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Molecular cloning and biochemical characterization of a serine threonine protein kinase, PknL, from *Mycobacterium tuberculosis*

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Abstract

PknL, a eukaryotic like serine threonine protein kinase from *Mycobacterium tuberculosis*, is predicted to be involved in transcriptional regulation and cell division. Attempts to clone and over-express the protein in *Escherichia coli* using pET43.1c as the vector were unsuccessful. The fusion protein was expressed as a truncated product and showed feeble autokinase activity. To overcome this technical glitch, the *pknL* ORF was cloned into a mycobacterial expression vector, pALACE and the purified His-tagged protein was evaluated for autokinase activity. Phosphorylation experiments with exogenous substrates like myelin basic protein (MBP) were performed. For the fast identification of protein phosphorylation sites, chromatographic methods of separating the [γ-^{32}P]phosphate radio labeled amino acids using thin-layer chromatography (TLC) on cellulose sheets was carried out. Thus, the activity of PknL was demonstrated using autophosphorylation and substrate phosphorylation experiments. Phospho amino acid determinations revealed that PknL was phosphorylated predominantly on serine and also on threonine residues. A single amino acid substitution of lysine to methionine in the active site completely abolished enzymatic action, thereby confirming the authenticity of the kinase function of the expressed protein.

Keywords: Kinase; Phosphorylation; Phosphoaminoacid analysis; Site targeted mutagenesis; Thin-layer chromatography

Protein kinases and phosphatases form a signaling network within the cell and enable the cell to translate extra cellular signals to intra cellular responses [1]. In bacteria, the molecular system responsible for stimulus response coupling involves the histidine kinase (HK)–response regulator (RR)[1] two component systems (TCS) [2]. Eukaryotes predominantly phosphorylate their targets on serine, threonine, or tyrosine residues [3]. This phylogenetic compartmentalization is not rigid and eukaryotic like serine/threonine kinases occur in a number of prokaryotic species [4]. The covalent modifications induced through phosphorylation have been shown to regulate cellular processes, such as mitosis, differentiation, stress response and nutrient acquisition [5]. Apart from regulating vital cellular responses, kinases have been implicated in the pathogenicity of certain bacterial species, namely, *Yersinia pseudotuberculosis* [6], *Listeria monocytogenes* [7] and *Pseudomonas aeruginosa* [8]. Modulation of the host cellular signaling pathways may be effected by the signaling particles present in these pathogenic bacteria.

Mycobacterium tuberculosis (*M. tuberculosis*) has 11 representatives each of the two component system and the eukaryotic like serine/threonine protein kinases (PknA to PknL) [3]. The presence of such a huge repertoire of the eukaryotic-like signaling modules may be due to the need to interact with and subvert the host signaling elements [9].

Characterization and function predictions of this family of biological macromolecules have gained impetus over the past decade. Many of the mycobacterial kinases have been

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[1] *Abbreviations used:* MBP, myelin basic protein; TLC, thin-layer chromatography; HK, histidine kinase; RR, response regulator; TCS, two component system.

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Fig. 1. PknL, a transmembrane protein, belongs to the family of eukaryotic-like serine/threonine protein kinases. The relative position of the kinase domain (19–278), the transmembrane segment (369–391) and the short extracellular domain have been shown. The critical nucleotide binding residue K (48) and the active site D residue (138–150) have been highlighted.

biochemically characterized [10–12] and functional predictions have been worked out for a few [13–15]. In the present study we have attempted to biochemically characterize PknL.

PknL protein has 399 amino acids. It has a single membrane spanning domain, which is closely followed by a carboxy-terminal region of variable length. A diagrammatic representation of the domain architecture of PknL is presented in (Fig. 1).

Initially T7-RNA polymerase based pET system was employed to express the mycobacterial protein in *Escherichia coli* (*E. coli*). Inability to express the recombinant fusion protein was reasoned as the effect associated with heterologous expression of membrane proteins [16]. To overcome the problems with unstable expression, *Mycobacterium smegmatis* (mc2 155) was opted as the expression host. *pknL* from *M. tuberculosis* ($H_{37}Rv$) was cloned into pALACE vector, in which the gene of interest was cloned downstream to highly inducible acetamidase promoter and tagged with a N-terminal His tag to allow easy purification (Fig. 2).

Herein, we report the over-expression of PknL as a His-tagged fusion protein using a mycobacterial-*E. coli* shuttle vector, pALACE. Phosphoaminoacid analysis and site targeted mutagenesis of the critical catalytic residue corroborate the *in silico* prediction that PknL belongs to the eukaryotic-like serine/threonine kinase super family.

Materials and methods

Bacterial strains and culture conditions

DH5α and BL21 were used as host strains for cloning and expression experiments in *E. coli.* The *E. coli* expression vector pET43.1c (Novagen) was used and the transformed strains were grown in Luria–Bertani (LB) broth or on LB agar (Hi-Media) supplemented with carbenicillin (100 μg/ml). *M. smegmatis* (mc2 155) used in this study was grown in Middle brook 7H9 medium (Difco) supplemented with 0.05% Tween and glucose (1%). Mycobacterial-*E. coli* shuttle vector, pALACE [17] was employed for the construction of the recombinant plasmids which were selected by using the antibiotic hygromycin (50 μg/ml) (Calbiochem). All strains were incubated at 37 °C with constant shaking (220 rpm). Restriction/modifying enzymes were obtained from New England Biolabs or Amersham Biosciences.

Cloning pknL into E. coli and mycobacterial expression vectors

The ORF encoding PknL was amplified from *M. tuberculosis* ($H_{37}Rv$) genomic DNA using the sequence informa-

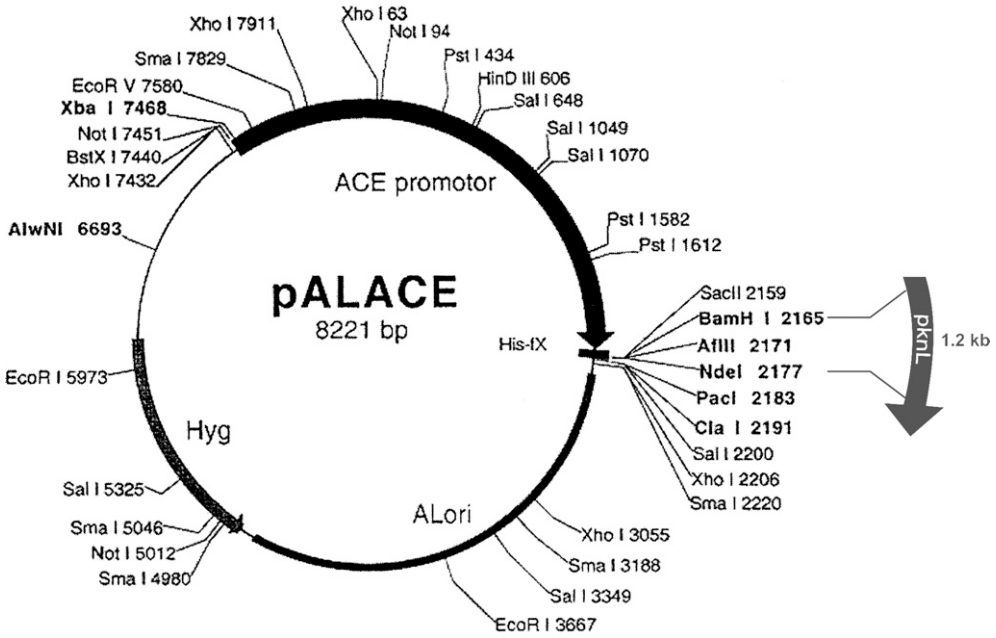

Fig. 2. Schematic representation of the pALACE vector that was used to express PknL as His fusion protein under acetamidase (ACE) promoter. The vector contains the following elements or encoding sequences: replication origin for *E. coli* (oriE); replication origin for mycobacteria derived from *M. fortuitum* (AL origin); the acetamidase promoter from *M. smegmatis* (ACE); multiple cloning site (MCS); the His-tag coding sequence.

tion available from the genome sequence database (http://www.tuberculist.org). The T7-RNA polymerase based expression vector pET43.1c was employed to express PknL in *E. coli*. The construct (*pknL* in pET43.1c) was named pET-L. For over-expression of PknL in *M. smegmatis* (mc2 155), the amplified gene was cloned into pALACE vector where protein expression is driven by the acetamidase promoter. The resulting plasmid was named pHL4 (*pknL-pALACE*). The strains, plasmids, oligonucleotide primer sequence and the overhangs used in this study have been summarized in (Table 1). The routine molecular techniques such as PCR, restriction digestion, ligation and electroporation were performed according to the standard protocol mentioned in Molecular Cloning manual Sam brook and Russell 2001, Cold spring Harbour Laboratory Press, Cold Spring Harbor, New York.

Protein over-expression

For expression in *E. coli*, recombinant cells were grown at 37 °C in LB medium containing carbenicillin (50 μg ml^{-1} final concentration) and induced at $OD_{600} = 0.8$ by adding isopropyl thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM. After induction, the cells were grown at 37 °C and collected at various time points (from 1 to 5 h) by centrifugation. Purification of protein from the crude lysate was carried out as mentioned below.

Subsequently, PknL was over-expressed in *M. smegmatis* harboring the pHL4 plasmid. The starter culture was diluted in fresh 7H9 broth (1:100 dil.) and incubated overnight. Excess carbon source was removed by washing the cells in plain 7H9 broth. The bacterial pellet was resuspended to an optical density at OD_{600} of 1.0 using 7H9 medium lacking glucose. The cells were then induced by adding acetamide to a final concentration of 0.02% and incubated overnight at 22 °C with constant agitation. Bacteria were acquired by spinning down the cells at 4000 rpm for 15 min and the cell pellet was stored at −20 °C until further processing. The cells were resuspended in lysis buffer (1:10 dil.) [20 mM Tris–HCl (pH 8), 10 mM NH_4Cl, 10% glycerol, 1 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride] and disrupted by sonication at 50 W with intermittent cooling on ice (until considerable clearing was observed). Cell debris was removed by centrifuging at 13,000 rpm for 30 min. Protein purification was carried out by immobilized metal affinity chromatography (IMAC) on a nickel–nitrilotriacetate resin (Qiagen), using the manufacturer's instructions.

Aliquots taken during the purification procedure were resolved by SDS–PAGE and the gel was stained using the silver staining protocol. The fusion protein was recognized by standard immunoblotting procedure using anti-HisG antibody.

Autophosphorylation and substrate phosphorylation of PknL

Autophosphorylation of PknL and myelin basic protein (Sigma), a general kinase substrate, was performed with an *in vitro* kinase assay. One to three micrograms of the purified His-tagged PknL was resuspended in 15 μl of 1× kinase buffer containing 25 mM Tris–Cl [pH 6.8], 1 mM DTT, 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 10 μCi of [γ-^{32}P]ATP ml^{-1}. The reactions were incubated at 30 °C for 30 min and terminated by addition of 5× SDS–PAGE loading buffer. The mixture was heated at 100 °C for 5 min. The proteins were resolved by 10% SDS–PAGE. The gels were dried and the autophosphorylated spots were visualized by exposing the gel to a phosphor imager screen.

Site directed mutagenesis of pknL (K48M)

To create a substitution of methionine for lysine at position 48 of *pknL* (nucleotide binding site) Quick-Change Site directed Mutagenesis kit (Stratagene) was used. The reactions were set up according to the directions provided by the supplier. Briefly, *pknL* was excised from pHL4 by digesting with BamHI and NdeI. The insert was ligated into pUC19, digested with the same set of enzymes.

Table 1
Primers used for cloning and expression of *pknL* in two different expression vectors

Primer sequence	Name	Purpose	Size (kb)	Annealing temperature (°C)
5′-TTCTGGATCCGTGGTCGAAG CTGGCACGAGGG-3′	S196-*pknL*-pAL-Fw-Bam	Cloning *pknL* coding seq into pALACE for expression	1.2	63–65
5′-CATCATTACATATGTTAG AGCAGGCCGCTCAGG-3′	S197-*pknL*-pAL-Re-Nde	Cloning *pknL* coding seq into pALACE for expression	1.2	63–65
5′-CCGTCGCGCTGATGGTGATG GATTC-3′	S242-*pknL*-K48M-Fw	To mutate lysine to methionine in the nucleotide binding site of *pknL*		55
5′-GAATCCATCACCATCAGCGC GACGG-3′	S243-pknL-K48M-Re	To mutate lysine to methionine in the nucleotide binding site of *pknL*		55
5′-CGCGGATCCGCGGTGGTCG AAGCTGGCACG-3′	HLN-1	Fw primer to clone *pknL* into pET43.1c	1.2	66
5′-CCCAAGCTTGGGGCCGTGG TCACCTTAATTCA-3′	HLN-2	Re primer to clone *pknL* into pET43.1c	1.2	62

The underlined nucleotides represent the restriction enzyme sites included while designing the primer.

The resulting plasmid pHL7 was used as a template for site targeted mutagenesis. The mutagenesis primers S242 and S243 (Table 1) were used to set up a PCR with pfu Turbo DNA polymerase (95 °C/30 s; 55 °C/1 min; 68 °C/4 min; 16 cycles). The PCR was treated with DpnI restriction enzyme to degrade residual methylated wild-type templates. The reaction mix was then used to transform XL1-Blue cells and the cells were plated on LB carb plates. The mutation was confirmed by sequencing using an automated DNA sequencer.

Abrogation of enzymatic action was confirmed by an *in vitro* phosphorylation assay as described in the preceding section.

Phosphoaminoacid analysis by two-dimensional thin-layer chromatography

To analyze the amino acids phosphorylated in the *in vitro* kinase reaction, phosphoaminoacid analysis was performed as described previously [18]. The $(His)_6$-PknL was kinased *in vitro* and subjected to acid hydrolysis at 110 °C for 1 h. Hydrolyzed samples were lyophilized and finally resuspended in 20 µl of $D{\cdot}H_2O$. An aliquot of hydrolyzed PknL along with the standard pSer, pThr and pTyr amino acids was spotted and resolved on a glass-backed cellulose thin-layer chromatography plate (TLC cellulose, Merck Inc.). The solvent used in the first dimension was a mix of isobutyric acid and 0.5 M NH_4 OH (5:3 v/v). Following separation for 10 h, the TLC plate was air dried and the resolution in the second dimension was carried out in 2-propanol, HCl, and water (at a ratio of 7:1.5:1.5, by vol.) for (8 h). The marker phosphoaminoacids were visualized by staining with ninhydrin (0.2% ninhydrin in acetone). The plate was exposed to a phosphoimager screen (Molecular Imager, Bio-Rad) and the labeled phosphor amino acids were identified by co migration with the standards.

Results

Expression of PknL in a heterologous expression host (E. coli)

Initially, *E. coli* BL21 (DE3) and pET43.1c (Novagen) were used as the host and the vector for protein overexpression, respectively. The predicted molecular mass of the fusion protein is ~100 kDa (http://bioinformatics.org/sms/prot_mw.html). However, when the crude extract and the purified fractions from induced *E. coli* cultures containing pET-L were analyzed by SDS–PAGE (Fig. 3A and B), the protein was consistently identified to migrate as a doublet of molecular mass ~85 kDa. The expression of the recombinant protein was confirmed by immunoblotting (Fig. 3C) using anti-HisG antibody.

Expression of PknL in M. smegmatis

To overcome the problems associated with overexpression in a heterogeneous system, we used *M. smegmatis* as a surrogate host to express PknL and its kinase inactive mutant. The extremely versatile pALACE vector was used for this purpose. The expression was identified by running the crude lysate and the elution fractions on a 10% SDS–PAGE gel and subsequently

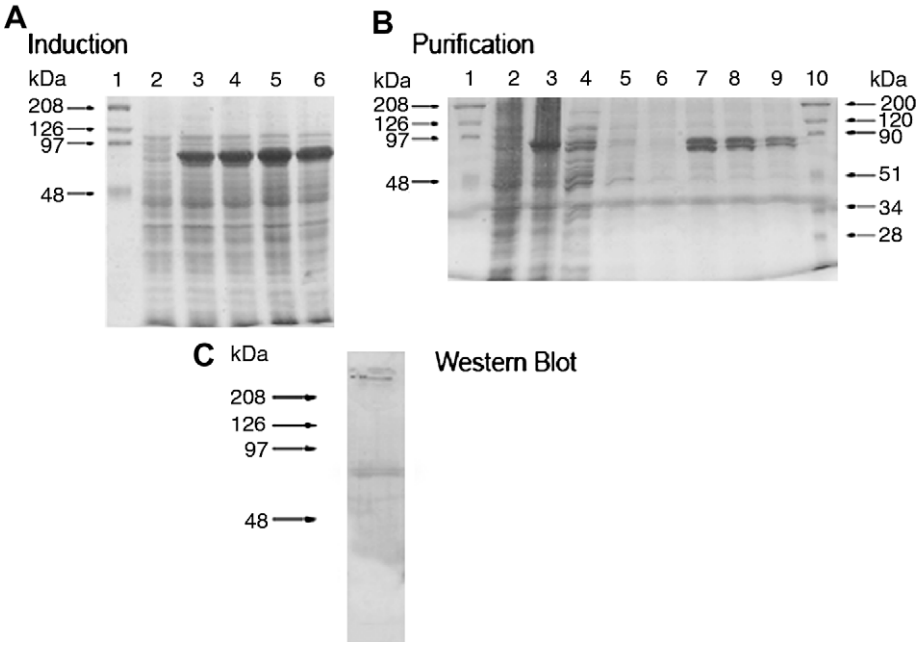

Fig. 3. Expression and purification of fusion protein in *E. coli* containing pET-L. (A) Lane 1, molecular weight standards; lane 2, uninduced culture; lanes 3–6, hourly induction samples (B) Purification of His-tagged recombinant protein by Ni–NTA metal affinity chromatography. Lane 1, molecular weight standards; lane 2, uninduced bacterial lysate; lane 3, IPTG induced culture; lane 4, column flow through; lanes 5 and 6, wash fractions; lanes 7–9, elution fractions; lane 10, molecular weight standards. (C) Western blot analysis of purified protein using mouse anti-HisG polyclonal antibody.

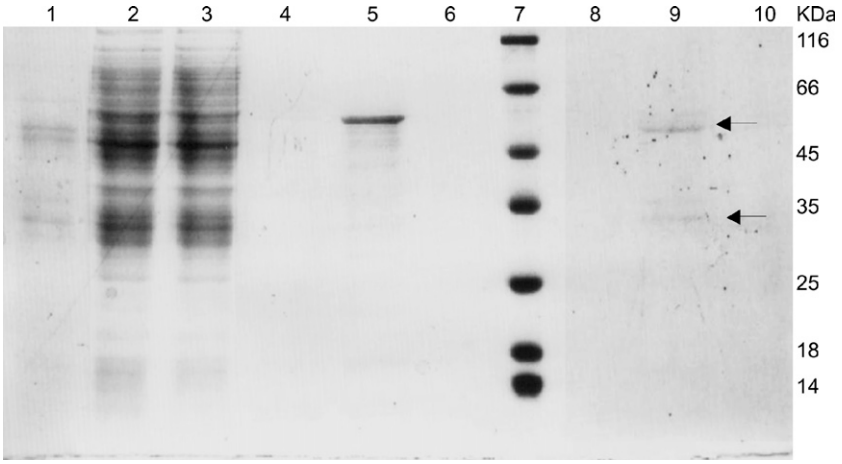

Fig. 4. SDS–PAGE analysis of His-tagged PknL fusion protein expressed from pALACE vector in *M. smegmatis* (mc2 155). The protein was concentrated from the crude extract by Ni–NTA chromatography under native conditions. Lane 1, pellet of induced bacterial lysate; lane 2, induced bacterial lysate; lane 3, run through; lanes 4–6, wash fractions; lane 7, protein molecular weight standards; lanes 8–10, elution fractions.

staining it with silver stain. The electrophoretic analysis revealed that the $(His)_6$-PknL chimeric protein migrated as a 55 kDa band (Fig. 4). The increased molecular mass of the expressed protein is ascribed to the chemical modifications seen in over expressed proteins [19]. The identity of the expressed protein was confirmed by mass spectrometry (Fig. 5). The mutated chimeric protein $(His)_6$-PknL (K48M), was also purified by the same procedure.

Kinase assay of PknL

To demonstrate that *pknL* gene encodes a functional protein kinase, the purified protein was examined for its

MATRIX SCIENCE Mascot Search Results

Protein View

Match to: **B70936** Score: **617**
probable serine/threonine-specific protein kinase (EC 2.7.1.-) 2 - Mycobacterium tuberculosis (stra

Nominal mass (M_r): **42921**; Calculated pI value: **5.84**
NCBI BLAST search of B70936 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Mycobacterium tuberculosis
Links to retrieve other entries containing this sequence from NCBI Entrez:
AAK46517 from Mycobacterium tuberculosis CDC1551
CAA17480 from Mycobacterium tuberculosis H37Rv
PKNL MYCTU from Mycobacterium tuberculosis

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **35%**

Matched peptides shown in **Bold Red**

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    1 MVEAGTRDPL ESALLDSRYL VQAKIASGGT STVYRGLDVR LDRPVALKVM
   51 DSRYAGDEQF LTRFRLEARA VARLNNRALV AVYDQGKDGR HPFLVMELIE
  101 GGTLRELLIE RGPMPPHAVV AVLRPVLGGL AAAHRAGLVH RDVKPENILI
  151 SDDGDVKLAD FGLVRAVAAA SITSTGVILG TAAYLSPEQV RDGNADPRSD
  201 VYSVGVLVYE LLTGHTPFTG DSALSIAYQR LDADVPRASA VIDGVPPQFD
  251 ELVACATARN PADRYADAIA MGADLEAIAE ELALPEFRVP APRNSAQHRS
  301 AALYRSRITQ QGQLGAKPVH HPTRQLTRQP GDCSEPASGS EPEHEPITGQ
  351 FAGIAIEEFI WARQHARRMV LVWVSVVLAI TGLVASAAWT IGSNLSGLL
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Fig. 5. Mass spectrometry to confirm the identity of the purified protein as PknL. The peptides whose mass was used to identify the protein have been indicated in bold font. MASCOT software was used to perform mass spectrometry data analysis.

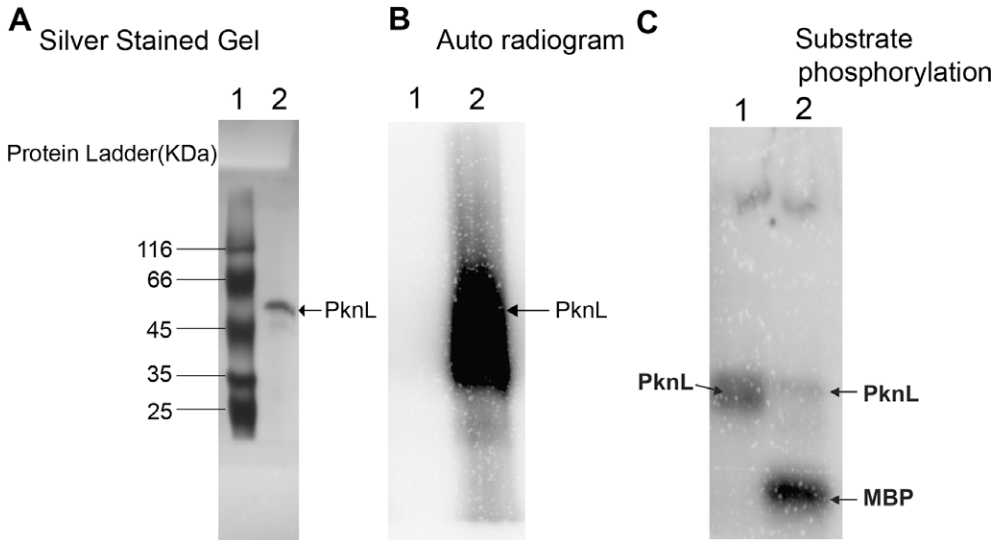

Fig. 6. *In vitro* phosphorylation assay on His_6-PknL (2.25 μg of purified protein) was performed with [γ-^{32}P]ATP for 30 min. Proteins were analyzed by (A) SDS–PAGE: lane 1, molecular weight standard; lane 2, purified PknL (B) radioactive bands were revealed by imaging in a phosphor imager system. (C) Substrate phosphorylation was performed by including 20 μg of MBP along with 2.25 μg of purified His-PknL.

ability to autophosphorylate (Fig. 6A and B) and phosphorylate exogenous substrates-like MBP (Fig. 6C) in the presence of [γ-^{32}P]ATP. A 55 kDa autophosphorylated protein was detected and was also capable of phosphorylating MBP, a common substrate for such kinases.

Site directed mutagenesis of PknL

To establish the authenticity of the purified protein kinase L as a Ser/Thr protein kinase and to rule out the possibility of contamination with endogenous kinases present in the cell extract of the expression host, the critical lysine residue (position 48) in the nucleotide binding site of PknL was mutated to methionine. The abrogation of kinase activity (Fig. 7A and B) following the substitution confirms that the phosphorylation detected in the kinase reactions is indeed catalyzed by PknL.

Phosphoaminoacid analysis of the PknL kinase

To identify the amino acid residue(s) that undergo phosphorylation, we performed a two-dimensional phosphor amino acid analysis using the modified Mark Kamp method. It is evident from the assay that PknL was labeled predominantly on serine and threonine (Fig. 8A and B).

Discussion

For the most part, serine threonine kinases in prokaryotes have been shown to be involved in three different processes, namely, regulation of growth and development, stress responses, and pathogenicity [3]. Annotation of the genome sequence of *M. tuberculosis* $H_{37}Rv$ [20] observed the presence of 11 putative eukaryote-like Ser/Thr kinases. Hitherto, 8 Ser/Thr protein kinases of *M. tuberculosis* have

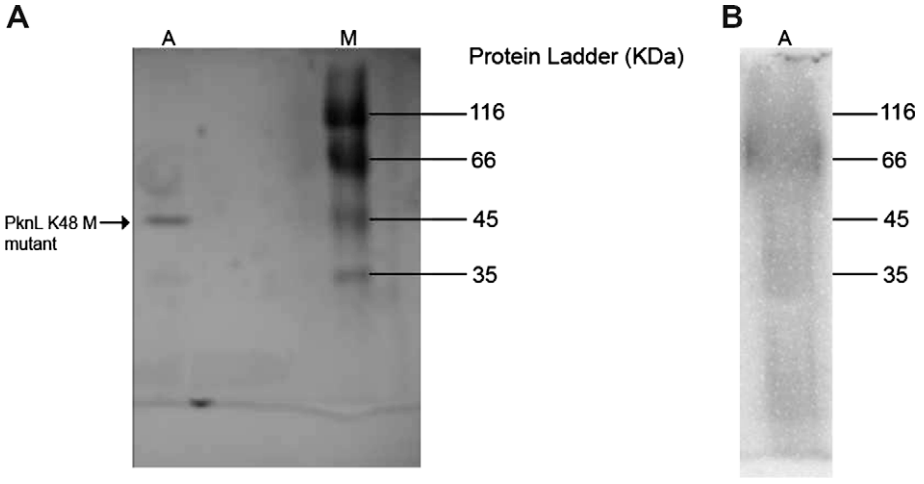

Fig. 7. Loss of kinase activity in PknL K48M mutant (A) SDS–PAGE analysis: lane A, purified PknL mutant; lane M, molecular weight standard (B) radiographic imaging. Lane A shows loss of kinase activity.

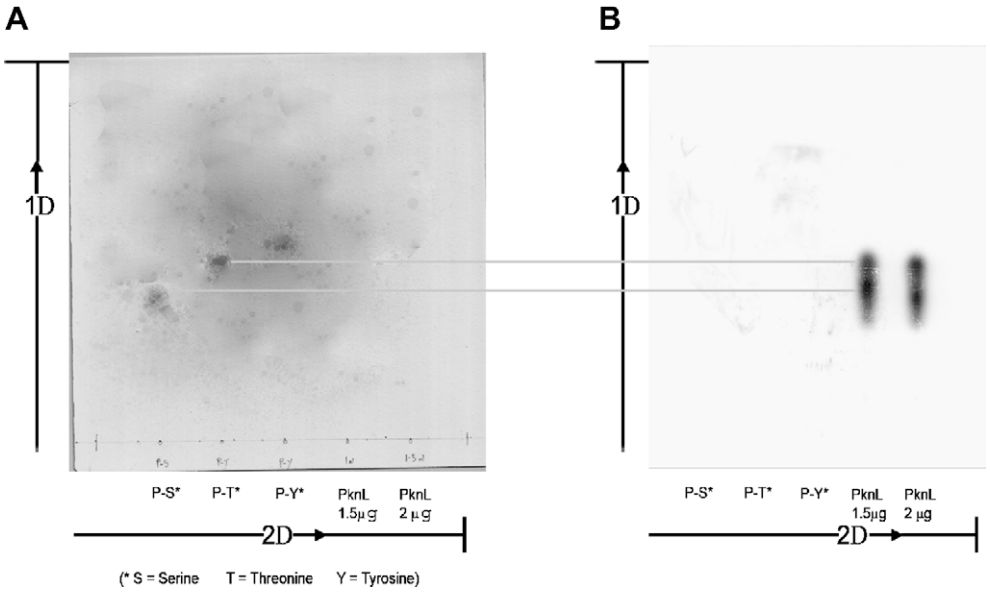

Fig. 8. Phosphoaminoacid analysis by two-dimensional thin-layer chromatography (TLC). (A) Identifying phosphor amino acid standards by ninhydrin staining (B) phospho amino acid pattern of acid hydrolyzed His-PknL after two-dimensional separation.

been cloned and characterized. PknL (M.wt = 42.6 kDa, pI = 6.19) is a trasmembrane signaling protein that retains the entire conserved Hank's signatures characteristic of Ser/Thr kinases. It has a very short c-terminal extracellular region. This casts doubts on the mechanism by which the kinase perceives external signals. The skepticism is ruled out by the fact that isolated catalytic domains of other kinases have proved to be catalytic active [21]. Previously, Molle et al., have reported the cloning and over-expression of the kinase core (1–376) of PknL [22]. The predicted association of PknL with the transcriptional system is a derivation of the proximal placement of *pknL* (Rv2176) with a gene coding for a putative transcriptional regulator [3]. A gene (ML0897c) sharing 75% identity with (Rv2176) exists in *Mycobacterium leprae* [23]. Though *pknL* was found to be non-essential based on transposon mutagenesis in H_{37}Rv and CDC1551 strains [24,25], it is possible that this kinase is concerned with subtle regulatory functions through protein interactions. In this article we describe the biochemical characterization of PknL.

Problems with heterologous expression hosts

Though *E. coli* appears to be an attractive host for protein over-expression, it presents several bottlenecks in the over-production, purification and stability of these proteins [26]. Heterologous expression of mycobacterial membrane proteins has been a knotty task Most of them form insoluble aggregates and some of these proteins also enter the membrane fraction [27]. Earlier attempts to over express and purify PknL in *E. coli* host proved unsuccessful. The protein was truncated, only 85 kDa as against the predicted molecular mass of 100 kDa (Fig. 3B). This suggests that expression of PknL may be toxic to *E. coli* cells.

It has been reported that high level expression of mycobacterium specific proteins requires a mycobacterial host [28]. Thus, pALACE over-expression vector was chosen as it offers the possibility for purification of native-like recombinant mycobacterial proteins in fast growing mycobacterial hosts, *M. smegmatis* [17]. The recombinant protein could be purified from the bacterial sonicate by virtue of the N-terminal 6-histidine tag (Fig. 4) However the expression levels were very poor.

Cleavage of a 35 kDa fragment from the purified $(His)_6$-PknL

When the purified protein was run on SDS–PAGE gels, a 55 kDa and a 35 kDa band was observed (Fig. 4). The 55 kDa band was cut and sequenced by mass spectrometry. The peptide mass fingerprint (Fig. 5) identified the purified protein as PknL. Such proteolytic cleavage has been documented for PKCμ, a phospholipid-dependent Ser/Thr protein kinase involved in regulating physiological processes like cell growth, differentiation and apoptosis. In apoptosis induced by genotoxic agents, PKCμ is cleaved by caspase 3 into catalytic and regulatory fragments. The catalytic fragment makes the cells more sensitive to apoptosis. The cleavage of the regulatory region relieves repression and increases the function of the catalytic region [29]. Acetamide induction of *M. smegmatis* cells harboring pHL4 was lethal as evidenced by a sharp dip in OD_{600} values from 0.709 (at time of induction) to 0.183 (at the time of harvest). Thus, it is speculated that the stress induced by PknL over-expression leads to its cleavage. Though caspases are absent in prokaryotes, the presence of evolutionary precursors of eukaryotic caspases has been documented by using bioinformatics tools such as DAB (Divide and Blast)

(http://www.bio.davidson.edu/old_site/student/karnik/discuss.html). *In vivo*, such proteolytic activation may be an important step in the signaling pathway involved in TNF-α induced apoptosis of infected macrophages [30]. This suggests a role for PknL in modulating apoptosis.

In summary, we have cloned, over expressed and purified the full-length *pknL* gene from *M. tuberculosis* $H_{37}Rv$. The use of a homologous expression host, *M. smegmatis* helped overcome the problem of truncation. We could demonstrate autophosphorylation of the His-tagged fusion protein, showing that it is a functional kinase. Phosphorylation of general substrates reaffirms the catalytic activity of the purified kinase. The legitimacy of the purified protein as a serine/threonine protein kinase was proven by abolishing the kinase activity through a site directed substitution of lysine to methionine in the conserved nucleotide binding site. The phosphoaminoacid analysis with two-dimensional TLC confirmed that the kinase is phosphorylated on serine and threonine residues.

Experiments to probe into the role of PknL in triggering macrophage apoptosis are underway. Creating deletion mutants of PknL in *M. tuberculosis* $H_{37}Rv$, and proteomic studies to identify the downstream interacting partners of PknL will further our understanding on the role of this protein in the physiology and pathology of this organism.

Acknowledgments

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