Stage-specific activity of the *Leishmania major CRK3* kinase and functional rescue of a *Schizosaccharomyces pombe cdc2* mutant

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Abstract

Cell cycle control by cdc2-related kinases (CRKs) is essential to the regulation of cell proliferation and developmental processes in many organisms. Alternating phases of growth, arrest, and differentiation are characteristics of the infectious cycle of many trypanosomatid parasites, raising the possibility that members of the trypanosomatid CRK gene family participate in the regulation of these essential processes. Here we describe properties of the *CRK3* gene from *Leishmania major*, which encodes a 36 kDa protein kinase showing 60% amino acid sequence identity with human CDK2, including several conserved sites implicated in regulation of kinase activity. *CRK3* mRNA was constitutively expressed throughout the parasite life cycle, but histone H1 kinase activity of an epitope tagged CRK3 protein was greater in log-phase than in stationary-phase promastigotes. When integrated into the genome and expressed at the optimal level, *CRK3* was able to rescue the growth defect of a *Schizosaccharomyces pombe cdc2* mutant (*cdc2-33*[ts]), indicating that *CRK3* is a functional homolog of *cdc2*. Mutants of *CRK3* at several key regulatory residues showed the expected dominant negative effects on the *S. pombe* mutant. This is the first example of functional expression of a trypanosomatid CRK in yeast, opening the way for further genetic studies within this amenable organism. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Cell cycle-dependent kinases are key regulators of cell division and mediate important steps in the regulation of developmental processes [1–3]. These two roles are intertwined in many protozoan parasites, because cell division and cellular differentiation are closely coordinated and linked to critical steps in the infectious cycle [4]. This is specifically the case for the trypanosomatid parasite *Leishmania*, the causative agent of cutaneous and visceral leishmaniasis. *Leishmania* alternates between two major developmental stages that undergo replication, the promastigote carried in the gut of the sand fly insect vector and the amastigote that resides within the phagolysosome of the macrophage [5]. The dividing procyclic promastigote is non-infective [6]. Upon exiting from the cell-cycle and entry into stationary phase in culture or in the sand fly, parasites differentiate into the non-dividing metacyclic promastigote, which is highly infective [6]. This coupling of cell division to differentiation makes it likely that cell-cycle dependent kinases play an important role in both the growth and infectious cycle of the parasite.

Cyclin-dependent kinases (CDKs) are serine/threonine protein kinases that play essential roles in eukaryotic cell cycle control [7,8]. In yeast, cell-cycle progression is predominantly regulated by a single CDK, $p34^{cdc2}$ in *Schizosaccharomyces pombe* or $p34^{CDC28}$ in *Sacchromyces cerevisiae* [9]. In higher eukaryotic cells, many CDKs act in a temporally or spatially-specific fashion [10], and an extensive network of regulatory actions serve to control them. CDK activity is activated by association with proteins such as cyclins [11,12], or reduced by binding to various CDK inhibitors [13–15]. CDK activity is also positively and negatively regulated through phosphorylation/dephosphorylation events at key regulatory sites [16,17].

The genomes of trypanosomatid parasites encode a family of CDKs [4,18], and thus resemble higher eukaryotes rather than yeast, which only contain one. In *Leishmania mexicana mexicana*, two CDK genes, *CRK1* and *CRK3* have been described [19,20]. The Lmm*CRK1* mRNA and

protein are present at similar levels in all parasite stages, but the kinase is only active during the promastigote stage, suggesting that regulation occurs post-translationally, as seen for other CDKs [19]. A distinct CDK activity able to bind yeast $p13^{suc1}$ was detected in dividing but not in non-dividing stages, and recently shown to be the product of the Lmm*CRK3* gene [19–22]. Both *CRK1* and *CRK3* have been shown to be essential in procyclic promastigotes [21].

While the known properties of trypanosomatid CDKs resemble those of other organisms, pursuit of their role in the *Leishmania* cell cycle and differentiation has been limited by the relative difficulty of these studies in protozoans. In other species, the study of CDKs has benefited greatly from the ability to express these proteins in heterologous systems such as yeast, where the extensive repertoire and knowledge of interacting genes and mutants has facilitated a detailed genetic dissection of CDK function. While trypanosomatid CDKs have been shown to bind to heterologous CDK-interacting proteins including cyclins and $p13^{suc1}$ [19–21,23], several well-controlled attempts to demonstrate activity in heterologous yeast systems have proven unsuccessful [19,20,23].

In the process of studying a region of *Leishmania* DNA implicated in virulence, we identified a sequence showing closest relationship to the *L. mexicana* and *T. brucei CRK3* genes [24]. We thus termed it *Leishmania major CRK3* (or Lmaj-*CRK3*), and here we describe its sequence, activity and developmental regulation. Notably, expression of *CRK3* in *S. pombe* at appropriate levels permitted rescue of a *cdc2*ts mutant, the first such example of heterologous rescue by a trypanosomatid CRK.

2. Materials and methods

2.1. Parasites

L. major Friedlin V1 promastigotes were grown in M199 medium at 25°C [25]. Promastigotes were harvested in logarithmic phase of growth (2.5×10^6 cells ml^{-1}), or allowed to grow to stationary

phase (2×10^7 cells ml^{-1}). Metacyclic forms were separated from stationary phase culture by the peanut agglutination method [26]. DNA transfections and plating on semi-solid media were performed as described [27]. Infections of BALB/c mice, monitoring of lesions by footpad size, and recovery and purification of amastigotes were performed as described [28].

2.2. Cloning and sequencing of the CRK3 gene

Several cosmids were isolated from a genomic library [29] in the process of identifying virulence genes involved in *L. major* infection. From cosmid I, an 8 kb *Eco*RV fragment including *CRK3* was cloned into pBluescript II (Stratagene) for sequence analysis (Beverley lab strain B2136). The CRK3 coding region was amplified by PCR with the primers SMB185 (5′-gcgagatctatgtacccatacgat-gttccagattacgctagcttgggatccacc-ATGTCTTCGT-TTGGCCGTGTTAC) and SMB186 (5′-gcgagatc-tggatcc-AACGCGTCAGCAAACTG; bases corresponding to *CRK3* are in upper case, added restriction sites are shown in lower case, and an N-terminal extension encoding the 11-amino acid influenza hemagglutinin (HA) epitope tag [30] is in underlined lower case). *Bgl*II-digestion of the PCR product yielded epitope-tagged *HA-CRK3* while *Bam*HI digestion yields the wild-type *CRK3*. The amplified *HA-CRK3* fusion was inserted into the *Bam*HI expression site of the *Leishamania* expression vector pXG [31], yielding pXG-HA-CRK3 (strain B2167). The accuracy of the PCR fragment was confirmed by sequencing, and the *CRK3* segment was removed by *Bam*HI digestion and then inserted into the *Bam*HI site of pXG, yielding pXG-CRK3 (strain B2163).

2.3. Site-directed mutagenesis

Oligonucleotide-mediated mutagenesis was performed with uracil-containing template DNA [32]. A 1.4 kb *Bgl*II + *Hin*dIII fragment containing *CRK3* was cloned into pGEM-7zf(+) vector (Promega; strain B2155) and used as the template for mutagenesis. Mutants were generated with the oligonucleotides SMB178 (5′-TTGGGAGAGGG-AGC GTTTGGTGTTGTGTATC; mutated nu-

cleotides are underlined) to yield *CRK3ON* (T33A/Y34F; strain B2157), or with SMB179 (5′-CATGCACACCTACGCGC ACGAGGTGGT-TAC) to yield *CRK3OFF* (T178A; strain B2158). The mutations were confirmed by sequencing.

2.4. Rescue of a S. pombe cdc2^{ts} mutant

Wild-type or mutant *L. major CRK3*s were amplified by PCR with primers SMB185 and SMB186, digested with *Bam*HI, and inserted into the *Bam*HI site of the *S. pombe* pREP3X expression vector, which contains a thiamine-repressible *nmt1* promoter [33,34]. These constructs (pREP3X-CRK3, -CRKON or -CRKOFF; strains B2172, B3353, and B3354) were introduced into haploid wild-type (*leu1-32, ura4-D18, h*$^-$) or *cdc2* mutant (*cdc2-33*^{ts}*, leu1-32, h*$^-$) *S. pombe* by either electroporation or LiOAc protocols [35]. For induction, cells were precultured in EMM medium [35] containing 5 μg ml^{-1} thiamine to repress the *nmt1* promoter, and then washed and either grown in thiamine-free liquid media or streaked on thiamine-free plates to derepress the promoter, at 36 or 25°C. Cells were observed and photographed using a Zeiss Axioskop microscope.

2.5. RT-PCR, DNA and RNA blot analyses

Total RNA was isolated from Friedlin V1 promastigotes (logarithmic and stationary phases), metacyclic cells, and lesion amastigotes by using the guanidinium method [36] with the RNAzol reagent purchased from Biotecx Laboratories. Complementary DNA was made from 5 μg of stationary promastigote RNA primed with oligo(dT) by SuperscriptII reverse transcriptase (Life Technologies). RT-PCR was performed for 30 cycles of 94°C, 30 s; 55°C, 1 min; and 72°C, 1 min, with a mini-exon specific primer (SMB145; 5′-ATCAGTTTCTGTACTTTATTG) and a *CRK3* specific primer (SMB144; 5′-ACATCAAG-CAAGTTCACAAT). The RT-PCR product was directly cloned into pGEM-T vector (Promega; strain B2152) and sequenced to determine the 5′ splice site of the *CRK3* transcript. Genomic DNA was digested by selected restriction enzymes and

separated on a 0.8% agarose gel. Total RNA isolated from different stages was denatured by 7 M glyoxal at 50°C for 1 h and resolved on a 1% gel with a 10 mM Na_2HPO_4 buffer system. The *CRK3* coding region probe was labeled by random priming. Southern and Northern blots were hybridized at 65°C for 16 h and washed at 65°C in $0.1 \times$ SSPE, 1% SDS.

2.6. Immunoprecipitation, protein kinase assay and immunoblotting

Leishmania protein extracts for immunoprecipitation were prepared as described [19]. Five hundred micrograms of protein from *L. major* transfected with pXG-HA-CRK3 were incubated with 20 μl of a polyclonal anti-HA antibody (Clontech) after incubation with preimmune rabbit serum for 1 h. One hundred micrograms of protein A-Sepharose was added and the incubation continued for 1 h. The beads were washed three times with 1 ml of lysis buffer, and used for H1 kinase assays after washing in $1 \times$ kinase buffer [19]. They were then preincubated at 30°C for 5 min, and then 20 μl of prewarmed 0.25 mg ml^{-1} histone H1, 2.5 μM ATP, 50 nCi of [α-^{32}P]ATP, were added and incubated for 15 min. The reaction was stopped by the addition of SDS-PAGE loading buffer, the samples were boiled for 2 min, and then electrophoresed on a 12.5% SDS-polyacryamide gel. Following electrophoresis, the gel was dried and autoradiographed at -80°C for overnight. For Western blots, 20 μl of the protein extract was mixed with SDS loading buffer, boiled for 5 min, and electrophoresed as above. The proteins were then blotted to immunoblot membrane (Amersham), and detection performed using the ECL method (Amersham). Results were quantitated by phosphorimaging (kinase activity) and scanning densitometry (protein).

3. Results

3.1. Identification of L. major CRK3

DNA sequence analysis [24] of a cosmid that partially rescued the virulence of the avirulent

Friedlin A1 line of *L. major* [37] revealed an ORF, potentially encoding a 311 amino acid polypeptide with similarity to the cdc2 family of protein kinases. The ORF showed 56% amino acid sequence identity to both *S. pombe* p34^{cdc2} [9] and *S. cerevisiae* p34^{CDC28} [38] (Fig. 1), and 60% identity to human CDK2 [10,39]. Comparisons with several trypanosomatid CRKs showed that the *L. major* ORF had 99, 82, and 82% sequence identity to CRK3s from *L. mexicana* [20], *Trypanosoma cruzi* [23] and *T. brucei* [40], respectively. We thus designated the *L. major* gene *CRK3* (Lmaj*CRK3*).

The predicted CRK3 protein shows conservation of key functional domains of the cdc2 protein kinase family (Fig. 1). Although there are some substitutions in the conserved PSTAIRE region implicated in cyclin binding, CRK3 retained the highly conserved ATP-binding site found in all functional cdc2/CDK kinases as well as several key residues implicated in cdc2 regulation such as T14, Y15 and T161 (Fig. 1B). The *Leishmania* and *Trypanosoma* CRK3 proteins have 10–11 additional amino acids at the N-terminus as compared to other cdc2 kinases, whose function is unknown.

Since *CRK3* was isolated from a cosmid identified by its ability to rescue a long-term culture passage avirulent line, we asked whether *CRK3* was the relevant gene identified in this genetic screen. *CRK3* was inserted into the *Leishmania* expression vector pXG, transfected into the avirulent *L. major* A1 line, and then tested for infectivity BALB/c mice. This test was negative; instead, another gene present within the cosmid gave rise to the restoration of virulence in the avirulent line (data not shown; LK Garrity, Y Wang, SM Beverley, in preparation). We emphasize that the failure of *CRK3* overexpression to rescue virulence of the A1 cell line does not rule out the possible involvement of *CRKs* in normal growth or pathogenesis of the parasite. This is most clearly shown by the fact that both *CRK1* and *CRK3* are essential in *L. mexicana* [20,21].

3.2. CRK3 rescues an S. pombe cdc2^{ts} mutant

In order to investigate the functional relatedness of *CRK3* to the family of cyclin-dependent

ATP-binding
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LmajCRK3     1  MSSFGRVTARSGDAGTRDSLDRYNRLDVLGEGTYGVVYRAVDKITG...QYVALKKVRLDRTEEGI
LmexCRK3     1  MSSFGRVTARSGDAGTRDSLDRYNRLDVLGEGTYGVVYRAVDKITG...QYVALKKVRLDRTEEGI
TcCRK3       1  MTTLTTLPSRPLSSNTREQFERYNRMDILGEGTYGVVYRAVDKITG...QIVALKKVRLDRTEEGI
TbCRK3       1  MTMLGALTGRQLSSGLKDQFDRYNRMDILGEGTYGVVYRAVDRATG...QIVALKKVRLDRTDEGI
ScCDC28      1  ...............MSGELANYKRLEKVGEGTYGVVYKALDLRPGQGQRVVALKKIRLESEDEGV
SpCDC2       1  ..................MENYQKVEKIGEGTYGVVYKARHKLSG...RIVAMKKIRLEDESEGV
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PSTAIRE domain

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LmajCRK3    64  PQTALREVSILQEFDHPN....IVNLLDVICSDG.KLYLVFEYVEADLKKAIEKQ.EGGYSGMD..
LmexCRK3    64  PQTALREVSILQEFDHPN....IVNLLDVICSDG.KLYLVFEYVEADLKKAIEKQ.EGGYSGMD..
TcCRK3      64  PQTALREVSILQEIHHPN....VVNLLDVICTDG.KLYLIFEYVDYDLKKAIEKR.GCTFTGVT..
TbCRK3      64  PQTALREVSILQEIHHPN....IVNLLDVICADG.KLYLIFEYVDHDLKKALEKR.GGAFTGTT..
ScCDC28     52  PSTAIREISLLKELKDDN....IVRLYDIVHSDAHKLYLVFEFLDLDLKRYMEGIPKDQPLGAD..
SpCDC2      45  PSTAIREISLLKEVNDENNRSNCVRLLDILHAES.KLYLVFEFLDMDLKKYMDRISETGATSLDPR
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T161-P
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LmajCRK3   122  .LKRLIYQLLDGLYFCHRHRIIHRDLKPANILLTSGNVLKLADFGLARAFQVPMHTYTHEVVTLWY
LmexCRK3   122  .LKRLIYQLLDGLYFCHRHRIIHRDLKPANILLTSGNVLKLADFGLARAFQVPMHTYTHEVVTLWY
TcCRK3     122  .LKKLVYQLLDGLFFCHRHRIVHSDLKPANILITSDNVLKLADFGLARTFQIPMHTYTHEVVTLWY
TbCRK3     122  .LKKIIYQLLEGLSFCHRHRIVHRDLKPANILVTTDNSVKIADFGLARAFQIPMHTYTHEVVTLWY
ScCDC28    112  IVKKFMMQLCKGIAYCHSHRILHRDLKPQNLLINKDGNLKLGDFGLARAFGVPLRAYTHEIVTLWY
SpCDC2     110  LVQKFTYQLVNGVNFCHSRRIIHRDLKPQNLLIDKEGNLKLADFGLARSFGVPLRNYTHEIVTLWY
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LmajCRK3   187  RAPEILLGEKHYTPAVDMWSVGCIFAELTRRKVLFRGDSEIGQLFEIFQVLGTPTDTEGSWPGVSR
LmexCRK3   187  RAPEILLGEKHYAPAVDMWSVGCIFAELARRKVLFRGDSEIGQLFEIFQVLGTPTDTEGSWPGVSR
TcCRK3     187  RAPEILLGEKHYTPAVDIWSVGCIFAELARGKVIFRGDSEIGQLFEIFQILGTPMDNEGSWPGVSS
TbCRK3     187  RAPEILLGEKHYTPAVDMWSIGCIFAELARGKVLFRGDSEIGQLFEIFQVLGTPMDAEGSWLGVSS
ScCDC28    178  RAPEVLLGGKQYSTGVDTWSIGCIFAEMCNRKPIFSGDSEIDQIFKIFRVLGTPN..EAIWPDIVY
SpCDC2     176  RAPEVLLGSRHYSTGVDIWSVGCIFAEMIRRSPLFPGDSEIDEIFKIFQVLGTPN..EEVWPGVTL
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LmajCRK3   253  LPDYRDVFPKWTAKRLGQVLPELHPDAIDLLSKMLKYDPRERISAKEALQHPWFSDLRW
LmexCRK3   253  LPDYRDVFPKWTAKRLGQVLPELHPDAIDLLSKMLKYDPRERISAKEALQHPWFSDLRW
TcCRK3     253  LPDYRDVFPRWAGKPLAQVIPQLDSEAIDLISRMLKYSPAERISAKEALQHSWFSEIRT
TbCRK3     253  LPDYRDVFPKWSGKPLTQVLPTLDGDAVDLLSQMLRYNPAERISAKAALQHPWFSDAMF
ScCDC28    242  LPDFKPSFPQWRRKDLSQVVPSLDPRGIDLLDKLLAYDPINRISARRAAIHPYFQES..
SpCDC2     240  LQDYKSTFPRWKRMDLHKVVPNGEEDAIELLSAMLVYDPAHRISAKRALQQNYLRDFH
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Fig. 1. Comparison of trypanosomatid CRK3 and yeast CDC2/CDC28 kinases. The alignment was produced using the ClustalX algorithm. Positions identical in four or more sequences are shown in gray, and gaps were introduced to maximize homology. Conserved domains are noted and phosphorylation sites are indicated (*). The sequence is deposited in GenBank (AF073381). Lmaj, *Leishmania major*; Lmex, *L. mexicana mexicana*; Tc, *Trypanosoma cruzi*; Tb, *T. brucei*; Sc, *Saccharomyces cerevisae*; and Sp, *Schizosaccharomyces pombe*.

protein kinases, we attempted to rescue the growth defect of a temperature sensitive *cdc2* mutant in *S. pombe*. *CRK3* was expressed from the strong thiamine-repressible *nmt1* promoter on a multicopy plasmid pREP3X [33,34], and introduced into *cdc2-33*[ts] mutant cells that arrest predominantly at the G₂-M transition at 36°C [7,41,42]. The *nmt1* promoter becomes highly

active in minimal medium [34].

Upon derepression of the *nmt1* promoter, the growth defect of the *cdc2-33*[ts]/pREP3X-CRK3 cells at the restrictive temperature of 36°C was not reversed. However, the cells did not grow at the permissive temperature of 25°C either. Microscopic observation revealed that the cells did not undergo cellular elongation, which is a character-
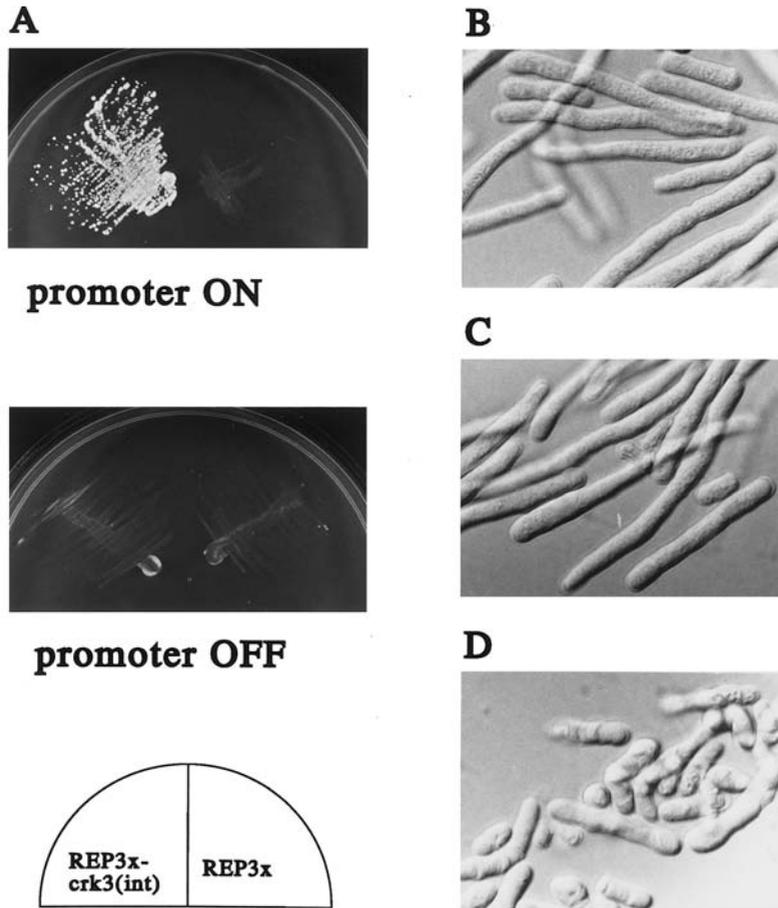
Fig. 2. Rescue of a *Schizosaccharomyces pombe cdc2* mutant by *L. major CRK3*. (A) *cdc2-33* [ts] cells transformed with the pREP3X vector, or with pREP3X-CRK3 integrated in the genome, were streaked on plates containing (promoter OFF) or lacking (promoter ON) thiamine, and incubated at 36°C for 3 days. (B–D) Nomarski-optic image of *cdc2-33* [ts] cells at the restrictive temperature transformed with the pREP3X vector (B), or pREP3X-CRK3 plasmid with promoter off (C) or promoter on (D).

istic of the *cdc2-33* [ts] cell cycle arrest at the restrictive temperature (data not shown). This suggested the possibility of a detrimental effect of *CRK3* on cell growth due to a high level of expression. This putative overexpression toxicity was observed only in the *cdc2-33* [ts] mutant at the permissive temperature, but not in wild-type cells, suggesting interference specifically with the function of the p34[cdc2] kinase.

We then asked whether a lower level of *CRK3* expression could rescue the *cdc2* mutant phenotype. This was accomplished by integrating the multicopy pREP3X-CRK3 plasmid into the yeast genome. 50 000 pREP3X-CRK3 transformants were plated on media lacking thiamine at 36°C. Two clones were obtained, which were able to grow at the restrictive temperature when the promoter was derepressed, but not when it was repressed (Fig. 2A). The strains were cultured under non-selective conditions to allow for plasmid loss, and then plated on selective media to ensure retention of the integrated plasmid marker. Chromosomal integration was confirmed by Southern blot analysis (data not shown), and demonstrating that the integrated leucine marker linked to *CRK3* segregated 2:2. For one strain, there were 85 *leu* [+]

and 79 *leu*⁻ colonies, in the other we obtained 48 *leu*⁺ and 60 *leu*⁻ colonies.

Morphological observation revealed that under conditions repressing *CRK3* expression, the integrated *S. pombe* transformants displayed cellular elongation (Fig. 2C) characteristic of *cdc2* mutants (Fig. 2B), which was not seen when expression of CRK3 was derepressed (Fig. 2D). Thus, integration of pREP3X-CRK3 allowed CRK3 production sufficient for the rescue of the *cdc2-33*[ts] mutation but not for exerting negative effects on the growth of the cells.

3.3. Effect of CRK3 mutations in essential regulatory and/or catalytic domains

We tested the activity of two engineered mutant *L. major* CRK3s. A constitutively active CRK3 (CRK3ON) was created by changing the threonine-tyrosine residues at positions 33–34 to alanine-phenylalanine (Fig. 1). These two residues are implicated in the down-regulation of kinase activity in other cdc2 family members [43]. An inactive CRK3 (CRK3OFF) was created by changing the threonine at position 178 to alanine (Fig. 1). In other cdc2 kinases, this residue is essential for activation [44–46]. Both CRK3ON and CRKOFF were inserted into pREP3X, and introduced into either wild-type or *cdc2-33*[ts] *S. pombe* strains in the presence of thiamine to repress *nmt1*-driven expression.

As for wild-type *CRK3*, no specific phenotype with regard to growth rate or cell morphology was observed when expression of either *CRK3* mutant was induced by the absence of thiamine in wild-type cells. However, expression of both *CRK3ON* and *CRK3OFF* in the *cdc2-33*[ts] mutant lead to growth inhibition, even at the permissive temperature (Fig. 3A). *cdc2-33*[ts] cells expressing *CRK3OFF* (Fig. 3C) had the elongated shape characteristic of loss of function c*dc2* mutants (Fig. 2B). In contrast, *cdc2-33*[ts] cells expressing *CRK3ON* (Fig. 3D) had a smaller cell size than wild-type cells (Fig. 3B), indicating a premature entry into mitosis, which is characteristic of the constitutively active dominant *cdc2* mutants [43].

Thus, the phenotypes of *CRK3ON* and *CRKOFF* over-expression resemble those of analogous dominant negative mutants of *S. pombe cdc2* [47,48] which are thought to be defective in their interactions with regulatory proteins or substrates. The fact that the mutated CRK3 proteins could only affect *cdc2-33*[ts] but not the wild-type cells suggests that the mutated p34[cdc2-33ts] is impaired in its ability to interact with regulatory molecules, making it possible for the heterologous CRK3ON and CRKOFF proteins to exert their dominant negative phenotypes. The same explanation applies to the lack of phenotype from overexpression of the wild-type *CRK3* in wild-type cells, as compared to the growth inhibition in the *cdc2-33*[ts] mutant strain at the permissive temperature. A similar phenotypic pattern has been observed in fission yeast expressing dominant negative mutants of an *Arabidopsis thaliana* CDK [49].

3.4. CRK3 is single copy and is expressed constitutively throughout the life cycle

Genomic Southern blot analysis, using a *CRK3* coding region probe and digests with several different enzymes lacking sites in *CRK3*, detected a single hybridizing band, indicating that *CRK3* is a single copy gene (data not shown). RT-PCR analysis with *CRK3* and mini-exon primers mapped the 5′ splice acceptor to position −364 bp relative to the presumptive translational start codon (Fig. 4). This splice site was also identified by an *L. major* EST from the LV39 strain (GenBank AA741770). Remarkably, this unexpectedly long 5′-untranslated region contains a 38 bp-long CA-dinucleotide repeats and several other nucleotide runs (Fig. 4). Generally, these simple sequence repeats are characteristic features of intergenic sequences in the *Leishmania* genome [50]. The potential role(s) of these sequences on *CRK3* transcription, processing and/or translation has not been tested.

When the same amount of total RNA from logarithmic phase promastigotes, stationary phase promastigotes, metacyclic promastigotes or amastigotes was probed with the *CRK3* coding region, a 1.7 kb mRNA was detected with equal intensity in each sample (Fig. 5A). The loading of RNA samples was standardized by hybridization
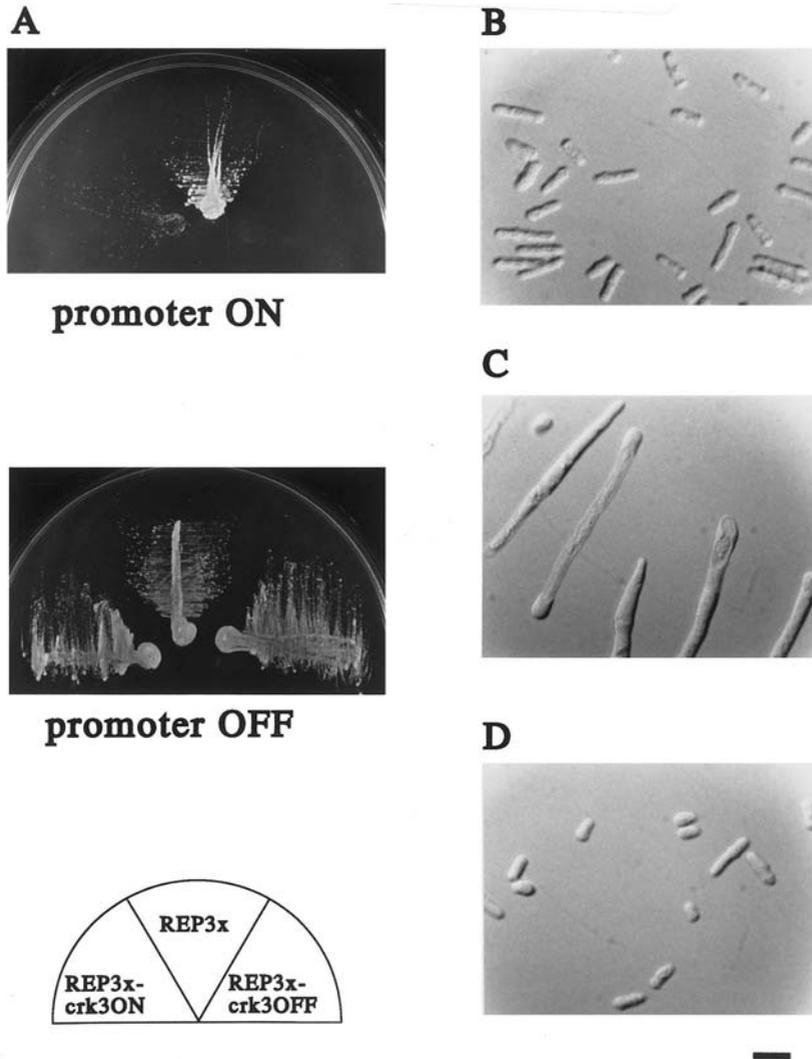
Fig. 3. Effects of *L. major* 'ON' or 'OFF' *CRK3* mutants expressed in *S. pombe*. (A) *cdc2-33*[ts] cells transformed with the vector pREP3X, pREP3X-CRK3OFF, or pREP3X-CRK3ON plasmids were streaked on a plate containing (promoter OFF) or lacking (promoter ON) thiamine, and incubated at 25°C for 3 days. (B–D) Nomarski optic image of *cdc2-33* [ts] cells at the permissive temperature transformed with pREP3X (B), pREP3X-CRK3OFF (C), or pREP3X-CRK3ON (D).

of the same blot to the *L. major* actin gene [51] (Fig. 5B). We conclude that the *CRK3* mRNA is expressed constitutively throughout the life cycle.

3.5. CRK3 has stage-regulated H1 kinase activity

In order to detect the kinase activity of CRK3, an N-terminal HA-tagged version of the CRK3 protein (HA-CRK3) was expressed in wild-type virulent *L. major* Friedlin V1. The activity of the tagged kinase was assayed by phosphorylation of histone H1, following immunoprecipitations with a polyclonal anti-HA antibody on protein extracts prepared from log and stationary phase parasites (Fig. 6A). The amount of the HA-CRK3 protein in these extracts was determined by Western blots with the same antibody (Fig. 6B). CRK3 H1-kinase activity within log phase promastigotes was

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  1  TTTTCTTCACATCTTTTTTTTCTCTTTTGTTCCTGATCTTGTGTTGTTCCGCTCAGAAGT
 61  AACAACGCTTCATAGTAAAGGCGTCCATTTGCCCAGCGTGGAGCTTTTTTTTTATCGGCT
121  CACGATCTAGAACTCGTCGGGAAAAAGGTATCCTGACAAAAAAAGAATACACACACTACA
181  CAAATACTTTTTCCCCCTTTAGCGCCTCTGCTGTTGCACTTTTCCGGCCCTTTCTCCGCG
241  CTACAACACACACACACACACACACACACACACACATACACAGATACAGGTGCCGAAAGCAC
301  TAGTTTCTGGTGCCTGCTCTTTTGTTGTTTGCGAGGAAAGGCGACGAAGGAATACCCTCC
361  TTTCTTTTGAAGTTTGCGGAGCCTCTGCTTTGAACAAAGCTTCTCCCTACAGCATTACAA
421  ATGTCTTCGTTTGGCCGTGTTACCGCCCGTAGCGGTGACGCTGGGACGCGTGACAGTCTT
      M   S   S   F   G   R   V   T   A   R   S   G   D   A   G   T   R   D   S   L
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Fig. 4. CRK3 has a long 5′-untranslated region containing many simple-sequence motifs. The AG in bold marks the 5′ splice site determined by RT-PCR. Underlining marks the location of CA dinucleotide repeats. The CRK3 coding region is shown with the translated protein sequence underneath. The sequence is deposited in GenBank (AF073381).

approximately 10-fold higher than stationary phase promastigotes (Fig. 6A), while there was relatively little change in HA-CRK3 protein levels (Fig. 6B).

4. Discussion

4.1. Functional rescue of yeast cdc2 mutants by Leishmania CRK3

We have identified and characterized the properties of a *L. major* CDK protein kinase, *CRK3*. By expressing *CRK3* in a *S. pombe cdc2*[ts] mutant, we were able to rescue the mutant phenotype. Several previously identified trypanosomatid *CRK*s failed to rescue mutants of *S. pombe* p34[cdc2] or *S. cerevisae* p34[CDC28] [19,20,23,52]. It seems likely that the reduction in *CRK3* expression levels achieved by chromosomal integration may account for the success of our experiments. It has been reported that overexpression of cdc2-related kinases can be toxic to cells [48,53,54]. Consistently, when we expressed *CRK3* from an episome in the *S. pombe cdc2* mutant, we found that overexpression of the gene rescued the cell morphology, but not the growth. Only the integration of *CRK3* was able to rescue growth of the mutant, indicating that a proper level of expression is crucial to the success of heterologous tests of trypanosomatid CDKs. This argues for considerable functional similarity between CRK3 and the cdc2 family protein kinases, and suggests that

studies of other *CRK3* mutants expressed heterologously in *S. pombe* could prove valuable (Fig. 3).

4.2. Regulatory sites of cdc2 kinases shared by CRK3

Many cell-cycle regulatory mechanisms are conserved in a wide range of eukaryotic organisms, and correspondingly, many important residues of p34[cdc2] are highly conserved as well. In all eukaryotic cells studied, cdc2 is phosphorylated on Y15 and to a lesser extent on the adjacent T14 during interphase, and dephosphorylated at mitosis [43,44,55]. Mutations at these sites result in premature activation of the kinase and entry into mitosis, suggesting that phosphorylation at the residues negatively regulates cdc2 activity [43]. cdc2 is also phosphorylated on T161 and phosphorylation on this site accompanies cdc2 activation during mitosis [44–46]. Mutation of this residue results in the loss of the kinase activity in vitro and the loss of *cdc2* function in vivo [46].

We tested the function of these three conserved residues in CRK3. CRK3 contains T33Y34 at the positions analogous to cdc2 T14Y15 within the ATP-binding domain, and has a conserved T178 analogous to cdc2 T161 (Fig. 1). Mutagenesis of these residues in CRK3, and tests of their functions in the *S. pombe cdc2*[ts] mutant, suggests that these residues have functions similar to those in the cdc2 kinase family. Specifically, expression of the CRK3ON (TY14/15AF) and CRK3OFF

(T178A) mutants had dominant negative effects on the *cdc2-33*[ts] mutant at the permissive temperature. This phenotype was similar to that observed with analogous *S. pombe* 'ON' and 'OFF' mutants [47,48], suggesting a possible interaction between regulatory molecules of *S. pombe* p34[cdc2] and *CRK3*.

The 16-amino acid sequence known as the PSTAIRE box is a highly conserved domain thought to be important for binding of cyclins. However, CRK3 has six nonconservative substitutions in this domain, which are shared by trypanosome CRK3s (Fig. 1). The only trypanosome cyclin identified so far has a highly diverged sequence compared to mitotic type cyclins in other eukaryotic organisms [56]. Recently, it was shown that the *T. cruzi* CRK3 was able to interact with
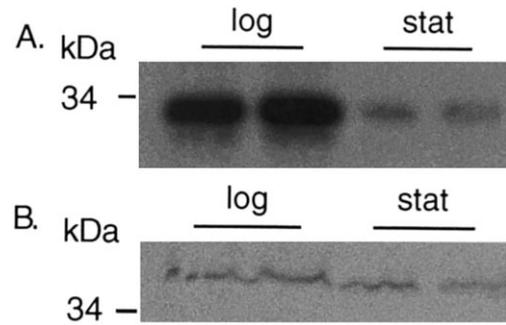
Fig. 6. Histone H1 kinase activity of *L. major* HA-CRK3. (A) H1 kinase activity. Duplicate protein extracts of *Leishmania major* promastigote cells transfected with the pXG-HA-CRK3 construct were immunoprecipitated with a polyclonal anti-HA antibody. The precipitates were assayed for H1 kinase activity for logarithmic promastigotes (log) and stationary promastigotes (stat). (B) Western blot. Protein extracts described above were separated on a 12.5% SDS-polyacryamide gel, blotted to a nylon membrane, and detected with anti-HA antibody.

mammalian cyclins [23], suggesting the possibility of families of cyclin genes as seen in higher eukaryotes. Thus, it appears that by genetic and biochemical tests, trypanosomatid CRKs show all of the classic properties of the cdc2 kinase family. The availability of heterologous assays in genetically amenable yeast will provide a powerful new approach for the future dissection of this important pathway.

4.3. Stage-specific kinase activities of CRKs

The presence of a CRK gene family in both *Leishmania* and trypanosomes suggests that, as in higher eukaryotes, these kinases may have distinct functions in regulation of complex differentiation pattern. The patterns of expression and activity of the known CRKs support this view. *L. mexicana* CRK1 kinase activity is highest in dividing procyclic promastigotes, declines in metacyclic promastigotes, and is absent from the amastigote phase, although the CRK1 protein is present throughout the life cycle [19]. *L. major* CRK3 is also regulated by post-translational mechanisms, as the H1-kinase activity declines dramatically in stationary phase (Fig. 6). Similar results were obtained with the p13[suc1]-binding kinase (SBCRK) of *L. mexicana* [19], recently shown to
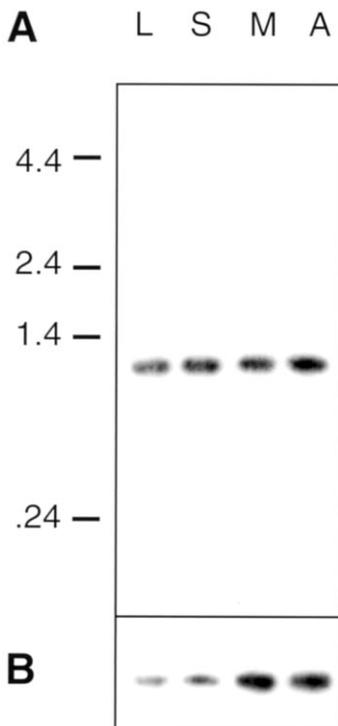

Fig. 5. Northern blot analysis. (A). Total RNA was separated on a 1% agarose gel, transferred to a nylon membrane, and hybridized with a *CRK3* coding region probe. (B). The same blot was reprobed with the *Leishmania major* actin gene. RNA samples were obtained from logarithmic-phase (L), staionary-phase (S), or metacyclic (M) promastigotes, and lesion amastigotes (A).

be *CRK3* [20]. Unlike CRK1, *L. mexicana* CRK3 kinase activity was found in both dividing stages of the parasite, making it a candidate for the key mitotic activator of the *Leishmania* cell cycle [19,20]. Gene-knockout studies of *L. mexicana CRK1* and *CRK3* have shown that both of these are essential for promastigote growth [20,21]. As in higher eukaryotes, *Leishmania* appears to possess a variety of CDKs with distinct temporal patterns of expression and activity, which are essential for cellular growth and possibly development.

Since *Leishmania* CRKs differ from their human host CDKs in their sensitivity to inhibitors such as olomoucine [20], it is possible to contemplate the design of species-selective inhibitors of this critical parasite regulatory enzyme. This goal could benefit from the intensive structural and pharmacological efforts already focused upon the host enzyme.

Acknowledgements

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