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Leishmania mexicana: expression, characterization and activity assessment of *E. coli*-expressed recombinant CRK3

N.O.M. ALI[1,2#], M.E. IBRAHIM[3], K.M. GRANT[2], J.C. MOTTRAM[2]

[1]Department of Parasitology, Faculty of Veterinary Medicine, University of Khartoum, Sudan
[2]Wellcome Trust Centre for Molecular Parasitology, Glasgow Biomedical Research Centre, University of Glasgow, Scotland, UK
[3]Department of Molecular Biology, Institute of Endemic Diseases, University of Khartoum, Sudan

Abstract. – OBJECTIVES AND METHODS: Previous studies have shown that CRK3 protein kinase of *Leishmania mexicana* is a potential drug target. Therefore, the aim of this study was to provide an active protein kinase for chemical inhibitors testing. A system was developed to express and affinity-purify recombinant *L. mexicana* CRK3 protein from *Escherichia coli.*

RESULTS: Biochemical analysis has confirmed the expression of the pure kinase. The bacterial-expressed kinase was found to be inactive as a monomer. The mutated CRK3-E178 protein kinase was also found to be inactive.

CONCLUSION: This study suggests that cyclin binding and phosphorylation status are both important for reconstituting protein kinase activity. Work presented by this paper has confirmed the usefulness of the prokaryotic system for production of pure homogenous recombinant protein kinase of *Leishmania* parasite, though this system is unable to produce active CRK3 protein kinase.

Key Words:
Leishmania mexicana, Bacterial-expression, Activity, cdc2-related kinase, CRK3, Drug target.

Abbreviations

CRK3 = cdc2-related kinase 3
E. coli = *Escherichia coli*
SDS = Sodium dodecyl sulphate
Ni-NTA = Nickel-nitrilotriacetic acid
Kb = kilobase

Introduction

The protozoan parasite of the genus *Leishmania* causes leishmaniasis in most tropical and sub-tropical areas of the world, affecting 12 million people and rendering 350 million at risk in 88 countries, 72 of which are developing countries[1]. The current treatments for kinetoplastid-caused diseases are not ideal: They are either very expensive, ineffective in many patients, or have severe side-effects. For instance, current treatments for the life-threatening, visceral form of leishmaniasis –VL -(antimonials or the expensive amphotericin B/lipid formulation) are administered by a series of injections over several weeks. However, recent clinical trials of an oral formulation of miltefosine have given promising results in treatment of VL[2]. Nevertheless, there is still a need for treatments, which have fewer side effects and shorter treatment courses. There are several target molecules or metabolic pathways of kinetoplastids that have been investigated including trypanothione reductase and polyamine metabolism, farnesyl transferase and cysteine proteinases, folate metabolism, topoisomerases[3], and cAMP signalling pathways. Other protozoa molecules such as cyclin-dependent kinases (CDKs) have recently investigated as novel drug targets[4].

A number of cdc2-related kinases (CRK) genes have been isolated from trypanosomatids, including *Crithidia fasciculata*[5], *Trypanosoma (T.) brucei*[6], *Trypanosoma cruzi*[7], *Leishmania (L.) mexicana*[8-9], *L. major*[10], and *L. donovani*[11]. *L. mexicana* cdc2-related kinase (*CRK3*) is a single copy gene with 5-fold higher mRNA in the replicative promastigote form than in both the non-dividing metacyclic and the mammalian amastigotes forms[9]. In addition, CRK3 from cell extracts of three life-cycle stages was found to bind the fission yeast $p13^{suc1}$ as well as the leishmanial homologue $p12^{cks1}$. Moreover, CRK3 fused with six histidines at the C-terminus was

Corresponding Author: Nahla O.M. Ali, MD; e-mail: dr_nahla2004@yahoo.com

expressed in *L. mexicana* and was shown to have suc-binfing cdc2-related kinase (SBCRK) histone H1 kinase activity[9].

Furthermore, CRK3 is essential to *L. mexicana*, as all attempts to disrupt both alleles in the promastigote form had resulted in changes in cell ploidy, and is expressed throughout the whole life cycle with its active status at the G2/M phase of the cell cycle[12]. The *L. mexicana* CRK3 has also been shown to be of 54% sequence identity to the human HsCDC2[9]. Taken all these together, CRK3 have a potential as drug target. Flavopiridol inhibits CRK3 kinase activity through studies done on recombinant (transgenic parasite) and native forms[12]. However, the specificity has not yet been confirmed, due to the low expression level of *CRK3* in *Leishmania.* In addition, Flavopiridol could probably inhibit other leishmanial CRKs. Therefore, recombinant CRK3 produced in prokaryotic system would be more suitable for this purpose.

The over-expression of CRKs in the bacterial as well as insect cells is well known approach to study the biochemical and physical characteristics of these molecules, and to evaluate their associated kinase activity. The recombinant *T. cruzi* CRK1 (TzCRK1) is a good example that has been shown to interact with the mammalian cyclins[7]. The main objective of this study was to provide an active CRK3 that could be used for inhibitory assays. In order to produce homogenous pure recombinant CRK3, *Escherichia (E.) coli* system was used in this study.

Materials and Methods

Expression of L. mexicana CRK3 Protein in E. coli

The *L. mexicana CRK3* gene was excised from the plasmid pGL343 by digestion with *Bam*HI, gel-purified and sub-cloned into pET5a (Novagen, Merck KGaA, Darmstadt, Germany) to give plasmid pGL448, that encodes a C-terminally His_6-tagged CRK3 (CRK3his). In order to confirm the presence of the *CRK3* fragment, a PCR screen was performed using the primers OL258 and OL261, which anneal to the 5′ and 3′ end of the *CRK3* gene and the correct orientation was checked by restriction digestion with *Bam*HI. *E. coli* BL-21 (DE3) expression strain was then transformed with plasmid pGL448. Overnight cultures of these cells were diluted 1:100 into fresh Luria-Bertani (LB) media supplemented with 100 μg/ml ampicillin, grown at 37°C to an OD_{600} of 0.4-0.6 and then induced (30°C, 0.2 mM IPTG [Isopropyl-1-thio-β-D-galactopyranoside] for 4 hours). Cells were harvested, resuspended in sonication buffer (SB: 50 mM NaPi, pH 8.0, 300 mM NaCl) and lysed by sonication as previously described[16]. The sonicated lysates were cleared by centrifugation. All samples were then, loaded into a 12.5% sodium dodecyl sulphate (SDS)-polyacrylamide gels (PAGE) and the separated proteins were visualized by Coomassie-Blue staining.

Purification of L. mexicana CRK3his from Escherichia coli

The supernatant was bound to nickel-nitrilotriacetil acid (Ni-NTA) agarose beads in 10 ml disposable column and, after washing with WB (50 mM NaPi, pH 6.0, 300 mM NaCl, 10% glycerol), the pure fusion CRK3his was eluted with SB that contains increased amount of imidazole. The Fast Protein Liquid Chromatography (FPLC) (BioCAD, San Diego, CA, USA) was also used to scale up the CRK3his purification and to obtain pure kinase. Originally, a 0.5 litre cultures were induced as usual and the cells lysates were filtered through a 0.2 μm filter. A volume of 12-20 ml of the *E. coli* cell lysates that contains the soluble *L. mexicana* CRK3his was passed over Ni-NTA column (BioCAD, San Diego, CA, USA), which was nickel chelated, primed with 500 mM imidazole and equilibrated by sonication buffer (SB). The column was washed with wash1 buffer (SB containing 0.5 mM imidazole) at a flow rate of 10 ml/min for 10 min, and then with wash 2 buffer (SB containing 20 mM imidazole). The elution was performed using a linear gradient of 20-200 mM imidazole contained in SB over 10 cv (column volume). The protein elution profile was monitored by measuring the absorbance at 280 nm spectrpphotometer (Jenway, NJ, USA). The purification steps were monitored by running all samples on 12.5% SDS-polyacrylamide gels (SDS-PAGE) and the separated proteins were visualized by Coomassie-Blue staining. The relative yield of CRK3his was determined using bicinchoninic Pierce BCA protein assay and was performed as per manufacturer. Briefly, standards were prepared as diluted bovine serum albumin (BSA) in wash buffer then 25 μl of each standard were mixed with 10 μl sample and 200 μl of the working reagent in 24-well microplate. The plate

was then incubated for 30 minutes at 37°C, and then cooled to RT. Finally, the absorbance was measured at 562 nm using Microplate Reader MRX_{II} (Dynex technologies, Kansas, USA). The readings were plotted to find out the correct concentration.

L. mexicana CRK3his Western blot Analysis

Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Little Chalfont, Buckinghamshir**,** UK) for 50 min at RT and 120 V in transfer buffer (48 mM Tris base pH 8.3, 39 mM Glycine, 0.037% SDS (w / v), and 20% (v / v) methanol). Blots were then pre-blocked with Blotto buffer (1 × TBST, 5% low fat milk, 10% horse serum) for 1 hour at room temperature (RT). Meanwhile, the primary antibody was prepared by adding 50 µl of anti-serum 528 (anti-sera against C-terminal peptide sequence from *L. mexicana* CRK3 [CQHPWFSDLRW]) to 5 ml Blotto, then divided into 2 parts, 5 µl of peptide solution were added to one part, and both were mixed for 1 hour at RT. Membranes were blocked for 3 hours at RT (or overnight at 4°C) with blocking peptides to a final dilution of 1:50 or without these peptides. After three washes with 1 × TBST buffer (10 mM Tris [pH 8], 150 mM NaCl, 0.05% Tween-20), membranes were probed with a 1 in 400 dilution of Biotin-conjugated anti-rabbit IgG-B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 45 min at RT, then were incubated for 10 min with a 1 in 100 dilution of avidin reagent (Santa Cruz Biotechnology), after which, the biotinylated horseradish peroxidase (HRP) (Santa Cruz Biotechnology) solution was added and incubated for 30 min. Washing as before, and detected using chemiluminescence system (Pierce SuperSignal, Thermo Fisher Scientific, Rockford, IL, USA).

Site-Directed Mutagenesis

Mutant CRK3his kinase was constructed using Quick Change™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Oligonucleotide primers OL877 (5′-CCCATGCACACCTACGAGCACGAGGTGGTTACG-3′ mutated nucleotides are underlined) and OL878 (5′-CGTAACCACCTCGTGCTCGTAGGTGTGCATGGG-3′) were used to produce Thr-178-Glu mutation. The plasmid pGL448 was used as template to create the plasmid pGL665 and mutagenesis was performed as recommended by the manufacturer. Expression and purification by selection on Ni-NTA agarose beads of the mutated CRK3hisE178 were performed as described previously and washed with washing buffer (50 mM sodium phosphate pH 6, 0.3 M NaCl, 20 mM imidazole, and 10% glycerol), and eluted with imidazole (0.1-0.5 M) added to washing buffer.

Kinase Assay of the Recombinant L. mexicana CRK3his

Assay of CRK3his kinase activity was carried out by binding 5 ml lysates of cells expressing either wild type or mutated CRKhis-E178 to 0.8 ml of 50% slurry Ni-NTA beads. Then by incubating 20 µl of the CRK3his-bound beads with KAM (Kinase Assay Mix) at 30°C for 20 min with 1 µCi [α-^{32}P]-γ-ATP (Amersham Radiolabel Chemicals, Saint Louis, MO, USA) in 20 µl total reaction volume. The unbound proteins were separated from the specifically bound ones by SDS-PAGE run, and the signals were detected by autoradiography (Typhoon phosphor imager, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Different amount of CRK3his preparations were tested as well. As a control, the kinase assay was performed by incubating the CRK3his with variable concentrations of *L. mexicana* cell lysates in order to supply a native cyclin for the recombinant CRK3his.

Results

Expression and Purification of L. mexicana CRK3his in E. coli

Expression of *L. mexicana* CRK3his in *E. coli* resulted in abundant protein of approximate 35 kDa (Figure 1, the arrow). This corresponds well

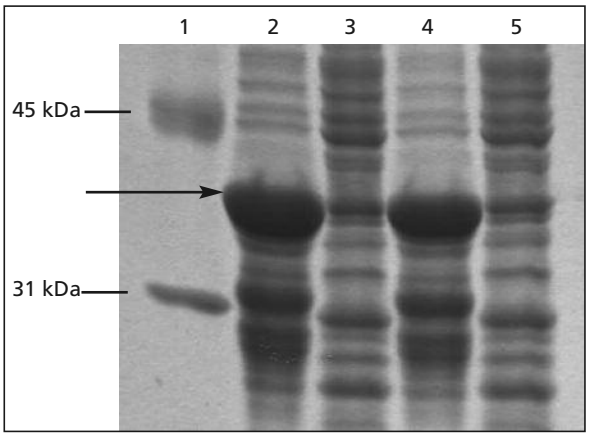

Figure 1. SDS-PAGE analysis of the expression of recombinant *L. mexicana*.

with the predicted molecular weight of *L. mexicana* CRK3. 75% of the expressed protein was detected in insoluble fractions (lanes 2 & 4) and 25% was present in soluble fractions (lanes 3 & 5). Nearly comparable level of expression observed upon using different induction temperatures (16°C, 30°C, and 37°C). Interestingly, using various concentrations of isopropyl β-D-thiogalactoside (IPTG) in a separate experiment has shown that the expression level of CRK3his is very high even with 0.05 mM (results not shown). The level of basal expression was not high as shown by the BL-21 (DE3) cells, no expression observed in the absence of the IPTG (not shown).

CRK3his Protein from BL-21 (DE3) Strain of E. coli

Lane 1 molecular weight marker, lane 2 insoluble fractions of *E. coli* lysates after 4 hr induction at 37°C, lane 3 soluble fraction of *E. coli* lysates after 4 hr induction at 37°C, lane 4 insoluble fractions of *E. coli* lysates after 4 hr induction at 30°C, and lane 5 soluble fraction of *E. coli* lysates after 4 hr induction at 30°C. The arrow shows the expression product.

The CRK3 was expressed as a His_6-tag fusion and was efficiently purified in the soluble form using Ni-NTA resin either in a batch or one-step purification (Figure 2). A single band was eluted, the size of which is consistent with the size of *L. mexicana* CRK3his protein. However, elution of CRK3his with a buffer containing 300 mM and 400 mM imidazole were the most efficient.

A volume of 5 µl of each fraction were loaded on 12.5% SDS-polyacrylamide gel and visualized by Coomassie-Blue staining. Lanes 2, 4, and 6 were, fractions eluted by 0.2 M, 0.3 M, and 0.4 M imidazole, respectively. Lanes 1, 3, and 5, fractions of non-induced BL-21 (DE3) cells used as controls, eluted by 0.2 M, 0.3 M, and 0.4 M imidazole, respectively. Lane 1 Molecular weight marker the arrow shows the eluted *L. mexicana* CRK3his.

In this study to obtain an active kinase, FPLC was used, in which washing and elution conditions were optimised using concentration gradient of imidazole (10 mM-20 mM). All steps were performed at pH 8.0 that is higher than the isoelectric point of the *L. mexicana* CRK3his protein (Ip = 6.8). The final CRK3 preparation is nearly homogeneous as judged by a single Coomassie Blue-stainable band on denaturing polyacrylamide gels (Figure 3). The concentration of recombinant CRK3his protein was determined by two methods: Coomassie brilliant blue and bicinchronic acid (BCA). The BCA methods gave results differing from the values derived from the Coomassie brilliant blue by up to 60% that the concentration of the purified recombinant *L. mexicana* CRK3his was found to be 1.633 mg/ml when the BCA method was used. Taken together, this result suggests that cultures of 0.5 litres are sufficient to prepare approximately up to 10 mg of purified homogenous CRK3his.

Coomassie-Stained SDS Gel

CRK3his (~ 35 kDa) was purified from 35 ml cleared lysates derived from 500 ml induced Bl-21 culture. Purification was performed on 10 ml of Ni-NTA in a1F size column at 10 ml/min in nondenaturing binding buffer containing 0.5 mM imidazole. Wash steps were performed with buffer containing 0.5 mM and 20 mM imidazole and elution with buffer containing gradient 20-200 mM imidazole. Lane 1 Molecular weight marker; lanes 2-8 fractions 32-38 eluted with 200 mM imidazole. The more concentration of imidazole in the elution buffer, the more clear the purified CRK3his.

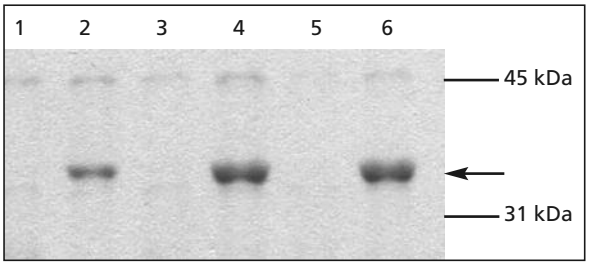

Figure 2. SDS-PAGE analysis of Ni-NTA-purified *L. mexicana* CRK3his.

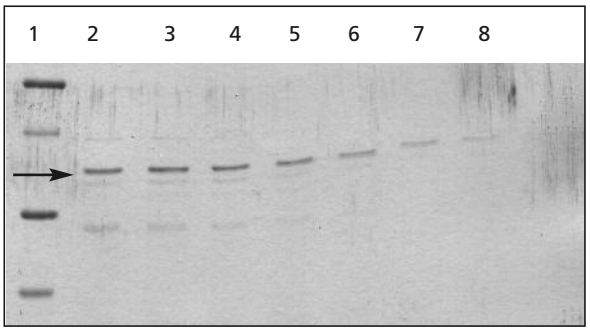

Figure 3. FPLC purification of *L. mexicana* CRK3his on BioCAD

Characterization of E. coli Expressed L. mexicana CRK3his by Immunoblotting

Western blot analysis was performed in order to characterize the recombinant *L. mexicana* CRK3his biochemically. Lysates from three different induction conditions (Figure 4); 16°C (lanes 1 and 4), 30°C (lanes 2 and 5), and 37°C (lanes 3 and 6) were analyzed. ECL (Enhanced Chemiluminescence) system has detected a strong signal of approximate 35 kDa in all the insoluble as well as the soluble fractions, the bands intensities reflects that the expression level is similar in both fractions, inconsistent with the SDS-PAGE analysis. The Ni-NTA purified CRK3his was confirmed further by immunoblotting, using either anti-CRK3 anti-bodies or anti $(His)_6$ anti-bodies, which recognized a single band of an approximate size of 35 kDa (Figure 5).

Cells were harvested after induction with 0.2 mM IPTG at 37°C (lanes 1 and 4), 30°C (lanes 2 and 5), and 20°C (lanes 3 and 6), proteins were extracted and supernatants lysates were applied to a 12.5% SDS-PAGE, transferred to PVDF nylon membranes, probed with anti-CRK3 antibodies and signals were detected by ECL. Samples in lanes 4-6 were probed with anti-CRK3 antibodies and with competing peptides to a final dilution 1:50.

L. mexicana CRK3his in the form of pGL448 was solubilized from BL-21 cells (100 ml) and purified by affinity chromatography on Ni-NTA resin. The protein fractions collected after different purification steps were analyzed by SDS-PAGE followed by immunoblotting using Antibodies directed against CRK3 (1:100 final dilution) (Not shown) and Anti- $(His)_4$ antibodies (1:1000 final dilution). Lanes 1-3 are fractions eluted from Ni-NTA by competition with 0.3 M, 0.4 M, and 0.5 M imidazole, respectively. A strong 35 kDa band (the arrow) was recognized by both Anti-$(His)_4$ and anti-CRK3 (not shown) antibodies, which represented the native purified recombinant *L. mexicana* CRK3his.

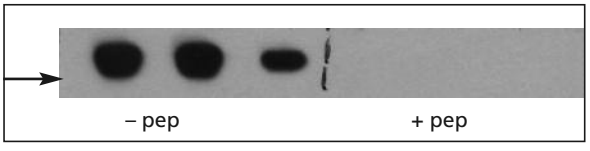

Figure 4. Western blot analysis using polyclonal antiserum raised against the C-terminal of L. mexicana CRK3

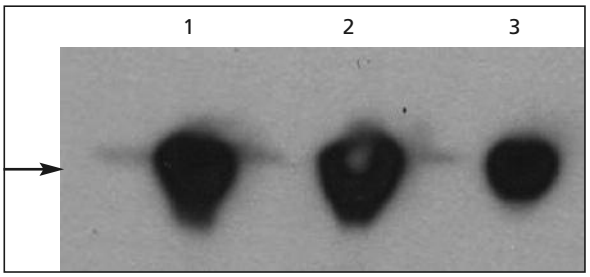

Figure 5. Western blot analysis of Ni-NTA-purified *L. mexicana* CRK3his.

Effect of L. mexicana CRK3 Mutation in its Expression in E. coli

If *L. mexicana* CRK3 is a true member of the CDK family, phosphorylation of Thr-178, the site in *L. mexicana* CRK3 corresponding to Thr 161/167 in the yeast cdc2/CDC28, should make the enzyme active. To testify this hypothesis this threonine residue, in the recombinant *L. mexicana* CRK3his was replaced by a glutamic acid using site directed mutagenesis. Based on the fact that the negatively charged amino acids when introduced at a position of a phosphorylatable residue can mimic a phosphorylation of this site[13]. When the mutated plasmid (pGL665) was expressed in *E. coli* a similar protein size was obtained compared to wild-type (WT) (not shown). However, the band intensity was stronger in the mutant one; this may be due to the effect of the single amino change in the T-loop that might play role in the solubility of the recombinant kinase. Replacement of Thr-178 with Glu-178 residue produced CRK3his recombinant protein in BL-21 strain of *E. coli* that was still soluble, as judged by its ability to induce under same conditions of temperature and IPTG concentration that used for WT recombinant CRK3his. Interesting-

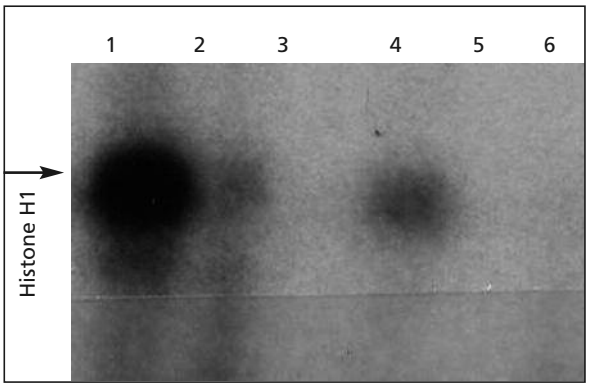

Figure 6. Western blot analysis of Ni-NTA-purified *L. mexicana* CRK3his.

ly, it was noticed that the gene expression from the pGL665 was about 5-fold higher than that detected in the wild-type pGL448. Taken all together, these results suggest that the exogenous expression of the recombinant CRK3his was practically unaffected by the Thr-178-E mutation change, and that the mutated CRK3his is not toxic to the *E. coli* cells.

Assessment of Histone H1 Kinase Activity of E. coli Expressed L. mexicana CRK3his

In this study the *in vitro* activity of WT and mutant CRK3his was assessed on the exogenous substrate histone H1, since it has been identified as a suitable substrate for the *L. mexicana CRK3*[14]. To determine if the recombinant *L. mexicana* CRK3his is active as a monomer, histone H1 kinase assay was performed following binding of cell lysates to Ni-NTA agarose beads (Figure 6). A signal of approximate 31 kDa corresponds to the histone H1, was detected by the transgenic *L. mexicana* expressing the wild-type *CRK3his*, nothing was detected by both WT and mutated CRK3his preparations. Active recombinant CRK3his can be isolated from transgenic *L. mexicana* as has been previously described[9]. In this study the bacterial-expressed CRK3his was found to be inactive as a monomer in the first time. However, faint band was detected on some samples, when changing assay conditions. Different amount of CRK3his preparations were tested, but most of them gave the same negative result (data not shown). When incubating the CRK3his with variable concentrations of *L. mexicana* cell lysates as a native source of cyclin for the activation of the recombinant CRK3his, weak signal appeared at all (data not shown).

The ability of the recombinant *Leishmania*-expressed WT and bacterial-expressed CRK3his to phosphorylate histone H1 as a substrate was assayed in the presence of [γ-^{32}P] ATP. Lane 1, transgenic *Leishmania* expressing CRK3his (beads); lane 2, wild-type *Leishmania* (beads); lane 3, BL-21 cells expressing CRK3his (beads); lane 4, transgenic *Leishmania* expressing CRK3his (eluate); lane 5, wild-type *Leishmania* (eluate); lane 6, BL-21 cells expressing CRK3his (eluate).

Discussion

Several lines of evidence have suggested that CRK3 protein kinase of *Leishmania mexicana* is a potential drug target; thus, chemical inhibitors could be tested against the enzyme. Specificity of the Flavopiridol through previous studies on recombinant (transgenic parasite) and native forms of CRK3 has not confirmed, as Flavopiridol can inhibit other leishmanial CRKs.

Previous studies have shown that the expression level of the transgenic CRK3his is low such that insufficient amount of recombinant kinase for assaying histone H1 kinase activity was obtained, together with the presence of others kinases. This is in consistent with the finding of the current study (very weak signal). Previous work on the attachment of histidine-tag at the amino terminus of *L. mexicana CRK3* suggested that such changes did not inactivate the kinase[9]. To this end, a bacterial-expressed recombinant *CRK3his* gene was engineered. The his-tag, consisting of six consecutive histidine residues, was added to the C-terminal end to allow purification of only the fully synthesized protein[15,16].

During this study, the CRK3 his was produced successfully in the soluble form in the bacterial cells and purified to nearly homogeneity using a one-step purification method. Since the translation of some genes may stop in advance, an *in vitro* transcription and/or translation experiment could be performed, to examine whether the translation product of *LmmCRK3his* gene is the same as predicted. However, in this study, the translation product of *LmmCRK3his* gene was about 35 kDa and accorded with the predicted 35.6 kDa. Analysis of the pGL448 construct showed the presence of another ATG codon downstream the T_7 promoter before the authentic ATG of the *CRK3* gene that is within the *Nde*I site. Therefore, transcription will begin from this false START codon instead, which will add 14 amino acid extra to the recombinant CRK3his protein (14-CRK3his) and may affect the activity (if there is any).

Several active mammalian[17-21] and non-mammalian[22] protein kinases were purified using FPLC. In this study to obtain a similar active kinase, FPLC was used. As the recombinant CRK3 had a six-histidine tag at the C-terminus, it was affinity purified on the Ni-NTA agarose beads and/or FPLC affinity chromatography. Analysis of the amino acids composition showed that *L. mexicana* CRK1 contains high ratio of cysteine residues than *L. mexicana* CRK3. This might explain why that the CRK3 was over-expressed in soluble form rather than CRK1, as the bacterial expression system is less efficient in dealing with the formation of the di-sulphide bonds that pre-

sent between the cysteine residues. This speculation also supports the findings from the current study. From these results we concluded that the *L. mexicana* CRK3his is a biphasic protein as it was over-expressed in both soluble and insoluble fractions; the level of expression was higher in the insoluble form. However, the high level of expression indicates that CRK3his is not toxic and the pGL448 construct is stable.

Structural studies on the cAPK protein kinase showed that the phosphorylation of Thr-197 contributed to the proper folding of the enzyme through interactions with multiple nearby side chains. However, the conformation of the T-loop in the CDK2 structure is very different[23]. The T-loop almost completely blocks the protein substrate binding cleft in CDK2, with the activating phosphorylation site Thr-160 found at its apex. Thr-160 phosphorylation contributes to the activation process by stabilizing the T-loop[23].

Phosphorylation of Thr161/167 in the fission/budding yeast cdc2/CDC28, respectively is a prerequisite for the CDK activity mainly for its interaction with cyclin. MO15 is responsible for this process in yeast[24]. Previous studies have shown that the kinase activity of the *Plasmodium falciparum* protein kinase 5 (PfPK5) stimulated 5-10 fold by the replacement of the threonine residue with glutamic acid[23], while replacement of this threonine residue by a valine, produced inactive kinase. It was suggested that because the threonine was replaced by the hydrophobic valine residue that might close the catalytic site and thus prevented the substrate binding.

Initial results obtained by this study, have suggested that the activation of *L. mexicana* CRK3 is a two-step process that needs cyclin binding first, then phosphorylation of Thr 161/167 (Thr-178 in CRK3) in the T-loop motif. The inability to activate the recombinant *L. mexicana* CRK3his by a mutation mimicking the phosphorylation step indicates that this enzyme is indeed a true member of the CDK family, which has to be bound to a cyclin regulator prior to be phosphorylated in Thr-178. There is a controversy in this, as it has been reported that recombinant TzCRK1 was active as a monomer[7].

CDKs are known as serine and threonine protein kinases because they phosphorylate their substrates specifically at these two residues and histone HI is the physiological substrate. In mammalian cells, up to six serines and threonines in histone HI are phosphorylated *in vivo* in a cell cycle dependent manner that has long been linked with chromatin condensation[24]. The histone HI kinase activity of cdc2 oscillates during the cell cycle[25]. Previous studies have demonstrated that *L. mexicana* CRK3his could phosphorylate some common substrates *in vitro*, such as histone H1, casein, the C- terminal domain of Rb and MBP (myelin basic protein)[9]. On the other hand, some endogenous proteins from the *L. mexicana* lysates could be co precipitated with the anti-LmmCRK3 anti-bodies specifically, suggesting that the *L. mexicana* CRK3 kinase activity may be associated with some endogenous regulatory partners (p13 ^{suc1}) or substrates[14].

A novel cyclin H-homologue from *Plasmodium falciparum* was able to activate recombinant PfPK5 *in vitro*, and that the PfPK5 could be activated by the mammalian p25 as well as cyclin H, even in the absence of phosphorylation by the *P. falciparum* CAK homologue; the Pfmrk[26]. Active recombinant CRK3his can be isolated from transgenic *L. mexicana*[9]. However, the bacterial-expressed CRK3his was inactive as a monomer. There are several possible explanations for this: Leishmanial CRK3 may be incorrectly folded. CDKs are normally only active as heterodimers, bound to their cyclin partner protein, and so perhaps CRK3his is inactive because it is monomer. Bacteria do not posses CDKs and do not, therefore, possess any of the processing enzymes required for activity, for example kinases (Wee1) and phosphatases (CDC25C) required regulating the phosphorylation of the CDKs themselves. Therefore, the phosphorylation status of the recombinant CRK3his may be responsible for the inactivity.

As *L. mexicana* CRK3his must be post-translationally modified (phosphorylated), eukaryotic system would be the best method for expression and also because 75% of *L. mexicana* CRK3his is expressed in the insoluble form. Moreover the newly identified cyclin[16] could possibly used to reconstitute the CRK3 activity, and hence inhibitors could be tested.

Acknowledgements

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References

1) Desjeux P. The increase in risk factors for leishmaniasis worldwide. Trans Royal Soc Trop Med Hygiene 2001; 95: 239-243.
2) Sundar S, Jha TK, Thakur CP, Engel J, Sindermann H, Fischer C, Junge K, Bryceson A, Berman J. Oral miltefosine for Indian visceral leishmaniasis. N Engl J Med 2002; 347: 1739-1746.
3) Barrett MP, Mottram JC, Coombs GH. Recent advances in identifying and validating drug targets in trypanosomes and leishmanias. Trends Microbiol 1999; 7: 82-88.
4) Doerig C, Meijer L, Mottram JC. Protein kinases as drug targets in parasitic protozoa. Trends Parasitol 2002; 18: 366-371.
5) Brown L, Hines JC, RAY DS. The *Crithidia fasciculata CRK* gene encodes a novel cdc2-related protein containing large inserts between highly conserved domains. Nucleic Acids Res 1992; 20: 5451-5456.
6) Mottram JC, SMITH G. A family of trypanosome cdc2-related protein kinases. Gene 1995; 162: 147-152.
7) Goméz EB, Kornblihtt AR, Tellez-Inon MT. Cloning of a cdc2-related protein kinase from *Trypanosoma cruzi* that interacts with mammalian cyclins. Mol Biochem Parasitol 1998; 91: 337-351.
8) Mottram JC, Kinnaird J, Shiels BR, Tait A, Barry JD. A novel CDC2-related protein kinase from *Leishmania mexicana*, Lmmcrk1, is post-translationally regulated during the life-cycle. J Biol Chem 1993; 268: 21044-21051.
9) Grant KM, Hassan P, Anderson JS, Mottram JC. The crk3 gene of *Leishmania mexicana* encodes a stage-regulated cdc2-related histone H1 kinase that associates with p12cks1. J Biol Chem 1998; 273: 10153-10159.
10) Wang Y, Dimitrov K, Garrity LK, Sazer S, Beverley SM. Stage-specific activity of the *Leishmania major* CRK3 kinase and functional rescue of a *Schizosaccharomyces pombe* cdc2 mutant. Mol Biochem Parasitol 1998; 96: 139-150.
11) Ali NOM, Ibrahim ME, Aradaib IE, Grant KM, Mottram JC. An investigation of CRK protein kinases of *Leishmania* and the assessment of their potential as drug targets. Cell Mol Biol Lett 2003; 8: 571-572.
12) Hassan P, Fergusson D, Grant KM, Mottram JC. The CRK3 protein kinase is essential for cell cycle progression of Leishmania mexicana. Mol Biochem Parasitol 2001; 113: 189-198.
13) Levin LR, Zoller MJ. Association of catalytic and regulatory subunits of cyclic AMP-dependent protein kinase requires a negatively charged side group at a conserved threonine. Mol Cell Biol 1990; 10: 1066-1075.
14) Mottram JC, Grant KM. *Leishmania mexicana* p12CKS1, a functional homologue of fission yeast p13SUC1, associates with a stage-regulated histone H1 kinase. Biochem J 1996; 316: 833-839.
15) Gomes FC, Ali Nom, Brown E, Walker RG, Grant KM, Mottram JC. *Leishmania mexicana* cyclin-dependent kinase complex CRK3:CYCA is active in the absence of T-loop phosphorylation. Mol Biochem Parasitol 2010; 171: 89-96.
16) Ali NOM, Ibrahim ME, Grant KM, Mottram JC. Molecular cloning, characterization and overexpression of a novel cyclin from *Leishmania mexicana*. Pakistan J Biol Sci 2010; 13: 775-784.
17) Kikkawa U, Go M, Koumoto J, Nishizuka Y. Rapid purification of protein kinase C by high performance liquid chromatography. Biochem Biophys Res Commun 1986; 135: 636-643.
18) Yokoyama M, Uesaka H, Ohtsuki K. Complete recovery of the phosphoenzyme-forming activity of nucleoside-diphosphate kinases after reconstitution of their subunits. FEBS Letters 1986; 206: 287-291.
19) Sasiak K, Saunders, PP. Purification and properties of a human nicotinamide ribonucleoside kinase. Arch Biochem Biophys 1996; 333: 414-418.
20) Lee HS, Moon BJ, Choi SY, Kwon OS. Human pyridoxal kinase: overexpression and properties of the recombinant enzyme. Mol Cells 2000; 10: 452-459.
21) Lim S, Zou Y, Friedman E. The transcriptional activator Mirk/dyrk1B is sequestered by p38alpha /beta MAP kinase. J Biol Chem 2002; 277: 49438-49445.
22) Janecek J, Dobrova Z, Moravec V, Naprstek J. Improved method for rapid purification of protein kinase from streptomycetes. J Biochem Biophys Methods 1996; 31: 9-15.
23) De Bondt HL, Rosenblatt J, Jancarik J, Jones HD, Morgan DO, Kim S-H. Crystal structure of Cyclin-Dependent Kinase. Nature 1993; 363: 595-602.
24) Pines J. Cyclins and cyclin-dependent kinases: A biochemical view. Biochem J 1995; 308: 697-711.
25) Graeser R, Franklin RM, Kappes B. Mechanisms of activation of the cdc2-related kinase PfPK5 from *Plasmodium falciparum*. Mol Biochem Parasitol 1996; 79: 125-127.
26) Le Roch K, Sestier C, Dorin D, Waters N, Kappes B, Chakrabarti D, Meijer L, Doerig C. Activation of a *Plasmodium falciparum* cdc2-related kinase by heterologous p25 and cyclin H. Functional characterization of a *P. falciparum* cyclin homologue. J Biol Chem 2000; 275: 8952-8958.