Leishmania mexicana p12^{cks1}, a homologue of fission yeast p13^{suc1}, associates with a stage-regulated histone H1 kinase

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We have isolated a *Leishmania mexicana* homologue of the fission yeast *suc1* gene using PCR with oligonucleotides designed to conserved regions of **c**dc2 **k**inase **s**ubunits (*cks*). The product of *cks1* is a 12 kDa polypeptide, which has 70% identity with human p9^{cks1} and 44% identity with fission yeast p13^{suc1}. p12^{cks1} was detected in the three life-cycle stages of *L. mexicana* by immunoblotting. Recombinant p12^{cks1} (p12^{cks1his}) bound to agarose beads was used as a matrix to affinity-select histone H1 kinase complexes from *Leishmania*, yeast and bovine extracts. Immunoblotting showed that yeast and bovine cdc2 kinase bound to p12^{cks1his}, thus demonstrating functional homology between *L. mexicana* p12^{cks1} and yeast p13^{suc1}. Histone H1 kinase activity was found at a high level in the proliferative promastigote and amastigote forms of *L. mexicana*, but at a low level in the non-dividing metacyclic form. These activities are likely to be the same as the leishmanial p13^{suc1} binding kinase (SBCRK) described previously [Mottram, Kinnaird, Shiels, Tait and Barry (1993) J. Biol. Chem. **268**, 21044–21051]. A distinct cdc2-related kinase, *L. mexicana* CRK1, was also found to associate with p12^{cks1his} but affinity-depletion experiments showed that CRK1 was not responsible for the histone H1 kinase activity associating with p12^{cks1his} in promastigote cell extracts. The finding that p12^{cks1} associates with at least two cdc2-related kinases, SBCRK and CRK1, is consistent with the presence of a large gene family of cdc2-related kinases in trypanosomatids, a situation thought to be more similar to higher eukaryotes than yeast.

INTRODUCTION

Cyclin-dependent kinases (cdks) are serine-threonine kinases that play pivotal roles in the control of the eukaryotic cell cycle (reviewed in [1]). In yeast, cell-cycle progression is predominantly regulated by one cdk, p34^{cdc2} in *Schizosaccharomyces pombe* or p34^{CDC28} in *Saccharomyces cerevisiae* [2,3], whereas in higher eukaryotes many cdks are involved (cdk1–8) [1,4]. cdk activity is post-translationally regulated by several mechanisms; positively and negatively by phosphorylation/dephosphorylation at various key residues [5–8], by association with cyclins (which are positive regulatory subunits) [9,10] and cdk inhibitors (reviewed in [11]). This cell-cycle regulatory mechanism is conserved throughout evolution with homologues of p34^{cdc2} and cyclins, determined both by sequence similarity and by complementation of yeast mutants, found in a wide range of eukaryotes.

The fission yeast *suc1* gene encodes an essential protein (p13^{suc1}) which interacts with p34^{cdc2} and was first identified as a plasmid-borne suppressor of certain temperature-sensitive cdc2 mutants [12]. The budding yeast homologue of *suc1*, *CKS1* (**c**dc2 **k**inase **s**ubunit 1), was identified in a similar manner, as an extragenic suppressor of some *CDC28* mutations, and encodes a protein with high sequence identity to p13^{suc1} [13]. Two human homologues of *suc1*, *ckshs1* and *ckshs2* [14], have been described. The human and fission yeast *cks* genes are capable of rescuing null mutations of the *Saccharomyces cerevisiae CKS1* gene [14], indicating their functional homology. More recently sequence homologues of *suc1* have been cloned from *Patella vulgata* [15] and the myxomycete *Physarum polycephalum* [16], and a protein which cross-reacts with anti-p13^{suc1} sera, and binds cdk4 and cdk5, has been isolated from starfish oocytes [17]. Thus cks proteins appear to be one of the highly conserved molecules involved in control of the eukaryotic cell cycle.

In yeast, p13^{suc1} is a component of the p34^{cdc2} kinase complex and the level of association between the two proteins is unaffected by entry into stationary phase [18]. Although p13^{suc1} has a high affinity for p34^{cdc2} *in vitro* [19] and the concentration of the *suc1* gene product does not vary through the cell cycle [18], only a small percentage of p34^{cdc2} is found in association with p13^{suc1} *in vivo* [18,20]. The high affinity with which p13^{suc1} binds p34^{cdc2} *in vitro* has been exploited in the isolation of cdc2 kinase complexes on p13^{suc1}–Sepharose affinity beads from a variety of organisms [18,21–23]. The specificity of binding is high and p13^{suc1} or p9^{cks1} has been shown to bind higher eukaryotic cdc2 and cdk2 strongly [24,25], cdk3 weakly [24], and other cdks apparently not at all [24]. The structures of p13^{suc1}, human p9^{cks1}, and human p9^{cks2} have recently been solved by X-ray crystallography, revealing some similar features between p13^{suc1} and human p9^{cks2}, but also some striking differences between the two human cks proteins [26–29]. Human p9^{cks1} has a different subunit conformation and assembly characteristics from p9^{cks2} but is surprisingly similar to the N-terminal fold of cdks [29]. p13^{suc1} can form several distinct conformational states, including a β-interchanged dimer and a β-hairpin single-domain monomer [28]. Changes in cks conformation during the cell cycle may be important for regulating cdk function [28,29].

Leishmania is a parasitic protozoan and the causative agent of a group of human diseases, the leishmaniases, which are prevalent in the tropics and subtropics. *Leishmania* has a biphasic life cycle: a motile extracellular form, found in the sandfly vector, and a non-motile intracellular form in the mammalian host [30]. After ingestion of infected blood by the sandfly, the parasite

Abbreviations used: cdk, cyclin-dependent kinase; *cks1*, *Leishmania mexicana* **c**dc2 **k**inase **s**ubunit **1** gene; p12^{cks1}, the protein product of *cks1*; p12^{cks1his}, recombinant p12^{cks1} which has a histidine tag at the C-terminus; *crk1*, *Leishmania mexicana* **c**dc2-**r**elated **k**inase **1** gene; CRK1, the gene product of *crk1*; FCS, fetal calf serum; Ni-NTA, Ni-nitrilotriacetic acid; ORF, open reading frame.

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Nucleotide sequence data reported in this paper are available in the EMBL database under the accession number Z49881.

initially develops into rapidly dividing promastigotes in the fly midgut from whence they migrate to the proboscis where they are found as metacyclic promastigotes, a non-proliferative infective form pre-adapted for survival in the mammalian host. After inoculation into the mammal, the metacyclics invade host macrophage cells where they differentiate into proliferative amastigotes within phagolysosomal vacuoles. The life cycle is completed when infected macrophages or amastigotes free in the blood are ingested by the sandfly. The metacyclic stage is cell-cycle arrested, probably in the G_1 phase, and release from this block occurs as the parasite differentiates to an amastigote, within the host macrophage.

The trypanosomatids have a large gene family of cdc2-related kinases [31,32] including two from *L. mexicana*, *crk1* [33] and *crk3* (J. C. Mottram, unpublished work). In addition, a distinct *L. mexicana* cdc2-related kinase (SBCRK), not apparently encoded by either of the *crk* genes, has been characterized biochemically [33]. SBCRK binds $p13^{suc1}$ and is active in dividing but not in non-dividing stages of the parasite and is potentially the functional *L. mexicana* cdc2 homologue. To further the analysis of SBCRK we have isolated an *L. mexicana* homologue of the *Schizosaccharomyces pombe suc*1 gene. $p12^{cks1}$ protein product of the *cks1* gene binds leishmanial SBCRK and cdc2 from yeast and bovine lymphocytes with high affinity.

MATERIALS AND METHODS

Parasites

Leishmania mexicana mexicana (MNYC/BZ/62/M379) wild-type promastigotes were grown in HOMEM medium [+10% fetal calf serum (FCS)] to mid-logarithmic phase of growth (5×10^6 cells/ml) [34], metacyclic promastigotes were grown at 25 °C in Schneider's medium (+20% FCS, pH 5.5) to stationary phase of growth (approx. 2×10^7 cells/ml) [35] and amastigotes were grown either as subcutaneous lesions in CBA mice, harvested and purified as described [34], or in culture in Schneider's medium (+20% FCS, pH 5.5) at 32 °C as amastigote-like forms [35]. BL20 bovine lymphocytes were grown in RPMI medium as described [36]. *Saccharomyces cerevisiae*, strain Y187 [37], was grown in YEPD medium.

Cloning of *L. mexicana cks1*

The oligonucleotide primers used in the PCR reactions for cloning the *cks1* gene were: 5′ primer 5′-AGAG*CT*CGAGTAYMGNCAYGTNATGYTNCC-3′; and 3′ primer 5′-GAGGAATTCAANARNARDATRTGNGGYTC-NGG-3′ corresponding to amino acid sequences EYRHVMLP and PEPHILLF (where N = G+A+T+C, Y = C+T, M = A+C, R = A+G and D = G+T+A). *Xho*I and *Eco*RI restriction sites are underlined. The PCR reactions were for 30 cycles of 94 °C for 1 min, 45 °C for 2 min and 72 °C for 2 min with 100 ng of *L. mexicana* genomic DNA using *Taq* DNA polymerase (Promega). PCR products of 200–250 bp were digested with *Xho*I and *Eco*RI, gel-purified and cloned into Bluescript plasmid. PCR clones were sequenced with Sequenase® (Amersham) to confirm their identity.

To isolate the full-length *cks1* gene an *L. mexicana* lambda EMBL 4 library [33] was screened with radiolabelled cloned PCR product. Positive lambda phage were taken through two rounds of plaque purification and DNA isolated (lambda Wizard miniprep, Promega). A 2.3 kb *Sal*I fragment (one *Sal*1 site of which was vector derived) containing the complete *cks1* gene was subcloned into Bluescript and parts of the insert containing *cks1* sequenced, with specific oligonucleotide primers and subclones, on both strands using Sequenase®. Sequence analysis was performed using the Wisconsin GCG package [38].

Southern blotting was performed as previously described [33] using the radiolabelled cloned PCR product as a probe.

Production of recombinant $p12^{cks1}$

cks1 was inserted into the pQE60 (Qiagen) vector by constructing unique *Nco*I and *Bgl*II sites on the 5′ and 3′ ends of the coding regions using PCR. Pfu polymerase (Stratagene) was used to generate the fragments. One of the cloned PCR fragments was sequenced to check that no PCR-induced mutations were introduced. M15 [pREP4] *Escherichia coli* (Qiagen) was transformed with the plasmid and recombinant $p12^{cks1his}$ purifed by metal chelate affinity chromatography, as follows. M15[pREP4] pQE60cks1 cells were induced with 1 mM isopropyl-β-thiogalactoside (5 h, 37 °C), harvested and resuspended in SB (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl). Cell suspensions were treated with lysozyme (1 mg/ml, 37 °C, 15 min), freeze/thawed (−80 °C) and subjected to sonication. Cellular debris was cleared by centrifugation (40000 ***g***, 30 min) and the supernatant applied to an Ni-nitrilo-triacetic acid (Ni-NTA)–agarose column (previously equilibrated with SB) at 0.2 ml/min. Unbound protein was washed from the column with SB, at 0.5 ml/min, and then with WB [50 mM sodium phosphate buffer, pH 6.0, 300 mM NaCl, 10% (v/v) glycerol] at 0.5 ml/min. Bound material was eluted with a linear gradient of 0 to 500 mM imidazole in WB at a flow rate of 0.5 ml/min. Fractions containing the majority of the $p12^{cks1his}$ were pooled, dialysed overnight against 50 mM sodium phosphate buffer, pH 6.0, 300 mM NaCl, and re-applied to the Ni-NTA–agarose column (equilibrated with WB) at 0.5 ml/min. The column was washed with WB, at 0.5 ml/min, and then bound $p12^{cks1his}$ was eluted with a linear imidazole gradient as before. $p12^{cks1his}$-containing fractions were pooled and dialysed extensively against 100 mM sodium phosphate buffer, pH 7.0, containing 0.05% NaN_3, prior to concentration and cross-linking to AminoLink beads (Pierce) at a concentration of 5 mg/ml. Control beads were prepared by cross-linking a solution of 1 M Tris, pH 7.4, in place of recombinant protein as described by the manufacturers (Pierce).

Production of $p12^{cks1}$-specific antiserum

Polyclonal antisera was raised in rabbits, using $p12^{cks1his}$ as an immunogen, following standard immunization protocols [39]. The serum was initially partially purified on a Protein A/G column using the ImmunoPure (A/G) IgG purification kit (Pierce), according to the manufacturer's protocol. Further purification was performed on a $p12^{cks1his}$ affinity column using the ImmunoPure Gentle Ag/Ab buffer system (Pierce) according to the manufacturer's protocol. Antibodies eluted from this column were desalted into TBS (50 mM Tris, pH 7.5, 137 mM NaCl, 0.02% NaN_3), using Presto® cross-linked dextran desalting columns (Pierce). A sample (20 μg) of S-100 cell extract prepared from each life-cycle stage (see below), or 100 ng of fusion protein were separated by Tricine SDS/PAGE [40] and transferred on to polyvinylidene difluoride membrane. Western blots were performed with affinity-purified $p12^{cks1his}$ antibodies, used at 1:50 dilution as described [33].

Histone H1 kinase assays

Leishmanial and bovine cell extracts were prepared by resuspending cells in lysis solution plus inhibitors (50 mM Mops, pH 7.2, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM NaF, 1% Triton X-100, 10%

glycerol, 1 mM 1,10-phenanthroline, 5 μg/ml pepstatin A, 100 μg/ml leupeptin, 500 μg/ml pefabloc SC), incubating on ice for 30 min and clearing by centrifugation (100000 ***g***, 45 min, 4 °C) to give an S-100 extract. Yeast were disrupted in the same lysis buffer by vortexing for 5 min with 50 % glass beads (50 μm-diam., Sigma). Cell extracts were normalized for protein concentration and incubated with affinity beads (4 °C, 2 h) and then unbound protein was removed by extensive washing with: lysis solution; high-salt lysis solution (500 mM NaCl); and lysis solution minus glycerol. The beads were finally washed with kinase assay buffer (KAB: 50 mM Mops, pH 7.2, 20 mM $MgCl_2$, 2 mM dithiothreitol, 10 mM EGTA). The kinase activity of bound proteins was assessed as described previously [33]. Aliquots of the stopped kinase reaction were separated by SDS/PAGE, and the gel was dried and exposed to autoradiography film, to assess the degree of histone H1 phosphorylation. The generation of *L. mexicana* Δ*crk1his2* mutants used for the depletion experiments will be described elsewhere. S-100 cell extracts were prepared from 10^8 mid-logarithmic Δ*crk1his2* or wild-type cells as described above (except in the presence of 0.1 mM EDTA/EGTA instead of 1 mM EDTA/EGTA). The extracts were incubated with an Ni-NTA–agarose (Qiagen) column for 30 min. The eluate from the column was incubated with p12^{cks1his} or control beads as described above and kinase assays or Western blots performed on material bound to the beads.

RESULTS

Isolation of the *L. mexicana cks1* gene

There is significant amino acid sequence identity between the cdk-associated proteins p9^{cks1} and p9^{cks2} from humans, p13^{suc1} of *Schizosaccharomyces pombe* and p18^{cks1} from *S. cerevisiae* [14]. On the basis of cks consensus sequences two degenerate oligonucleotides were designed to the peptide sequences EYRHVMLP and PEPHILLF for use in PCR. A 240 bp fragment was amplified from genomic *L. mexicana* DNA, subcloned and sequenced. This revealed an open reading frame (ORF) with significant identity to both human and yeast cks1 protein sequences. The cloned PCR fragment was used to screen an *L. mexicana* lambda EMBL4 genomic library and the complete gene, *cks1*, was isolated on a 2.3 kb *Sal*I fragment, of which a limited portion, containing the ORF, was sequenced (Figure 1A).

The sequence of *cks1* revealed an ORF of 99 amino acids encoding a predicted protein of 11.8 kDa with an isoelectric point of 10.1. p12^{cks1} has a high level of identity with other cks1 proteins within the conserved central region (residues 31–99 of p12^{cks1}). Human p9^{cks1} has the highest overall identity (70 %) followed by human p9^{cks2} (64 %), *Patella vulgata* p9^{cks1} (64 %), *S. cerevisiae* p18^{cks1} (46 %) and *Schizosaccharomyces pombe* p13^{suc1} (44 %) (Figure 1B). p12^{cks1} has a 25 amino acid N-terminal extension relative to p9^{cks1}. N-terminal extensions are also observed with the two yeast cks proteins, although there is no sequence identity between the yeast proteins and p12^{cks1} in this region. A 9 amino acid insertion in the middle of the two yeast cks proteins is absent from all other cks proteins, including p12^{cks1}. All of the highly conserved residues within the central region that are thought to be important for forming the tertiary structure of the cks protein are present in p12^{cks1} (Figure 1B, [26–28,41]). These include the two domains that were used in the design of the degenerate oligonucleotides for cloning the *cks1* gene, albeit with conserved substitutions at positions 48 (Ile for Met) and 91 (Val for Ile). Conservation extends to residues that form two important surface features of p13^{suc1} proteins [26,27,41], the 'charged cluster' (equivalent p12^{cks1} residues Lys-36, Met-41, Glu-43, Arg-45, Ser-76, Trp-81, Arg-104, marked with a + in Figure 1B) and the 'hydrophobic cluster' thought to be a site of protein–protein interaction (equivalent p12^{cks1} residues Leu-32, Tyr-37, Phe-42, Tyr-44, His-46, Tyr-82, His-85, Pro-87, Glu-88, His-89, marked with an * in Figure 1B). The five important residues of p9^{cks1} that form a shallow pocket and are involved in phosphate binding [29] are totally conserved in p12^{cks1} (equivalent p12^{cks1} residues Lys-36, Arg-45, Ser-76, Trp-81, Arg-104) and are part of the 'charged cluster'. In addition, the HVPEPH motif that is part of the β-strand exchange in the p9^{cks2} and p13^{suc1} dimers [26,28] is conserved, with a lysine residue at position 86 of p12^{cks1} giving the sequence HKPEPH. In p9^{cks1} this sequence forms a β-hinge region that folds back to form a β-hairpin loop [29].

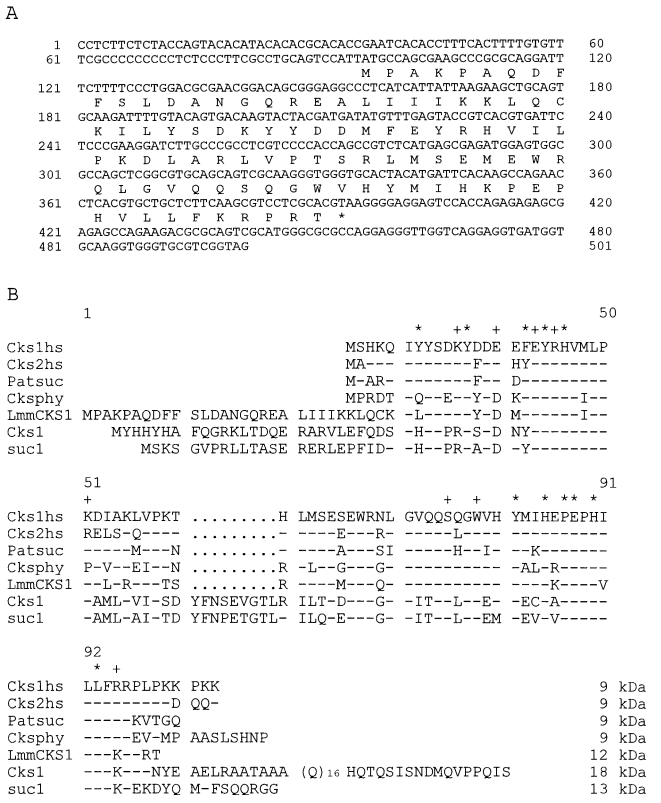

Figure 1 Nucleic acid and deduced amino acid sequence of *cks1* from *L. mexicana* (A) and alignment of cks proteins (B)

(**A**) The top line shows the numbered nucleotide sequence and the bottom line shows the predicted amino acid sequence. (**B**) *L. mexicana* p12^{cks1} compared with human p9^{cks1} and p9^{cks2} [14], *Patella vulgata* p9^{cks1} [15], *Physarum polycephalum* p9^{cks1} [16], *Schizosaccharomyces pombe* p13^{suc1} [12] and *Saccharomyces cerevisiae* p18^{CKS1} [13]. Positions identical to human p9^{cks1} are indicated with a dash (–) and gaps (.) are introduced to maximize homology. Numbers refer to the CKS1 sequence. The C-terminus of the p18^{cks1} is incomplete. Residues that form part of the 'charged cluster' (+) and 'hydrophobic patch' (*) are indicated.

Genomic organization and expression of *cks1*

The genomic organization of *cks1* was investigated by Southern blotting of *L. mexicana* genomic DNA (Figure 2). For each of six restriction digests a single band was detected when hybridized with the cloned PCR fragment containing the majority of the *cks1* gene, indicating a single-copy genomic locus. This was confirmed by restriction analysis of the lambda clone and plasmid subclones which revealed a single copy of *cks1* within the locus.

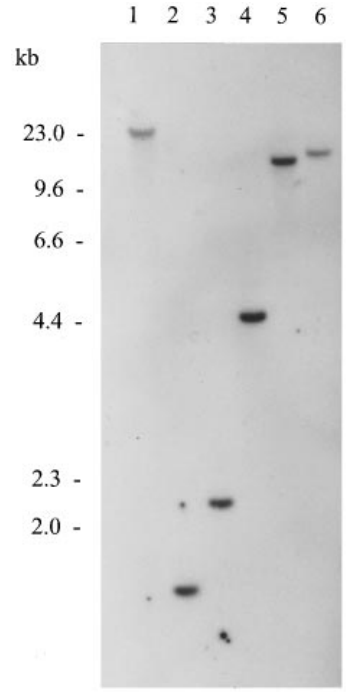

Figure 2 Southern blot analysis

L. mexicana DNA was digested with restriction enzymes, separated on a 0.8% agarose gel, blotted on to nylon membrane and hybridized with the cloned PCR fragment containing the *cks1* gene. Lanes 1–6, *Eco*RI, *Hin*cII, *Pst*I, *Sal*I, *Bam*HI and *Hin*dIII.

The complete ORF of *cks1* was inserted by PCR cloning into the pQE60 expression vector in order to generate recombinant $p12^{cks1}$ with a six histidine tag at the C-terminus ($p12^{cks1his}$). As a result of this cloning strategy a mutation was introduced at the N-terminus of the protein where a G residue replaced a C residue immediately after the ATG start codon, resulting in the replacement of the proline residue at position 2 with an alanine. $p12^{cks1his}$ was purified by FPLC with two rounds of Ni-chelate affinity chromatography. This produced a mix of monomer and dimer which formed under the non-denaturing conditions used to purify the protein. The dimerization could be significantly reduced by removal of the phosphate buffer by dialysis against a buffer containing 50 mM Mops, pH 7.2, 100 mM NaCl, 10 mM EDTA and 10 mM β-mercaptoethanol. Some of the $p12^{cks1his}$ was used to immunize rabbits to raise $p12^{cks1}$-specific antiserum. A Western blot was performed with affinity-purified $p12^{cks1his}$ and cell extracts from three life-cycle stages of *L. mexicana*; promastigote, metacyclic and amastigote (Figure 3). The antiserum recognized a doublet in the recombinant protein preparation, corresponding to a monomer of 13 kDa and dimer of 26 kDa. In addition, two major proteins were detected in the three life-cycle stages of *L. mexicana*, an abundant approx. 80 kDa protein and an approx. 12 kDa protein of the size expected for $p12^{cks1}$. Both proteins were specifically blocked by pre-incubating the antibody with recombinant $p12^{cks1his}$ (results not shown).

Histone H1 kinase binding assays

One of the features of $p13^{suc1}$ and its human homologues is their ability to bind cdc2 and cdc2-related kinases from diverse organisms [14,18,21] including *L. mexicana* [33]. To determine if

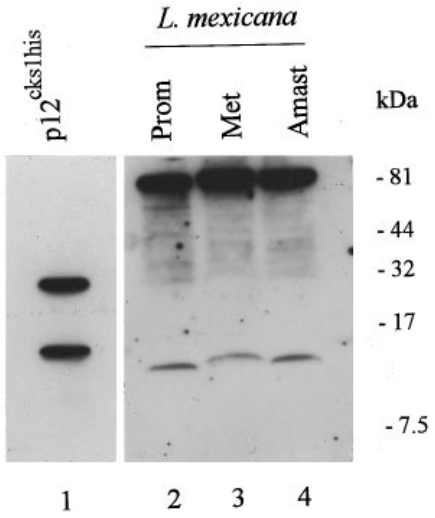

Figure 3 Western blot analysis

Purified recombinant $p12^{cks1his}$ (lane 1) and cell extracts from three life-cycle stages of *L. mexicana*, promastigote (lane 2), metacyclic (lane 3) and amastigote (lane 4), were separated on a 12.5% Tricine SDS/PAGE gel, transferred on to polyvinylidene difluoride membrane and probed with affinity-purified anti-$p12^{cks1}$ serum. Equivalent amounts of each cell extract (as determined by protein concentration) were applied to the gel.

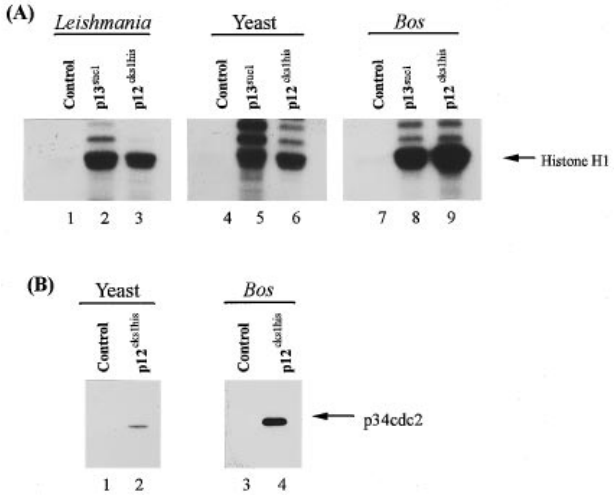

Figure 4 Binding of histone H1 kinase to $p12^{cks1his}$ beads

(**A**) Cell extracts from *L. mexicana* promastigotes (lanes 1–3), *S. cerevisiae* (lanes 4–6) and bovine BL-20 cells (lanes 7–9) were incubated with control (lanes 1,4 and 7), $p13^{suc1}$ (lanes 2,5 and 8) and $p12^{cks1his}$ (lanes 3,6 and 9) beads. Kinase activity bound to the beads, assayed by the ability to phosphorylate histone H1, was detected after SDS/PAGE and autoradiography. (**B**) Western blot of eluates from control (lanes 1 and 3) or $p12^{cks1his}$ (lanes 2 and 4) beads probed with an anti-PSTAIR monoclonal antibody [42].

$p12^{cks1}$ is a functional homologue of $p13^{suc1}$, histone H1 kinase assays were performed following binding of cell extracts to $p12^{cks1his}$ immobilized on AminoLink beads. S-100 cell extracts were prepared from *L. mexicana* promastigotes, *S. cerevisiae* and bovine BL20 lymphocytes [36] and bound to $p12^{cks1his}$ beads. After extensive washing the proteins bound to the beads were assayed for kinase activity by their ability to phosphorylate exogenous histone H1. Phosphorylation was observed following

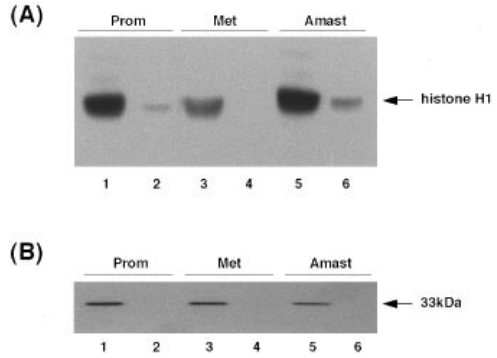

Figure 5 Stage-regulated histone H1 kinase activity in *L. mexicana*

Cell extracts from three life-cycle stages of *L. mexicana*, promastigote (lanes 1 and 2), metacyclic (lanes 3 and 4) and amastigote (lanes 5 and 6), were incubated with p12^{cks1his} beads (lanes 1, 3 and 5) or control beads (lanes 2, 4 and 6). (**A**) Histone H1 kinase activity bound to the beads was detected after SDS/PAGE and autoradiography. (**B**) CRK1 bound to the beads was detected by Western blotting with anti-CITAA serum. Equivalent amounts of each cell extract (as determined by protein concentration) were incubated with equal volumes of each bead slurry.

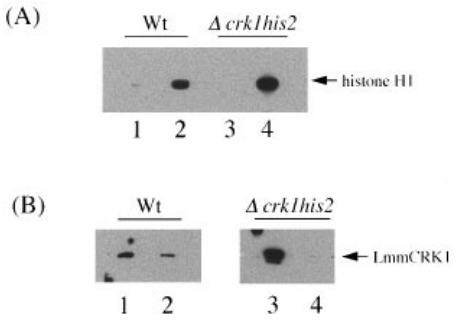

Figure 6 Histone H1 kinase activity isolated from CRK1-depleted extracts

Cell extracts from wild-type (lanes 1 and 2) or Δ*crk1his2* (lanes 3 and 4) *L. mexicana* were applied to a Ni-NTA–agarose column and the eluate subsequently incubated with p12^{cks1his} or control beads. (**A**) Histone H1 kinase activity bound to p12^{cks1his} beads (lanes 2 and 4) or control beads (lanes 1 and 3) was detected after SDS/PAGE and autoradiography. (**B**) CRK1 in the cell extract (lanes 1 and 3) or after Ni-NTA–agarose depletion (lanes 2 and 4) was detected by Western blotting with anti-CITAA serum.

SDS/PAGE and autoradiography (Figure 4A). Histone H1 kinase activity bound to the p12^{cks1his} beads was detected for each of the three cell extracts (Figure 4, lanes 3, 6 and 9) and on p13^{suc1} beads (Figure 4, lanes 2, 5 and 8) but not control beads (Figure 4, lanes 1, 4 and 7). To confirm that the histone H1 kinase activity from bovine and yeast lysates arose from the binding of cdc2 and not an unrelated kinase, Western blots were performed with protein eluted from the p12^{cks1his} beads and a monoclonal antibody to the PSTAIR region of cdc2 [42] (Figure 4B). The anti-PSTAIR serum is specific for yeast cdc2 but cross-reacts with closely related cdks such as cdk2 and cdk3 in higher eukaryotes. The PSTAIR monoclonal antibody recognized a 34 kDa protein eluted from p12^{cks1his} beads for both yeast (Figure 4B, lane 2) and bovine lysates (lane 4) but not for control beads (lanes 1 and 3).

A leishmanial histone H1 kinase activity which binds to p13^{suc1} (SBCRK) has previously been demonstrated to have stage-regulated activity, being active in the dividing promastigote form but inactive in the non-dividing metacyclic form [33]. Histone H1 kinase actvity bound to p12^{cks1his} beads was assayed in cell extracts from the different life-cycle stages (Figure 5A). High activity was detected bound to p12^{cks1his} beads in the promastigote (lane 1) and amastigote (lane 5) dividing forms and a low, but significant, activity in the non-dividing metacyclic form (lane 3) in comparison with controls which had a background level of non-specific binding (Figure 5A, lanes 2, 4 and 6). This pattern of kinase activity from the three life-cycle stages was reproduced in a number of independent experiments (results not shown). Western blots were performed with protein eluted from the beads (Figure 5B). Anti-CITAA serum [33], which is specific to CRK1, detected the protein bound to p12^{cks1his} beads in all life-cycle stages (Figure 5B), including the metacyclic form (lane 3) which has low histone H1 kinase activity (Figure 5A). However, the affinity with which CRK1 bound to the p12^{cks1his} beads was low since only a small proportion of the total CRK1 in the cell extract bound to the beads (results not shown). To address the question of whether CRK1 was responsible for the SBCRK activity, promastigote cell extracts were depleted of CRK1 and then assayed for SBCRK activity (Figure 6). The experiment was performed with a *L. mexicana* mutant (Δ*crk1his2*) in which *crk*1 had been deleted from the genome and *crk1* was expressed from an episome with a six-histidine tag (J. C. Mottram and K. M. Grant, unpublished work) and wild-type cells. S-100 cell extracts were incubated with Ni-NTA–agarose for 30 min to allow the histidine-tagged CRK1 protein to bind to the column. The eluates were collected and analysed by Western blotting (Figure 6B) and for SBCRK activity after affinity selection on p12^{cks1his} beads (Figure 6A). Western blot analysis showed that following Ni-NTA–agarose selection negligible CRK1 remained in the Δ*crk1his2* eluate (Figure 6B, lane 4) in comparison with the eluate from wild-type extracts in which CRK1 was not depleted as it did not contain a His tag (Figure 6B, lane 2). SBCRK activity bound to p12^{cks1his} beads, as assessed by phosphorylation of histone H1, remained high in both Δ*crk1his2* eluates and wild-type eluates (Figure 6A, lanes 2 and 4) compared with controls (lanes 1 and 2). This experiment indicates that CRK1 is not responsible for the majority of the kinase activity that binds p12^{cks1his} beads since cell extracts from which CRK1 has been selectively removed contain levels of p12^{cks1his} -binding SBCRK activity comparable with wild-type extracts.

DISCUSSION

L. mexicana is a member of the family trypanosomatidae, which diverged from the eukaryotic tree early in evolution [43]. The molecular mechanisms that control the cell cycle are thought to be highly conserved in evolution and this has been substantiated by the discovery of genes in trypanosomatids which show significant similarity to essential components of the cell-cycle machinery, for example cdc2-related kinases [31–33] and a cyclin [44]. In this paper we describe the isolation of an *L. mexicana* homologue of the cks family. cks genes are essential for cell viability in yeast [45,46] and the encoded proteins, which have no apparent enzymic activity, are components of the cdc2 kinase complex [18]. cks proteins are highly conserved at the primary amino acid level, with sizes varying from 9 kDa in the two human isoforms [14] to 18 kDa in CKS1 from *S. cerevisiae* [13]. The *L. mexicana cks1* encodes a 12 kDa protein which surprisingly has a much higher level of primary amino acid sequence identity with the human cks isoforms ($\sim 70\%$) than with either budding or fission yeast ($\sim 45\%$) (Figure 1B).

The determination of the structures of the human $p9^{cks1}$ [29], $p9^{cks2}$ [26] and *Schizosaccharomyces pombe* $p13^{suc1}$ [27,28] by X-ray crystallography has revealed some clues about the biological role of the proteins. The structures of $p9^{cks2}$ and $p13^{suc1}$ are very similar. $p9^{cks2}$ has a β-strand exchange that creates a dimer which can oligomerize to form a stable hexameric ring [26] and $p13^{suc1}$ can form several distinct thermodynamically stable forms: a monomer [27,28], a β-strand exchanged dimer [28] and a zinc-mediated dimer [27]. Of the four residues (Asp-23, His-26, His-40 and Glu-91) that are important for zinc ion binding in $p13^{suc1}$, only two (His-40 and Glu-91) are conserved in $p12^{cks1}$. Consequently it is unlikely that leishmanial $p12^{cks1}$ can undergo a similar zinc-mediated oligomerization. A significant proportion of the purified $p12^{cks1his}$ protein exists in a dimeric state which is resistant to β-mercaptoethanol and SDS. This dimerization can, however, be reduced by replacing the phosphate buffer with a Mops-based buffer containing EDTA. This suggests that the dimerization of recombinant $p12^{cks1his}$ is promoted by the presence of bivalent metal cations and phosphate ions [26]. The type of dimerization cannot be predicted for $p12^{cks1}$, however, as the subunit conformation of $p9^{cks1}$ is very different from $p9^{cks2}$ and $p13^{suc1}$, yet the primary sequences are very similar. The yeast proteins have two additional features that distinguish them for the human cks proteins; an N-terminal extension that forms an α-helix and a 9 amino acid insertion that forms an exposed surface loop in the 3- and 4-helical regions [27]. The leishmanial $p12^{cks1}$ also has an N-terminal extension of about the same length as the two yeast cks proteins, which accounts for the 3 kDa size difference when compared with human $p9^{cks1}$, but $p12^{cks1}$ does not have the 9 amino acid insertion found in the two yeast proteins. At present it is not possible to say if the $p12^{cks1}$ is structurally more similar to the yeast or human cks proteins, but the oligomerization state of $p12^{cks1}$ could be important for its biological function (see below).

Many of the residues that are highly conserved between species are found on the surface of the structure and have been implicated in the biological function of the protein. These include a number of aromatic residues that form a hydrophobic patch and a cluster of positively charged residues (see Figure 1B). Residues that form these conserved domains are present in $p12^{cks1}$. In the $p9^{cks2}$ hexamer these positively charged residues bind a sulphate ion, which has been suggested to mimic the binding of the phosphorylated residue Thr-161 on $p34^{cdc2}$ [26]. Phosphorylation of Thr-161 is essential for cdk activity [8]. Modelling of cdc2 binding to $p13^{suc1}$, however, suggested that Thr-161 may not be the residue through which this interaction occurs [41]. Rather the interaction may occur via another phosphorylated residue, Tyr-15 [41] which is located in the ATP-binding domain of cdks and is dephosphorylated by the cdc25 phosphatase to activate the cdc2 kinase complex. The similarity of the $p9^{cks1}$ structure to the N-terminal domain fold of cdks, together with the presence of the five conserved residues in the 'charged cluster' that bind a phosphate ion, suggest that this might be a specific recognition site for a phosphorylated cdk residue [29]. Members of the four classes of trypanosomatid cdc2-related kinases (CRKs 1–4) all have equivalent residues to human Tyr-15 and Thr-161 [31–33,47], suggesting the kinases are regulated through their phosphorylation status in a similar way to yeast and higher eukaryotes and indicating that an interaction between these conserved tyrosine and threonine residues and $p12^{cks1}$ would be possible for those trypanosomatid crks which bind $p12^{cks1}$.

Despite the structural differences between the human $p9^{cks2}$ and *Schizosaccharomyces pombe* $p13^{suc1}$ proteins they share some common functions. The human *cks* genes and *suc1* from *Schizosaccharomyces pombe* are able to rescue a null mutation in the *CKS*1 gene of *S. cerevisiae* [14] and cdc2 from a variety of unicellular and multicellular organisms bind recombinant $p13^{suc1}$ [21–23]. Likewise the leishmanial homologue $p12^{cks1}$ binds cdc2 kinases from yeast and higher eukaryotes, thus demonstrating the high level of structural and functional conservation of this protein during evolution.

Western blot analysis with anti-cks1 serum, although not quantitative, indicates that $p12^{cks1}$ levels do not vary extensively between the three different life-cycle stages. The antiserum also cross-reacts with higher-molecular-mass proteins, particularly one of about 80 kDa. A cross-reacting high-molecular-mass component was also detected in *Schizosaccharomyces pombe* extracts immunoprecipiated with an anti-$p13^{suc1}$ serum [18], but the identity of this protein is unknown. The major histone H1 kinase activity associated with $p12^{cks1}$ has the same characteristics as SBCRK, a $p13^{suc1}$-associated activity predicted to be a cdc2-related kinase [33], in that it showed a similar stage-regulation with high activity in dividing forms (promastigote and amastigote) and low activity in the metacyclic form (Figure 5A). Given the structural and functional similarity between $p13^{suc1}$ and $p12^{cks1}$ it is likely that the same kinase binds both yeast and leishmanial cks proteins. We demonstrated previously that SBCRK was not encoded by the cdc2-related kinase *crk1* as CRK1 did not bind $p13^{suc1}$ [33]. CRK1, however, does associate, at least *in vitro*, with the homologous $p12^{cks1his}$ (Figure 5B). CRK1 binding does not account for the majority of the histone H1 kinase activity bound to $p12^{cks1his}$, since a cell extract in which CRK1 had been depleted still had comparable SBCRK activity with wild-type extracts in which CRK1 had not been depleted (Figure 6) and histone H1, the preferred substrate for SBCRK, was found to be a poor substrate for CRK1 (J. C. Mottram and K. M. Grant, unpublished work). The histone H1 kinase activity from the non-dividing metacyclic cell extracts that bound $p12^{cks1his}$ was relatively high (Figure 5A, lane 3) and comparable with the activity detected previously in association with $p13^{suc1}$ [33]. The binding of CRK1 to $p12^{cks1his}$ may in part account for some of this activity. It is more likely, however, that this activity is due to the presence of a small proportion of the dividing promastigote form in the metacyclic preparation, especially as $p13^{suc1}$ does not bind CRK1 but does bind similar amounts of kinase activity from metacyclic extracts. Although CRK1 is not part of the SBCRK activity it is possible that the histone H1 kinase activity binding to the $p12^{cks1}$ beads is composed of more than one kinase. We are in the process of purifying the complex to address this question.

The oligomerization state of cks proteins has important implications for function [16,26,29]. Six kinase subunits can be modelled to interact with the hexameric $p9^{cks2}$ without steric hindrance, leading to the hypothesis that $p9^{cks2}$ can act as a hub for the binding of cdc2 and other cdks. The presence of two human cks isoforms, each of which might form dimers or multimeric structures, together with a family of cdks that bind the cks proteins [24], gives a range of combinatorial interactions [26] that may be important in higher eukaryotes where cell-cycle control is linked to complex differentiation patterns in different cell types. The finding that trypanosomatids have a large family of cdc2-related kinases and that $p12^{cks1}$ is capable of binding at least two of these kinases, SBCRK and CRK1, mirrors the situation in higher eukaryotes and may reflect the complex controls of their cell cycles that are required during differentiation of these unicellular parasites during their life-cycle.

We thank G.H. Coombs, J. Kinnaird and K. Djinovic for their critical reading of our manuscript. This work was supported by the Medical Research Council. J.C.M. is an MRC Senior Fellow.

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Received 18 December 1995/15 February 1996; accepted 23 February 1996