The *crk3* Gene of *Leishmania mexicana* Encodes a Stage-regulated cdc2-related Histone H1 Kinase That Associates with p12<sup>cks1</sup>*

(Received for publication, November 6, 1997, and in revised form, February 10, 1998)

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A cdc2-related protein kinase gene, *crk3*, has been isolated from the parasitic protozoan *Leishmania mexicana*. Data presented here suggests that *crk3* is a good candidate to be the leishmanial cdc2 homologue but that the parasite protein has some characteristics which distinguish it from mammalian cdc2. *crk3* is predicted to encode a 35.6-kDa protein with 54% sequence identity with the human cyclin-dependent kinase cdc2 and 78% identity with the *Trypanosoma brucei* CRK3. The trypanosomatid CRK3 proteins have an unusual, poorly conserved 19-amino acid N-terminal extension not present in human cdc2. *crk3* is single copy, and there is 5-fold higher mRNA in the replicative promastigote life-cycle stage than in the non-dividing metacyclic form or mammalian amastigote form. A leishmanial suc-binding cdc2-related kinase (SBCRK) histone H1 kinase, has previously been described which binds the yeast protein, p13<sup>suc</sup><sup>1</sup>, and that has stage-regulated activity (Mottram J. C., Kinnaird, J., Shiels, B. R., Tait, A., and Barry, J. D. (1993) *J. Biol. Chem.* 268, 21044–21051). CRK3 from cell extracts of the three life-cycle stages was found to bind p13<sup>suc</sup><sup>1</sup> and the leishmanial homologue p12<sup>cks1</sup>. CRK3 fused with six histidines at the C terminus was expressed in *L. mexicana* and shown to have SBCRK histone H1 kinase activity. Depletion of histidine-tagged CRK3 from *L. mexicana* cell extracts, by Ni-nitrilotriacetic acid agarose selection, reduced histone H1 kinase activity binding to p13<sup>suc</sup><sup>1</sup>. These data imply that *crk3* encodes the kinase subunit of SBCRK. SBCRK and histidine-tagged CRK3 activities were inhibited by the pu- rine analogue olomoucine with an IC<sub>50</sub> of 28 and 42 μM, respectively, 5–6-fold higher than human p34<sup>cdc2</sup><sup>k</sup>(cyclinB).

cdc2, one of the cyclin-dependent kinase (CDK) family of serine-threonine protein kinases, is an essential mitotic acti- vator in yeast and animals and plays a crucial role in controlling the cell cycle in all eukaryotes (1, 2). In mammals, a large number of CDKs have been described, some of which have been shown to be directly involved in controlling cell cycle transitions, e.g. CDKs 1–4 and CDK6 (1, 3), while others have less defined roles, e.g. CDK5 (4, 5). In contrast, yeast have only one CDK (*Schizosaccharomyces pombe* cdc2 or *Saccharomyces cerevisiae* CDC28), which is responsible for transition through both G<sub>s</sub>/S and G<sub>2</sub>/M boundaries (6, 7). The activity of CDKs is controlled post-translationally by a number of different mechanisms: the association of the kinase subunit with its positive regulatory partner, one of the family of cyclins; phosphorylation of conserved sites which can stimulate or inhibit kinase activity; or binding of an inhibitory partner, a CDK inhibitor protein such as members of the p16<sup>ink4</sup> or p21<sup>cip1</sup> families (1). Another group of proteins known to interact with cdc2 are the cdc2 kinase subunit proteins, such as human p9<sup>ck2</sup> and p9<sup>ck1</sup> (8) and *S. pombe* p13<sup>suc1</sup> (9, 10). The function of these proteins in the kinase complex is as yet unknown but the high specificity with which they interact with cdc2 has been exploited in the affinity purification of cdc2 from a variety of organisms on p13<sup>suc1</sup>-Sepharose (9, 11, 12).

Members of the order Trypanosomatidae, which includes *Leishmania* and the closely related parasite *Trypanosoma brucei*, possess a large family of cdc2-related kinase genes, *crk1–4* (13–16), and thus in this respect appear to be more similar to multicellular organisms than to yeast. This is not surprising since, although it is unicellular, *Leishmania* has a complex life cycle during which the cell cycle is switched on and off as the parasite differentiates between proliferative forms (the promastigote in the sandfly midgut and the amastigote in the mammalian host) and the cell cycle arrested metacyclic stage, which is the human infective form found in the fly proboscis. Thus, there is an integral link between the cell cycle and the developmental life cycle of this parasite. The large evolutionary divergence between protozoa and yeast or higher eukaryotes has made it difficult to determine which of the trypanosomatid *crk* genes might be the functional cdc2 homologue, as each of the genes have a similar level of homology to yeast or human cdc2, and they do not complement yeast cdc2 temperature-sensitive mutants (15, 16).

In *Leishmania*, one cdc2-related kinase gene has been described, *crk1* (15). The CRK1 protein was found in all leishmanial life cycle stages, but its histone H1 kinase activity was only detected in the promastigote and metacyclic forms, indicating post-translational control of kinase activity (15). Evidence from gene-targeted disruption experiments indicated that the *Leishmania mexicana* *crk1* gene is essential to the promastigote form of the parasite (17). A distinct histone H1 kinase activity has been identified biochemically from *L. mexicana* (15, 18). This activity, known as the suc-binding cdc2-related kinase (SBCRK), is thought to be a candidate for the functional cdc2
homologue from *L. mexicana* since it shares a number of attributes with cdc2 from other eukaryotic species: it binds the S. *pombe* protein p13′*wcl* and its *L. mexicana* homologue p13′*sk*; it phosphorylates the cdc2 substrate histone H1, and its activity correlates with the division status of the parasite, being active in the proliferative life cycle stages (promastigote and amastigote) and inactive in the cell cycle-arrested metacyclic stage (15, 18). In the closely related protozoan *T. brucei*, three cdk genes have been cloned and analyzed (16). *tbcrk1*—*3* encode proteins of 34, 39, and 35 kDa, respectively, which share 49–54% sequence identity with human cdc2.

Here, we describe a second cdk gene from *L. mexicana*, *crk3*. This gene is single copy, is expressed in all life leishmanial life cycle stages, and is closely related to the *T. brucei* cdk gene. *Crk3* has stage-regulated histone H1 kinase activity that binds p13′*wcl*, features of the leishmanial SBCRK and mammalian cdc2.

**EXPERIMENTAL PROCEDURES**

**Parasites—Leishmania mexicana mexicana** (MNYC/B2/62/M379) promastigotes were grown in HOMEM medium as described previously (17). Promastigotes were harvested in the mid-log phase of growth (5–10 × 10^6 cells/ml). Metacyclic and axenic amastigote-like forms (ALFs) were grown in Schneider’s *Drosophila* media at 25 and 32 °C, respectively, as described previously (19). Metacyclics were harvested in the stationary phase of growth (5–5 × 10^6 cells/ml) and ALFs were harvested in the mid-log phase of growth (5–10 × 10^6 cells/ml). Cells were washed twice in phosphate-buffered saline, and the cells were either used immediately or stored at −70 °C until required.

**Cloning and Sequencing of the *L. mexicana* *crk3* Gene—**A fragment of the *crk3* gene was obtained by PCR using degenerate oligonucleotide primers designed to conserved regions of cdc2-related kinases and *L. mexicana* genomic DNA as described previously for the *T. brucei* cdk3 gene (16). The full-length *crk3* gene was isolated by screening an *L. mexicana* a DashII genomic library as described previously (19). A 2-kb *HindIII* fragment containing the complete *crk3* open reading frame (pGL89) was subcloned into Bluescript for sequencing.

**Western Blots—**Preparation of *p12csk1* and *p13suc1* Beads—

**Immunoblotting—**200 μl of *S*. *lystes* of wild-type *L. mexicana* (1.3 mg/ml) were incubated with 20 μl of p12′*wcl*, p13′*sk*, or control beads as for immunoblotting above. Removal of nonspecifically bound protein and the histone H1 kinase assay were performed as described previously (15, 18). Phosphorylated histone H1 was quantified by scintillation counting or by phosphorimaging.

**Complementation of *S. cerevisiae* cdc28 Mutants—**The *crk3* gene was expressed in three *Saccharomyces cerevisiae cdc28* temperature-sensitive alleles using the yeast vector pRS416-met (22). pRS416-met is a 2-μm based plasmid containing the *URA3* gene as a selectable marker and *cassettes* were transformed into M15[pREP4] cells. Recombinant p13*sk* protein lane was isolated from the three life cycle stages of *L. mexicana*. 4% of the total metabolism resulted in binding to the 5′′ probe was used as a control (19).

**Transfection of *L. mexicana*—**The *crk3* gene was fused to a 6-histidine tag using the pQ660 vector (VI.AGEN Inc.) by constructing unique *NcoI* and *BglII* sites on the 5′ and 3′ ends of the coding region using PCR with the plasmid clone pGL89 and the following primers (with engineered restriction sites underlined): 5′-GCCCATGGCTTCGTTTG-3′ and 5′-GCCGAGATCTCCAAACGAGTGTGCTGAACC-3′. PCR was performed with *Pfu* polymerase (Stratagene). The PCR product was digested with *NcoI* and *BglII* and cloned into pQ660/II-digested pQ660 to give the plasmid pQ691. This encodes CRK3 protein with a 6-histidine tag at the C-terminal end (WRSHHHHHHH-COOH), where the underlined W is the last authentic CRK3 amino acid). The PCR product was cloned into the *S. cerevisiae* centromere plasmid pYLO to generate CRK3 sequence at the second amino acid position, changing the authentic serine to an alanine residue (resulting in an inactive kinase). To subclone the *crk3his* gene into the pX shuttle vector (25) and repair this amino acid change, the following protocol was used. An EcoRI/SalI fragment, corresponding to the 5′ end of the *crk3* gene, was excised from the plasmid clone pGL89, and an *Stul/HindIII* fragment, corresponding to...
the 3′ end of the crk3his gene, was excised from the pGL91 plasmid. These two fragments were ligated together and cloned into BlueScript (pGL92). The repaired crk3his gene was then excised with SmaI and XhoI, the XhoI site was filled-in using Klenow, and the fragment was cloned into the shuttle vector, pXneo (25), restricted with SmaI. A plasmid with the insert in the correct orientation was designated pGL95. Transfections were carried out as described previously (17, 26).

The cell line expressing CRK3his from the pX vector in wild-type L. mexicana genetic background was named wt[pXCRK3his].

Depletion of CRK3his from Cell Extracts—1.5 ml of S-100 cell lysates were prepared from 1 × 10^8 L. mexicana or wt[pXCRK3his]. The extracts were made up to 50 mM imidazole and 500 μl incubated with 200 μl of Ni-NTA agarose at 4 °C. Both the original lysate and the eluate from the column (CRK3-depleted) were then incubated with p13suc1 or control beads, as described above, and the bound protein was assayed for histone H1 kinase activity together with the washed Ni-NTA agarose beads.

Inhibition by Olomoucine—SBCRK was bound to p13suc1 beads, as described above, and eluted with 2 mg/ml free p13suc1. CRK3his was purified on Ni-NTA agarose, as described above, and eluted by incubation with 100 mM EDTA in lysis buffer for 30 min at 4 °C. CRK3his was then bound to p13suc1 beads and assayed in situ. Samples of enzyme were incubated with increasing concentrations of olomoucine (Alexis Corporation UK (Ltd)), and kinase activity was assayed as described. Recombinant human cdc2/cyclin B (New England Biolabs) was used as a positive control.

**RESULTS**

Characterization of the crk3 Gene from L. mexicana—The L. mexicana crk3 gene was isolated using the same PCR strategy and degenerate oligonucleotide primers that proved successful in the cloning of the T. brucei crk3 gene (16). The full-length gene (Fig. 1) was isolated from a λ Dash II genomic library (19) and subcloned into Bluescript on a 2-kb HindIII DNA fragment. Mapping of the λ clone revealed that a mitochondrial elongation factor G gene homologue was located about 3.5-kb downstream of crk3. Interestingly, a homologous gene is located immediately downstream of the crk3 gene in T. brucei (16). The predicted protein encoded by the leishmanial crk3 gene shows the greatest degree of homology to the cdc2 family of serine/threonine protein kinases when compared with protein sequence databases. The predicted amino acid sequence is compared in Fig. 2 with that of human cdc2 and of the homologous proteins from T. brucei (TbCRK3) and T. cruzi (TcCRK3). The three trypanosomatid CRK3s each have an unusual 19-amino acid N-terminal extension, which is not highly conserved in sequence and has only one conserved residue, an arginine at position 10. Overall, the leishmanial CRK3 has 78% identity with TbCRK3,
Genes have, however, been isolated from *T. brucei* major life-cycle stages of *L. mexicana*. The *crk3* gene is single copy and expressed in the three major life-cycle stages of *L. mexicana*. A Southern blot analysis of *L. mexicana* DNA was digested with restriction enzymes, electrophoresed on a 0.7% agarose gel, blotted onto nylon membrane, and hybridized with a radiolabeled *crk3*-specific probe under high stringency. Lanes 1-5, SalI, BglII, EcoRI, HindIII, and XbaI. B. Northern blot analysis, 2 μg of poly(A)+ mRNA from mid-log promastigotes (lanes 1 and 4), stationary phase metacyclics (lanes 2 and 5), or axenic amastigotes (lanes 3 and 6) was separated on a 1.4% formaldehyde-agarose gel, transferred onto nylon membrane, and hybridized with a *crk3*-specific probe (lanes 1-3) or an α-tubulin-specific probe (lanes 4-6).

77% identity with TcCRK3, and 54% identity with human cdc2. The leishmanial CRK3 contains all the domains and residues characteristic of the serine/threonine protein kinase family. In addition, CRK3 also contains the conserved residues and domains which are important for the regulation of cdc2 activity. This includes equivalent residues to human cdc2 at Thr-14 and Tyr-15, in the ATP-binding domain, and Thr-161 (Fig. 2). These three residues are highly conserved both within the CDK family and between species. This implies that CRK3 activity may be controlled by similar post-translational mechanisms as exist in other eukaryotes (through a positively regulating kinase, wee1, and a negatively regulating phosphatase, cdc25), although no homologues of these regulatory enzymes have been identified in *Leishmania* to date.

The “PSTAIRE” box, consisting of the 16-amino acid sequence EGVPSTAIRESLLKE, is a highly conserved domain in the leishmanial kinase suggests that cyclin binding is important in regulating kinase activity through cyclin binding. CRK3 binds specifically to a protein of approximately 35 kDa (Fig. 4, lane 1). A number of higher molecular mass proteins were also detected with this antiserum, but these were also present in the control (lane 2). In a parallel experiment, recombinant CRK3 protein was blotted onto a PVDF membrane and probed with p12<sub>cks1</sub>. Detection of bound p12<sub>cks1</sub> by Western blotting with an anti-p12<sub>cks1</sub> specific antibody (18) revealed that p12<sub>cks1</sub> binds specifically to a protein of approximately 35 kDa (Fig. 4, lane 1). A number of higher molecular mass proteins were also detected with this antiserum, but these were also present in the control (lane 2).

**Recombinant p12<sub>cks1</sub> Binds to a 35-kDa Protein in Leishmanial Cell Lysates and to Recombinant CRK3his—** The S. pombe protein, p13<sub>suc1</sub>, interacts with cdc2 and cdk2 from many species and has been widely used as a highly specific affinity matrix for the purification of cdc2 when covalently cross-linked to a solid support, such as Sepharose beads (9, 11, 12, 31–33). This matrix, however, does not bind other CDK proteins such as cdk4, cdk5, and cdk6 (32). We have previously described a leishmanial homologue of p13<sub>suc1</sub>, p12<sub>cks1</sub>, which is also capable of binding p34<sub>cdc2</sub> from yeast and bovine cell extracts (18). Both p13<sub>suc1</sub> and p12<sub>cks1</sub> bind a histone H1 kinase activity from leishmanial cell extracts, SBCRK, which is thought to be the functional cdc2 homologue from this parasite (18). To investigate proteins that bind p12<sub>cks1</sub> and are components of the SBCRK complex, promastigote cell lysates were separated by SDS-PAGE and blotted onto a PVDF membrane, which was then incubated with recombinant p12<sub>cks1</sub> protein. Detection of bound p12<sub>cks1</sub> by Western blotting with an anti-p12<sub>cks1</sub> specific antibody (18) revealed that p12<sub>cks1</sub> binds specifically to a protein of approximately 35 kDa (Fig. 4, lane 1). A number of higher molecular mass proteins were also detected with this antiserum, but these were also present in the control (lane 2).

**CRK3 Binds p12<sub>cks1</sub> and p13<sub>suc1</sub>—** Binding of CRK3 from leishmanial cell extracts to p13<sub>suc1</sub> and p12<sub>cks1</sub> beads was assessed by Western blot using an anti-CRK3 antibody, raised against the C-terminal 12 amino acids of CRK3. This antibody did not recognize reproducibly a band of the predicted molecular mass from leishmanial whole cell extracts. However, when CRK3 from promastigote cell extracts was concentrated by selective affinity binding to p13<sub>suc1</sub> and p12<sub>cks1</sub> beads, the anti-CRK3 antibody recognized a band of 35 kDa (Fig. 5A) which was abolished by the presence of competing peptide (not shown). When cell extracts of wild type *L. mexicana* were incubated with p13<sub>suc1</sub> and p12<sub>cks1</sub> beads, it was found that CRK3 bound to both proteins from all three life cycle stages (Fig. 5A). Less CRK3 protein bound to p13<sub>suc1</sub> and p12<sub>cks1</sub> from metacyclic cell extracts than from promastigotes or amastigotes (Fig. 5A) correlating with a reduced histone H1 kinase activity detected from the metacyclic form, in comparison to the
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CRK3his, purified on Ni-NTA beads and eluted with 100 mM EDTA, was incubated with p13\textsuperscript{ carcin } beads (lane 3). The detection of histone H1 kinase activity bound to the beads confirmed that CRK3his could bind p13\textsuperscript{ carcin } and retain histone H1 kinase activity.

**Inhibition of SBCRK and CRK3his by Olomoucine**—Olomoucine is a purine analogue that is a potent inhibitor of mammalian cdc2 with a narrow range of selectivity (34). Olomoucine was tested for its effects against SBCRK and CRK3his. Dose response curves gave IC\textsubscript{50} values for the inhibition of histone H1 kinase activity by olomoucine of 28 \mu M for SBCRK and 42 \mu M for CRK3his (Fig. 7).

**crk3 Is Unable to Complement CDC28 Temperature-sensitive Mutants**—To test if crk3 could complement an \textit{S. cerevisiae} CDC28 ts mutant, the crk3 gene was introduced on a low copy plasmid into three strains containing different ts alleles. The plasmid contained a promoter repressible by methionine, allowing the expression levels of the CRK3 protein to be regulated (22). At the restrictive temperature, the \textit{cdc28–13} mutant arrests at the G\textsubscript{1}/S boundary of the cell cycle while the other two mutants tested (\textit{cdc28–4} and \textit{cdc28–IN}) block at the G\textsubscript{1}/M transition (7, 24). The crk3 gene was unable to restore growth at the restrictive temperature for any of the strains using a variety of expression levels. The expression of CRK3 was confirmed by Western blotting of yeast extracts with anti-CRK3 antibodies (not shown). The \textit{ncc-1} gene of \textit{C. elegans} was used as a positive control for complementation of the ts phenotype (23).

**DISCUSSION**

We have previously described a histone H1 kinase that binds fission yeast p13\textsuperscript{ carcin } and whose activity is stage-regulated during the \textit{Leishmania} life cycle (15, 18). This cdc2-related kinase activity (SBCRK) is not encoded by the leishmanial \textit{crk1} gene as CRK1 does not bind p13\textsuperscript{ carcin } (18). Attempts to purify this activity and obtain amino acid sequence for the components of the kinase complex (predicted to be kinase and cyclin partners) proved problematic due to the low concentration of SBCRK in the cell. As an alternative approach, we used PCR and oligonucleotides designed to conserved regions of cdc2-related kinases to amplify \textit{crk} genes. This approach had proved successful previously with the cloning of several \textit{crk} genes from the closely related trypanosomatid \textit{T. brucei} (16), and it also proved successful with \textit{L. mexicana} as described in this paper. Several lines of evidence suggest that crk3 encodes a p13\textsuperscript{ carcin } binding kinase (SBCRK, (15)).

(a) \textit{crk3} is predicted to encode a protein of 35 kDa, and p13\textsuperscript{ carcin } binds a similar size protein in leishmanial cell extracts (Fig. 4).

(b) Antibodies can detect CRK3 from leishmanial cell extracts bound to p13\textsuperscript{ carcin } (Fig. 5).

(c) CRK3 tagged with 6-histidine residues, expressed in \textit{L. mexicana} and

![Fig. 5. CRK3 binding to p12\textsuperscript{ carcin } or p13\textsuperscript{ carcin } from the three different life cycle stages parallels histone H1 kinase activity. S-100 lysates prepared from promastigotes, metacyclics, or amastigotes were incubated at saturating concentrations with a limited number of p12\textsuperscript{ carcin } or control beads. The proteins bound to the beads were assayed for the presence of CRK3 by Western blotting (panel A, lanes 1–9) or for histone H1 kinase activity (panel B). Dark gray bars, p12\textsuperscript{ carcin }; black bars, p13\textsuperscript{ carcin }; light gray bars, control.](image)
affinity purified on Ni-NTA agarose, has p13\textsuperscript{cyc} binding histone H1 kinase activity (Fig. 6). (d) Depletion of CRK3h3 from leishmanial cell extracts reduces p13\textsuperscript{cyc} binding histone H1 kinase activity (Fig. 6). (e) SBCRK and CRK3h3 have a similar \( I_{C_{50}} \) for histone H1 kinase inhibition with the ATP analogue, olomoucine (Fig. 7). Although these experiments provide strong evidence that CRK3 is the major cdc2-related kinase activity that binds p13\textsuperscript{cyc}, and hence encodes the stage-regulated kinase previously described (15, 18), it cannot be ruled out that other minor activities might bind p13\textsuperscript{cyc}. For instance, CRK1 can bind p12\textsuperscript{kbs}, the leishmanial homologue of p13\textsuperscript{cyc} (18), but not p13\textsuperscript{cyc}, and so a number of leishmanial CRKs might bind p12\textsuperscript{kbs} in vivo, as has been demonstrated for human CDKs and pGSK3 (32, 33). Further evidence that CRK3 encodes the SBCRK might have come from the generation of null mutants for crk3 by targeted gene disruption. However, attempts to create crk3 null mutants resulted in changes in cell ploidy, a phenomenon that has been used as a positive criterion for determining if a gene is essential in Leishmania (17, 35). Thus it would appear that crk3 is essential.3 Attempts to create a mutant cell line in which crk3h3 was expressed in a null mutant background, similar to that described previously for crk1 (17), also failed, possibly because the histidine tag on the C terminus of the protein interfered with some aspect of the function of the kinase.

Despite the high level of sequence identity between the leishmanial crk3 gene and CDC28, crk3 was unable to complement any of the three S. cerevisiae CDC28 mutants tested. The mutants were selected for alleles that caused conditional blocks at either the G1/S or the G2/M boundary; however, CRK3 could not function as a CDC28 replacement at either of these checkpoints. We had previously shown that the T. brucei crk3 and L. mexicana crk1 were unable to complement an S. pombe cdc2 mutant (15, 16). It is possible that the large sequence divergence in the PSTAIR region of CRK3 (only 10/16 amino acid identity), which is a cyclin binding site (29), precludes the binding of yeast cyclins. Clearly the large phylegnetic distance between Leishmania and yeast (36) makes negative cross-species complementation tests such as this difficult to interpret.

Northern analysis shows differential expression of crk3 mRNA in the three different life cycle stages of L. mexicana, with 5-fold more crk3 mRNA in promastigotes than in either amastigotes or metacyclics. This ratio does not reflect CRK3 activity (as assessed by SBCRK histone H1 kinase activity, (15, 17)). The level of CRK3 activity does, however, parallel the amount of CRK3 protein which binds to p13\textsuperscript{cyc} beads from cell extracts prepared from the three life cycle stages. When equivalent experimental conditions were used for SBCRK activity, and for the anti-CRK3 Western blot in which p13\textsuperscript{cyc} was in excess, no CRK3 could be detected bound to p13\textsuperscript{cyc} from the metacyclic extract (data not shown). Only when cell extract was in excess could CRK3 be detected by Western blot bound to p13\textsuperscript{cyc} from metacyclic extracts. (a) CRK3 could be translated at a low level in the metacyclic stage. crk3 mRNA was detected in metacyclics by Northern analysis, but it cannot be ruled out that it is either not recruited onto the ribosome or is translated with poor efficiency in metacyclics compared with the proliferative life cycle stages. (b) CRK3 protein is unstable and quickly degraded in metacyclics. (c) A putative inhibitor protein binds to the CRK3 kinase in metacyclic cells and interferes with the binding interaction between CRK3 and p13\textsuperscript{cyc}. Our anti-CRK3 antisera cannot detect CRK3 on Western blots of wild-type lysates, presumably due to the low cellular levels of this kinase, and therefore it has proved difficult to determine the relative concentrations of CRK3 protein in the three life cycle stages of wild-type L. mexicana. However, the low levels of CRK3 protein in the cell cycle-arrested metacyclic stage, but not a concomitant reduction in mRNA levels, might represent an unusual mechanism for controlling cdc2-related kinase activity. In mammalian cells, cdc2 is generally stable throughout the cell cycle (37, 38), although both cdc2 mRNA levels and cdc2 protein decline to almost undetectable levels following cell growth arrest and then increase again when the cell re-enters the cell cycle (37, 39). The mechanism by which the activity of leishmanial CRK3 is regulated during the life cycle is currently under investigation.

Acknowledgments—We are grateful to S. Reed for providing the yeast mutants and P. Sternberg for the ncc1 gene.

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