipitated from cell lysates were separated on a silver-stained SDS-PAGE gel and a protein corresponding to the expected size of HA tagged CYCA was immuno-precipitated from *L. major* [pXG-HA-CYCA] (Figure 3B 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 3B, 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 3C, 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 [31], 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 threonine in the regulation of CRK3 protein kinase activity.

Phosphorylation of the T-loop threonine in CDK1, CDK2 and CDK4 is required for full activation [13,14] and is associated with a dramatic increase in protein kinase activity [34]. This increased activity is explained by the conformational change

elicited by phosphorylation, which creates the substrate binding site and orientates ATP for phospho-transfer [15,35]. Mutation of the T-loop threonine to glutamic acid is thought to mimic phosphorylation at this site and has been shown to result in the activation of certain protein kinases that require T-loop phosphorylation, including a CDK from *Plasmodium falciparum* [30,36]. Mutation of the T-loop residue of CRK3 to glutamic acid ($CRK3^{T178E}$), however, did not activate the enzyme; instead it abolished protein kinase activity both in the absence and presence of CYCA. This result is consistent with what is observed for other CDKs; the T-loop threonine is essential for catalytic activity in CDK1 [37], CDK2 [38] and *S. cerevisiae* CDC28 [39]. 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 $CRK3^{T178E}$, confirming T178 as 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 [34], 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 2C, CRK3 72% identical to CDC28) and CRK3 is a 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 [40].

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 T-loop phosphorylation. 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 [41,42]. In humans phosphorylation of Y15 by the wee1 kinase is a negative regulator of protein kinase activity [43] 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 [41] and no CAK-like protein kinases have been identified in either the trypanosome or *Leishmania* genomes [4,5]. Thus 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 threonine residue is essential for kinase activity and that T-loop phosphorylation 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.

Acknowledgements. We thank Jane Endicott for the Civ1-GST construct. This work was funded by the Medical Research Council [grant numbers G9722968 and G0400028]. FG was a recipient of a Coordenação de Aperfeiçoamento de Passoal de Nivel Superior (CAPES) Fellowship from the Brazilian Government.

Figure legends

Figure 1. *L. mexicana* CRK3:CYCA (A) SDS PAGE of CRK3his (lane 1), CYCAhis (lane 2) and CRK3^{T178E}his (lane 3) purified from *E. coli* and stained with Coomassie blue R-250. (B) CYCAhis binds CRK3. Ni-NTA beads, with (lane 1) and without (lane 2) bound CYCAhis, were incubated with *L. mexicana* promastigote cell extracts, 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 (0.5μg-3μg in 0.5μg increments from lanes 2-8) to a fixed amount of CRK3his (4μg, lanes 1-7) in an *in vitro* kinase assay buffer containing 2.5μg of histone H1 per reaction and γ-P^{32}-ATP. Phosphorylated histone H1 was detected following SDS-PAGE and autoradiography. (D) CRK3^{T178E}his kinase assay. 4 ug of CRK3his (lane 1) or CRK3^{T178E}his (lane 2) was incubated with 3ug of CYCAhis and histone H1 kinase activity assessed as in panel C. H1; histone H1.

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 (3μg, lanes 1-7) or CRK3^{T178E}his (3μg, lane 8) were incubated with increasing concentrations of Civ1-GST (lanes 1, 0μg, lanes 2-6, 0.5μg increasing in 0.5μg increments, lanes 7 and 8, 2.5μg) 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 (4μg) was preincubated with 0.5 μg Civ1-GST (lanes 1-5) or control buffer (lanes 6-10) prior to addition of histone H1 substrate. Samples were taken at 5, 10, 15 and 20 mins and analysed by SDS-PAGE and autoradiography. (C) 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 (*) (D) Phosphorylation of *L. major* CRKs by Civ1-GST. Left panel: purified recombinant histidine-tagged CRK proteins. Right panel: Phosphorylation by Civ1-GST.

Figure 3. 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

Reference List

[1] Mottram, J. C. and Grant, K. M. *Leishmania mexicana* $p12^{cks1}$, a functional homologue of fission yeast $p13^{suc1}$, associates with a stage-regulated histone H1 kinase. Biochem. J. 1996;316:833-839.

[2] Grant, K. M., Hassan, P., Anderson, J. S. and Mottram, J. C. The *crk3* gene of *Leishmania mexicana* encodes a stage-regulated cdc2-related histone H1 kinase that associates with $p12^{cks1}$. J. Biol. Chem. 1998;273:10153-10159.

[3] Siman-Tov, M. M., Ivens, A. C. and Jaffe, C. L. Identification and cloning of *Lmairk*, a member of the Aurora/Ipllp protein kinase family, from the human protozoan parasite *Leishmania*. Biochimica et Biophysica Acta: Gene Structure and Expression 2001;1519:241-245.

[4] Naula, C., Parsons, M. and Mottram, J. C. Protein kinases as drug targets in trypanosomes and *Leishmania*. Biochimica et Biophysica Acta: Proteins and proteomics 2005;1754:151-159.

[5] Parsons, M., Worthey, E. A., Ward, P. N. and Mottram, J. C. Comparative analysis of the kinomes of three pathogenic trypanosomatids; *Leishmania major*, *Trypanosoma brucei* and *Trypanosoma cruzi*. BMC. Genomics 2005;6:127.

[6] Mottram, J. C., McCready, B. P., Brown, K. P. and Grant, K. M. Gene disruptions indicate an essential function for the LmmCRK1 cdc2-related kinase of *Leishmania mexicana*. Mol. Microbiol. 1996;22:573-582.

[7] Hassan, P., Fergusson, D., Grant, K. M. and Mottram, J. C. The CRK3 protein kinase is essential for cell cycle progression of *Leishmania mexicana*. Mol. Biochem. Parasitol. 2001;113:189-198.

[8] Malumbres, M. and Barbacid, M. Mammalian cyclin-dependent kinases. Trends Biochem Sci 2005;30:630-641.

[9] Fung, T. K. and Poon, R. Y. A roller coaster ride with the mitotic cyclins. Semin. Cell Dev. Biol 2005;16:335-342.

[10] Parker, L. L. and Piwinica-Worms, H. Inactivation of the p34(cdc2)-cyclin-b complex by the human WEE1 tyrosine kinase. Science 1992;257:1955-1957.

[11] Mueller, P. R., Coleman, T. R., Kumagai, A. and Dunphy, W. G. Myt1: A membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. Science 1995;270:86-90.

[12] Nilsson, I. and Hoffmann, I. Cell cycle regulation by the Cdc25 phosphatase family. Prog. Cell Cycle Res. 2000;4:107-114.

[13] Larochelle, S., Merrick, K. A., Terret, M. E., Wohlbold, L., Barboza, N. M., Zhang, C., Shokat, K. M., Jallepalli, P. V. and Fisher, R. P. Requirements for Cdk7 in the assembly of Cdk1/cyclin B and activation of Cdk2 revealed by chemical genetics in human cells. Mol Cell 2007;25:839-850.

[14] Kato, J.-Y., Matsuoka, M., Strom, D. K. and Sherr, C. J. Regulation of cyclin D-dependent kinase 4 (cdk4) by cdk4- activating kinase. Mol. Cell. Biol. 1994;14:2713-2721.

[15] Bartova, I., Otyepka, M., Kriz, Z. and Koca, J. Activation and inhibition of cyclin-dependent kinase-2 by phosphorylation; a molecular dynamics study reveals the functional importance of the glycine-rich loop. Protein Sci 2004;13:1449-1457.

[16] Bockstaele, L., Bisteau, X., Paternot, S. and Roger, P. P. Differential regulation of cyclin-dependent kinase 4 (CDK4) and CDK6, evidence that CDK4 might not be activated by CDK7, and design of a CDK6 activating mutation. Mol Cell Biol 2009;29:4188-4200.

[17] Devault, A., Martinez, A. M., Fesquet, D., Labbé, J. C., Morin, N., Tassan, J. P., Nigg, E. A., Cavadore, J. C. and Dorée, M. MAT1 ('*menage a trois*') a new RING finger protein subunit stabilizing cyclin H-cdk7 complexes in starfish and *Xenopus* CAK. EMBO J. 1995;14:5027-5036.

[18] Thuret, J. Y., Valay, J. G., Faye, G. and Mann, C. Civ1 (CAK in vivo), a novel Cdk-activating kinase. Cell Cell 1996;86:565-576.

[19] Kaldis, P., Pitluk, Z. W., Bany, I. A., Enke, D. A., Wagner, M., Winter, E. and Solomon, M. J. Localization and regulation of the cdk-activating kinase (Cak1p) from budding yeast. J. Cell Sci. 1998;111:3585-3596.

[20] Kaldis, P., Russo, A. A., Chou, H. S., Pavletich, N. P. and Solomon, M. J. Human and yeast Cdk-activating kinases (CAKs) display distinct substrate specificities. Mol. Biol. Cell 1998;9:2545-2560.

[21] Fesquet, D., Labbé, J.-C., Derancourt, J., Capony, J.-P., Galas, S., Girard, F., Lorca, T., Shuttleworth, J., Dorée, M. and Cavadore, J.-C. The *MO15* gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. EMBO J. 1993;12:3111-3121.

[22] Aprelikova, O., Xiong, Y. and Liu, E. T. Both p16 and p21 families of cyclin-dependent kinase (CDK) inhibitors block the phosphorylation of cyclin-dependent kinases by the CDK-activating kinase. J. Biol. Chem. 1995;270:18195-18197.

[23] Foley, E., O'Farrell, P. H. and Sprenger, F. Rux is a cyclin-dependent kinase inhibitory (CKI) specific for mitotic cyclin-Cdk complexes. Curr. Biol. 1999;9:1392-1402.

[24] Brown, N. R., Noble, M. E., Lawrie, A. M., Morris, M. C., Tunnah, P., Divita, G., Johnson, L. N. and Endicott, J. A. Effects of phosphorylation of threonine 160 on cyclin-dependent kinase 2 structure and activity. J. Biol. Chem. 1999;274:8746-8756.

[25] Wang, Y. X., Dimitrov, K., Garrity, L. K., Sazer, S. and Beverley, S. M. Stage-specific activity of the *Leishmania major CRK3* kinase and functional rescue of a *Schizosaccharomyces pombe cdc2* mutant. Mol. Biochem. Parasitol. 1998;96:139-150.

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

[27] Besteiro, S., Tonn, D., Tetley, L., Coombs, G. H. and Mottram, J. C. The AP3 adaptor is involved in the transport of membrane proteins to acidocalcisomes of *Leishmania*. J. Cell Sci. 2008;121:561-570.

[28] LeBowitz, J. H., Coburn, C. M., McMahon-Pratt, D. and Beverley, S. M. Development of a stable *Leishmania* expression vector and application to the study of parasite surface-antigen genes. Proc. Natl. Acad. Sci. USA 1990;87:9736-9740.

[29] Robinson, K. A. and Beverley, S. M. Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite *Leishmania*. Mol. Biochem. Parasitol. 2003;128:217-228.

[30] Levin, L. R. and Zoller, M. J. Association of catalytic and regulatory subunits of cyclic AMP-dependent protein kinase requires a negatively charged side group at a conserved threonine. Mol Cell Biol 1990;10:1066-1075.

[31] Grant, K. M., Dunion, M. H., Yardley, V., Skaltsounis, A.-L., Marko, D., Eisenbrand, G., Croft, S. L., Meijer, L. and Mottram, J. C. Inhibitors of *Leishmania mexicana* CRK3 cyclin-dependent kinase: chemical library screen and antileishmanial activity. Antimicrob. Agents Chem. 2004;48:3033-3042.

[32] Losiewicz, M. D., Carlson, B. A., Kaur, G., Sausville, E. A. and Worland, P. J. Potent inhibition of CDC2 kinase activity by the flavonoid L86-8275. Biochem. Biophys. Res. Commun. 1994;201:589-595.

[33] Hoessel, R., Leclerc, S., Endicott, J. A., Nobel, M. E. M., Lawrie, A., Tunnah, P., Leost, M., Damiens, E., Marie, D., Marko, D., Niederberger, E., Tang, W. C., Eisenbrand, G. and Meijer, L. Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. Nature Cell Biology 1999;1:60-67.

[34] Connell-Crowley, L., Solomon, M. J., Wei, N. and Harper, J. W. Phosphorylation independent activation of human cyclin-dependent kinase 2 by cyclin A in vitro. Mol. Biol. Cell 1993;4:79-92.

[35] Stevenson, L. M., Deal, M. S., Hagopian, J. C. and Lew, J. Activation mechanism of CDK2: role of cyclin binding versus phosphorylation. Biochemistry 2002;41:8528-8534.

[36] Graeser, R., Franklin, R. M. and Kappes, B. Mechanism of activation of the cdc2-related kinase PfPK5 from *Plasmodium falciparum*. Mol. Biochem. Parasitol. 1996;79:125-127.

[37] Ducommun, B., Brambilla, P., Felix, M. A., Franza, B. R., Karsenti, E. and Draetta, G. Cdc2 phosphorylation is required for its interaction with cyclin. EMBO J. 1991;10:3311-3319.

[38] Abbas, T., Jha, S., Sherman, N. E. and Dutta, A. Autocatalytic phosphorylation of CDK2 at the activating Thr160. Cell Cycle 2007;6:843-852.

[39] Cross, F. R. and Levine, K. Genetic analysis of the relationship between activation loop phosphorylation and cyclin binding in the activation of the *Saccharomyces cerevisiae* Cdc28p cyclin-dependent kinase. Genetics 2000;154:1549-1559.

[40] Nolen, B., Taylor, S. and Ghosh, G. Regulation of protein kinases; controlling activity through activation segment conformation. Mol Cell 2004;15:661-675.

[41] Nett, I. R., Martin, D. M., Miranda-Saavedra, D., Lamont, D., Barber, J. D., Mehlert, A. and Ferguson, M. A. The phosphoproteome of bloodstream form *Trypanosoma brucei*, causative agent of African sleeping sickness. Mol Cell Proteomics 2009;8:1527-1538.

[42] Nett, I. R., Davidson, L., Lamont, D. and Ferguson, M. A. Identification and specific localization of tyrosine-phosphorylated proteins in *Trypanosoma brucei*. Eukaryot. Cell 2009;8:617-626.

[43] Morgan, D. O. Principles of CDK regulation. Nature 1995;374:131-134.

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**CACGAGGTGGTTACG
***L. mexicana* CRK3**	Mutagenesis primer	OL878	CGTAACCACCTCGTG**CTC**GTAGGTGTGCATGGG
***L. mexicana* CYCA**	pET21a	OL813	CATATGGCGGTCCCACTGCGAATG
***L. mexicana* CYCA**	pET21a	OL814	CTCGAGCGCAGAAGTTGAAATGAA
***L. major* CRK1**	pET15b	OL1783	CCATATGACCAGCCGGTACGAGCGGCAGGAGAAGATC
***L. major* CRK1**	pET15b	OL1784	CGGATCCCTAAAACTGGAGGCTAAAGTACGGGTG
***L. major* CRK2**	pET15b	OL1785	CCATATGCGGAGCAGCGGCCCCACCCCAGCGC
***L. major* CRK2**	pET15b	OL1786	CGGATCCTTACGACTGCTGCTGCTGCTGCTGCTG
***L. major* CRK3**	pET15b	OL1787	CCATATGTCTTCGTTTGGCCGTGTTACCGCCC
***L. major* CRK3**	pET15b	OL1788	CGGATCCCTACCAGCGAAGGTCACTGAACCACGGG
***L. major* CRK4**	pET15b	OL1789	CCATATGTCGACGGCGGGTCGGTACAAGCACG
***L. major* CRK4**	pET15b	OL1790	CGGATCCTCATAGCAAGTGGCAGGCCTCCATCGTC
***L. major* CRK6**	pET15b	OL1791	CCATATGTCCGCGTCAGTGAACGACTTGGATG
***L. major* CRK6**	pET15b	OL1792	CGGATCCCTACGCATCCTTCATAAAGGGGTGTTCC
***L. major* CRK7**	pET15b	OL1793	CCATATGGACAAGTACGCGTTGGGGCCGGTTATC
***L. major* CRK7**	pET15b	OL1794	CGGATCCTCATGCACGCAGCAAGGTATCTGAGAG
***L. major* CRK8**	pET15b	OL1795	CCATATGGGAGGGGAACTGGATAACCAGAAC
***L. major* CRK8**	pET15b	OL1796	CGGATCCTCAATGCTCCAGCTCCTTCCGCTTGACC
***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 AAA GG
Restriction endonuclease sites are underlined			
Mutagenesis sites are in **bold**			

Figure 1

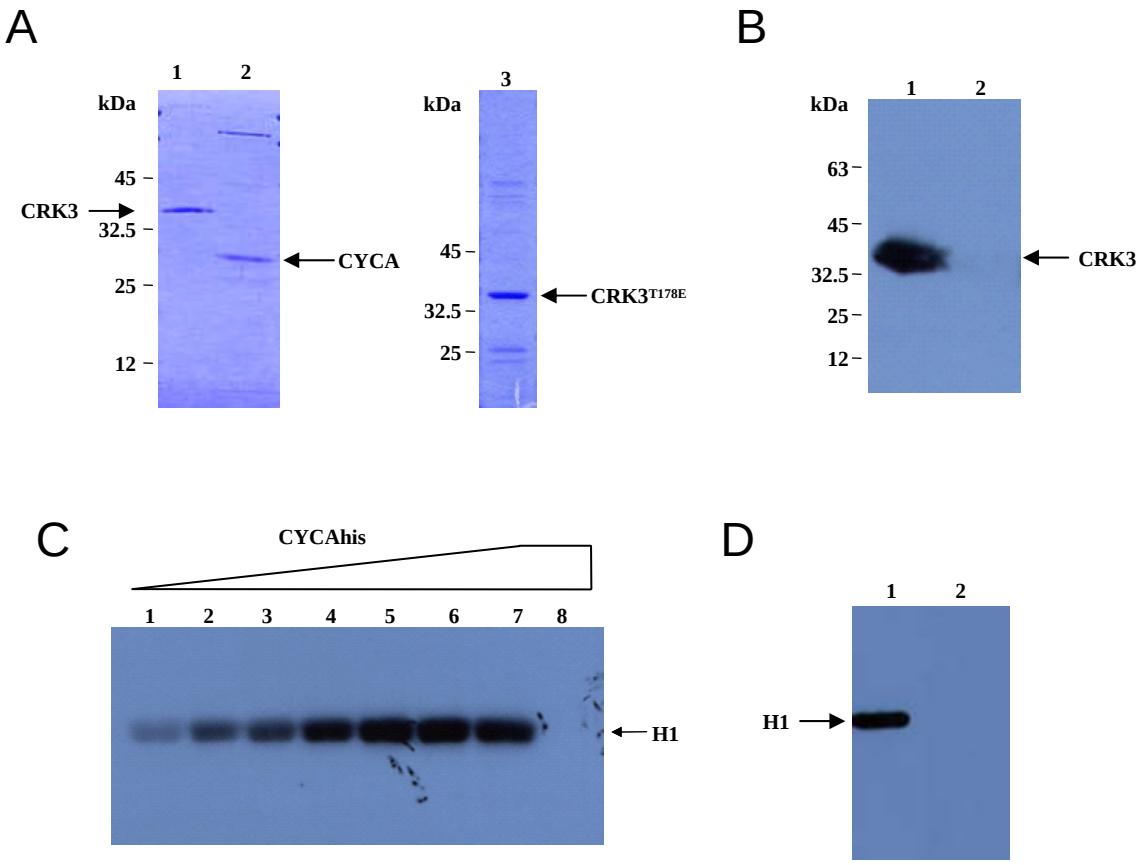
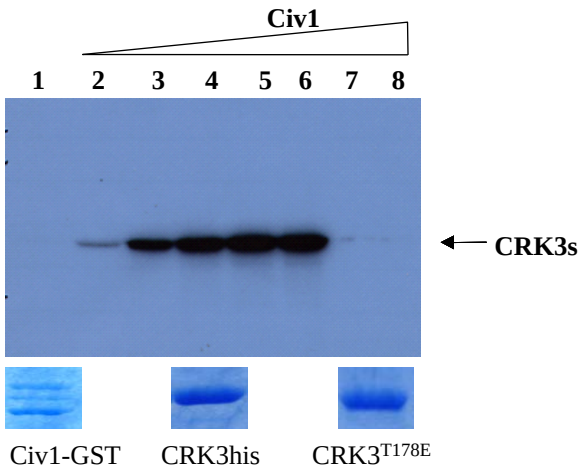
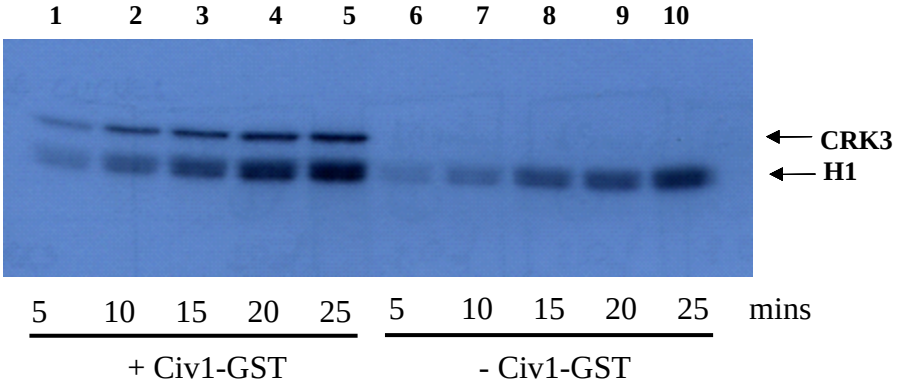

A
1
2
kDa
45
CRK3
32.5
25
12
CYCA
3
kDa
45
32.5
25
CRK3T178E
B
1
2
kDa
63
45
32.5
25
12
CRK3
C
CYCAhis
1
2
3
4
5
6
7
8
H1
D
1
2
H1

Figure 2

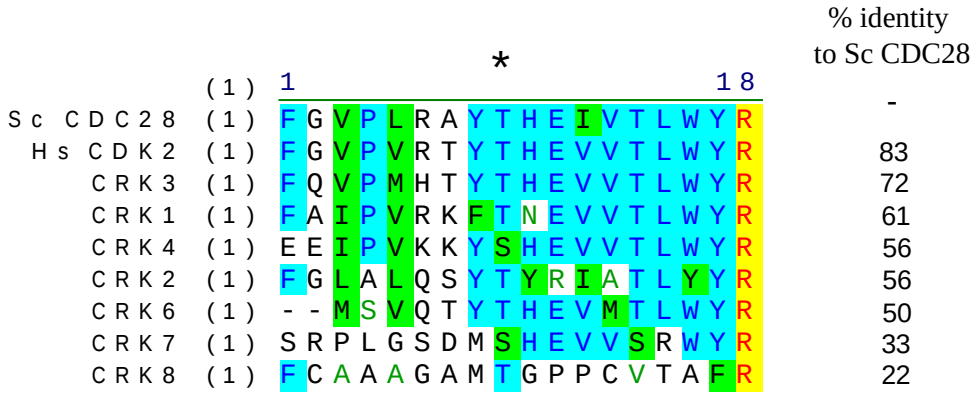
A



B



C



% identity to Sc CDC28

Sc CDC28	-
Hs CDK2	83
CRK3	72
CRK1	61
CRK4	56
CRK2	56
CRK6	50
CRK7	33
CRK8	22

D

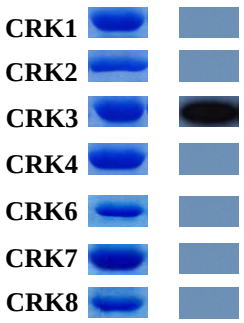
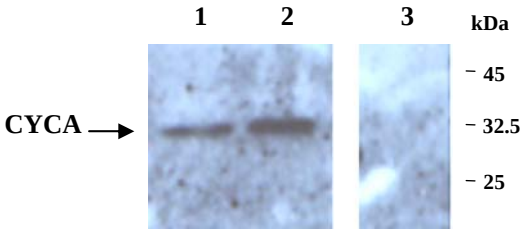
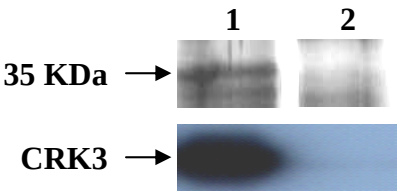


Figure 3

A



B



C