



Protein kinase I of *Mycobacterium tuberculosis*: Cellular localization and expression during infection of macrophage-like cells

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Accepted 11 March 2005

KEYWORDS

Protein kinase A;
Protein kinase I;
Mycobacterium tuberculosis;
Molecular beacons;
Transcription

Summary Protein kinase I of *Mycobacterium tuberculosis*, which has an unusual amino acid composition in its catalytic loop, displayed autophosphorylation and transphosphorylation activity. Immunoblot analysis of sub-cellular fractions of *M. tuberculosis*, using anti-PknI antibodies raised in rabbits, showed that PknI localizes to the bacterial cytosol. In contrast, PknA was membrane-bound. Relative expression of *pknI*, when measured by combining molecular beacons and RT-PCR, decreased during infection of THP-1 human macrophages. Expression of *pknA* and *pknB* was upregulated during infection. Thus PknI represents a group of protein kinases that is distinct from the more extensively studied enzymes PknA and PknB.

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Introduction

The genome of *Mycobacterium tuberculosis* contains eleven serine/threonine protein kinase genes, some of which may be involved in pathogenesis.¹ Analysis of predicted amino acid sequences reveals that PknI is unique in having an asparagine at the active site.² The genes for protein kinases A (*pknA*), I (*pknI*), and B (*pknB*) are located within operons containing genes that are known to be involved in

cell division in *Escherichia coli* and *Bacillus subtilis*; thus it has been suggested that one or more of the kinases may be involved in the regulation of bacterial cell division.² As expected, *pknB* is continuously transcribed during infection of murine macrophages and in alveolar macrophages of a tuberculosis patient.³ Little else is known about expression patterns or functions of these enzymes.

In the present work properties of PknI were compared with those of more typical serine/threonine protein kinases, PknA and PknB.^{3,4} Recombinant PknI and PknA were examined for autophosphorylation and transphosphorylation

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activity, antibodies were prepared for determination of sub-cellular localization in *M. tuberculosis*, and the genomic sequence of *M. tuberculosis* was used to design hybridization probes for comparative transcription profiling following a shift from broth culture to infection of THP-1 cells. PknI was distinct from PknA in being largely cytosolic and down-regulated upon infection.

Methods and materials

Bacterial growth and infection of macrophages

M. tuberculosis strain H₃₇Rv was grown at 37 °C in Middlebrook 7H9 medium supplemented with ADC.⁵ Human monocytic cell line THP-1 was cultured in RPMI-1640 medium containing 10% fetal calf serum in 5% CO₂ at 37 °C and stimulated to differentiate into macrophage-like cells by treatment with phorbol myristic acetate.⁶ Cells were plated at a density of 5 × 10⁵ cells/ml in RPMI medium in 24-well tissue culture plates. Infection with *M. tuberculosis* was as described⁷ at a multiplicity of

infection of 1. Prior to RNA extraction, the extent of infection was determined by acid-fast staining of cells washed twice in phosphate-buffered saline.

Expression of protein kinases as fusion proteins

M. tuberculosis H₃₇Rv genomic DNA was used as template for amplification of *pknI* (Rv2914c) and *pknA* (Rv0015c) by the polymerase chain reaction (PCR, see Table 1 for primers). *M. tuberculosis* PknI and PknA proteins, expressed as glutathione-S-transferase (GST)-fusion proteins in *E. coli* DH5 α , were purified using Glutathione-Sepharose 4B resin (Amersham Biosciences, Sweden). Protein purity, estimated following gel electrophoresis and staining by Coomassie Blue, was greater than 95%.

PknI and PknA kinase activities

Kinase activity was measured as phosphorylation of myelin basic protein (MBP).⁸ Purified GST-PknI and GST-PknA (2 μ g each) were incubated with MBP (6 μ g) at 21 °C in kinase buffer (25 mM Tris-Cl, pH 7.4 containing 5 mM MgCl₂, 2 mM MnCl₂, 1 mM

Table 1 Sequences of primers and beacons*.

Primer or beacon	Sequence
For cloning in pGEX-5X-3 <i>pknI</i> (Forward) (Reverse)	5'-GCGGTTACTCTCGAGAATGGCGTT-3' 5'-CTGGTCAAGCTCGAGATCAGCGACC-3'
For RT-PCR <i>pknI</i> (Forward) (Reverse) (Beacon)	5'-CCGCGATGGGTGAGGTCTAC-3' 5'-CTCCAGGATGTGCGGGTGAA-3' 5'-CCGTGC-ATTTCCGCCGCCGATTTC AACGGG-GCACGG-3'
For cloning in pGEX-5X-3 <i>pknA</i> (Forward) (Reverse)	5'-GCACTGCAGGGATCCCCATGAGC-3' 5'-GGTGGGAAGGAATTCTCATTGCGC-3'
For RT-PCR <i>pknA</i> (Forward) (Reverse) (Beacon) <i>pknB</i> (Forward) (Reverse) (Beacon)	5'-AGCCACTAAATTCGGTGCTC-3' 5'-GGGGTGATCAAGATGTTGCC-3' 5'-CGTCGC-TGCTCGAGCAGACCGGCCGC-CGACG-3' 5'-AAACTGACTGCCGCCGGATTTC-3' 5'-CCGACCAGAGCCAACGATGA-3' 5'-CGCACG-TCCACCCGGAAGTGGTG-CGTGCG-3'
<i>sigA</i> (RT) (Forward) (Reverse) (Beacon)	5'-CGGACGAGACCATGGTGCGGC-3' 5'-GGCCAGCCCGGCACCCTTGAC-3' 5'-AGGTAGTCGCGCAGGAC-3' 5'-CCTCGC-GTCGAAGTTGCGCCATCCGA-GCGAGG-3'

*Where an RT primer is not indicated, the reverse primer was used for RT. All beacons had tetrafluorescein attached to the 5' end and dabcyI (quencher) attached to the 3' end.

dithiothreitol, 200 μ M orthovanadate, and 10 μ Ci of [γ - 32 P] ATP). The reactions were terminated by addition of SDS sample buffer, and the products were detected by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was electroblotted to a nitrocellulose membrane, and autoradiography revealed phosphorylation of MBP. Specific activity of the PknI fusion protein was approximately 10^{-5} fmol/min/ μ g protein at 21 °C; PknA fusion protein was about five times more active.

Preparation of antisera and subcellular localization

Polyclonal antisera directed against PknA or PknI were obtained by immunization of rabbits with 200 μ g of purified GST-PknA or GST-PknI fusion protein that was solubilized in 0.5 ml Freund's Incomplete Adjuvant and injected into rabbits twice at 15-day intervals. Seven days after the final injection, blood was drawn, and titers of anti-GST-PknI and anti-GST-PknA were determined using enzyme-linked immunosorbent assay.⁹ Antiserum raised with PknA recognized GST-PknA but not GST-PknI. Likewise, antiserum raised against PknI recognized GST-PknI, not GST-PknA. Subcellular fractions of *M. tuberculosis* were obtained from John T. Belisle, Colorado State University under the Tuberculosis Research Material and Vaccine Testing Program of the NIAID, NIH, Colorado, USA (Contract number: NO1-AI-75320). Equal amounts of protein (40 μ g each) from whole cell lysate, cell wall, cell membrane, culture filtrate, and cytosolic fractions of *M. tuberculosis* H37Rv were separated by 12% SDS-PAGE. The proteins were then electroblotted onto a nitrocellulose membrane at 100 V for 1 h and incubated with either anti-GST-PknA antibodies or with anti-GST-PknI antibodies. A protease inhibitor (phenylmethylsulfonyl fluoride) was added during the purification of GST proteins and during preparation of the fractions received from Colorado State University.

Nucleotide sequences, primers, and molecular beacon structures

Nucleotide sequences internal to the coding sequence of the three kinase genes and *sigA* were taken from the National Center for Biotechnical Information (NCBI) database and were used to design primers and beacons for real-time PCR with molecular beacons. Primers (Table 1) were designed using the Oligo 6.7 program (NBI/Genovus Inc., Plymouth, MN) to amplify DNA segments in an RT-PCR reaction.

Molecular beacons were designed using the Zuker mfold DNA folding program (available at <http://www.ibt.wustl.edu/zuker/dna/form1.cgi>). The molecular beacons were designed such that the hairpin stem would be closed in the absence of target; in the presence of target, the molecular beacon forms a stable probe-target hybrid, and the stem denatures.¹⁰ Each of the molecular beacons had the fluorophore tetrachlorofluorescein (TET) attached to the 5' end and the quencher dabsyl attached to the 3' end. Molecular beacons for *sigA* and *pknB* were obtained commercially from Biosearch Technologies, Inc. (Novato, CA), and the molecular beacons for *pknA* and *pknI* were a kind gift from Dr. Sanjay Tyagi at the Public Health Research Institute. All beacons were obtained in their crude form and were purified using high-pressure liquid chromatography with a C-18 reverse phase column (Waters, Milford, MA) utilizing a linear elution gradient of 20–70% buffer B (0.1 M triethylammonium acetate in 75% acetonitrile, pH 6.5) in buffer A (0.1 M triethylammonium acetate, pH 6.5). Chromatography was performed for 25 min at a flow rate of 1 ml/min. Two wavelengths (260 nm for DNA and 522 nm for TET) were used to monitor the coupling of the fluorophore to the beacon and purification. Fractions corresponding to the highest absorbance at 260 nm were pooled, and samples were precipitated and dissolved in 100 μ l TE. Yield was determined by measuring the absorbance at 260 nm. Working stocks of 100 ng/ μ l were prepared for each beacon and stored at –20 °C.

Measurement of transcript abundance

RNA was extracted from exponentially growing cultures of *M. tuberculosis* H₃₇Rv at about 10^7 cells/ml. The same culture was used to infect THP-1 cells, and RNA was extracted from infected THP-1 macrophages at 2, 24, 48 and 72 h post-infection as described.⁷ The RNA was used for synthesis of cDNA in an RT-PCR reaction using *C. therm* Polymerase for Reverse Transcription with a Two-Step PCR kit (Roche Applied Science, Indianapolis, USA). Control samples that were not treated with *C. therm* RT polymerase were also prepared. The cDNA templates were detected using PCR and molecular beacons⁷ (see Table 1 for primers and beacons).

Results

Kinase activities of recombinant PknI and PknA

Recombinant *M. tuberculosis* H₃₇Rv PknI was expressed in *E. coli* and purified using Glutathione-

Sepharose 4B matrix. The fusion protein migrated as a single 90 kDa band during electrophoresis in a 10% SDS-polyacrylamide gel, consistent with the expected size of 91 kDa. Kinase activity of GST-PknI was detected by the ability of the protein to become autophosphorylated and to phosphorylate MBP (Fig. 1, Panel A). No change in electrophoretic mobility was associated with autophosphorylation (not shown). During the course of this work PknI was expressed and purified by a different method.¹¹ In that work two protein bands were observed, each having a greater molecular weight than expected.

For comparison, the *M. tuberculosis* *pknA* gene was also cloned, and the GST-PknA fusion protein was purified. This protein also phosphorylated itself and MBP (Fig. 1, Panel B), as has been reported previously.⁴

The amino acid specificity of the phosphorylating activity of PknI and PknA was determined by subjecting the autophosphorylated enzymes to acid hydrolysis and resolving the resulting amino acids by two-dimensional thin-layer chromatography.¹² Both enzymes phosphorylated serine and threonine, and neither phosphorylated tyrosine. Thus PknI is a Ser-Thr protein kinase, as predicted by nucleotide sequence analysis.

Cellular localization of PknI and PknA

Antisera raised against purified GST-PknI and GST-PknA were used to localize the proteins within mycobacterial subcellular fractions. Most of PknI, seen as a 61 kDa protein, was found in the cytosolic fraction (Fig. 1, Panel C), although small amounts were also seen in the cell wall and cell membrane fractions. PknI in the membrane fraction migrated more slowly, presumably due to an uncharacterized modification and/or an interaction with other membrane components. PknA was seen as a 45 kDa protein present in the whole cell lysate, cell wall, and cell membrane fractions of *M. tuberculosis* H₃₇Rv (Fig. 1, Panel D). The size difference for PknI between cytosolic and membrane fractions was probably not due to generalized proteolysis, since no degradation was observed with PknA and since the same lysates were used for analysis of PknA and PknI. We conclude that PknI differs from PknA with respect to subcellular localization by being more abundant in the cytosol than in the cell membrane.

Expression of *pknI*, *pknA*, and *pknB*

To compare expression levels, RNA was extracted from *M. tuberculosis* growing exponentially in

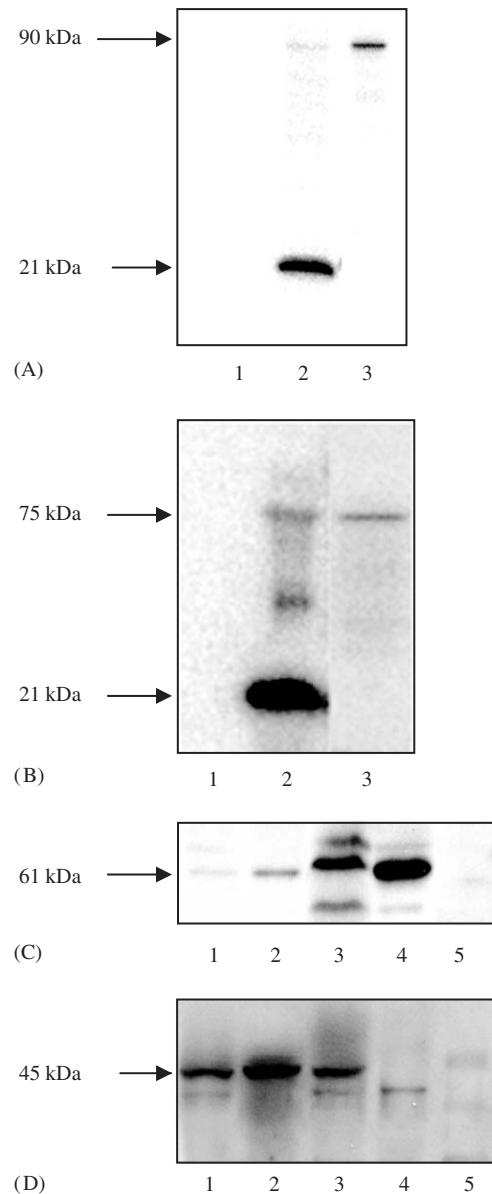


Figure 1 Protein kinase activity and cellular localization of PknI and PknA. Panel A. Kinase activity of PknI. Lane 1: 6 μg myelin basic protein alone; lane 2: 6 μg myelin basic protein plus 2 μg GST-PknI; lane 3: 2 μg GST-PknI alone. Panel B. Kinase activity of PknA. Lane 1: 6 μg myelin basic protein alone; lane 2: 6 μg myelin basic protein plus 2 μg GST-PknA; lane 3: 2 μg GST-PknA alone. Panel C. Cellular localization of PknI. Subcellular fractions of *M. tuberculosis*, 40 μg each, were separated by 12% SDS-PAGE, electroblotted, and incubated with anti-GST-PknI antiserum as described in Methods. Lanes: 1, whole cell lysate; 2, cell wall; 3, cell membrane; 4, cytosol; and 5, culture filtrate. Panel D. Cellular localization of PknA. Subcellular fractions of *M. tuberculosis*, 40 μg each, were prepared as in panel C. Lanes: 1, whole cell lysate; 2, cell wall; 3, cell membrane; 4, cytosol; and 5, culture filtrate.

broth and from intracellular bacilli at 2, 24, 48, and 72 h after infection of human THP-1 macrophages. RNA was then subjected to reverse transcription using antisense primers specific to each gene, and the resulting cDNAs were amplified by PCR in the presence of molecular beacons. Amplification was followed in real time by fluorescence produced after molecular beacon hybridization to the PCR product.¹⁰ Samples containing known amounts of H₃₇Rv chromosomal DNA were included to generate a standard curve for each gene measured. All samples exhibited fewer than 10 copies of genomic DNA contamination when measured using RNA that was untreated with reverse transcriptase. Within broth cultures of exponentially growing *M. tuberculosis*, the relative expression was *pknB*: *pknA*: *pknI* = 2.8: 2.6: 0.5 (Fig. 2). The absolute amounts of these mRNAs varied from 1.3×10^3 to 5.8×10^3 copies per ng of RNA. These values were similar to that for *sigA* mRNA, which is considered moderately abundant.¹³ Thus in broth culture *pknI* expression is about 20% that of *pknA* and *pknB*.

Immediately after infection, expression of *pknI*, *pknA*, and *pknB* dropped to 37%, 8%, and 20%, respectively, relative to levels in broth when normalized to *sigA*. Normalization to *sigA* mRNA is commonly used to correct for sample-to-sample variation,⁷ since *sigA* mRNA accumulates in proportion to bacterial replication inside THP-1 macrophages. Over the course of infection, *pknI* expression decreased 30-fold (Fig. 2, Panel A), *pknA* expression increased 12-fold (Fig. 2, Panel B), and *pknB* expression increased 14-fold (Fig. 2, Panel C). Thus, the expression of *pknI* starts low and drops during infection, while expression of *pknA* and *pknB* increases.

Discussion

Protein kinases are thought to contribute to *M. tuberculosis* biology in two general ways. First, secreted kinases, such as PknG of *M. tuberculosis*, interfere with phagosome–lysosome fusion and thus affect intracellular survival.¹ Second, the membrane-bound protein kinases may transmit environmental signals to internal signaling elements. Indeed, nine out of 11 *M. tuberculosis* kinases have transmembrane domains. Of those, PknB, PknD, PknE, PknF, and PknH have been shown to be membrane-bound.^{3,8,14–16} The present work adds PknA to that list (Fig. 1, Panel D). PknI is one of the proteins with a transmembrane domain,² but surprisingly it is localized predominantly in the cytosol (Fig. 1, Panel C). We were also surprised to

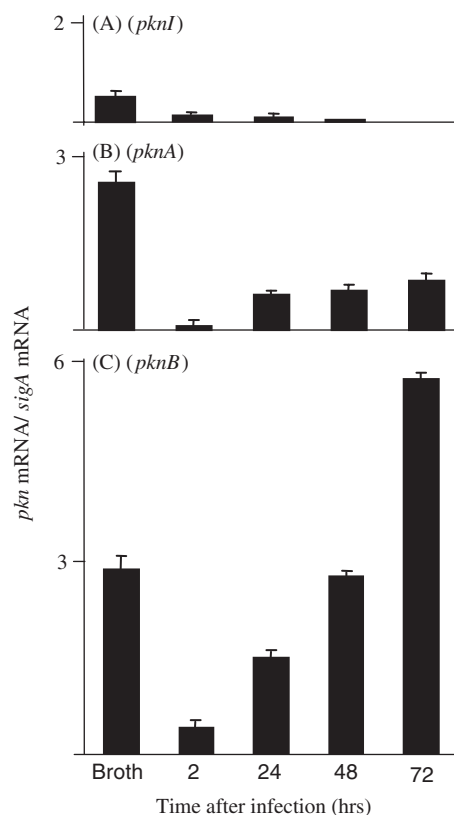


Figure 2 Changes in relative transcript copy number upon infection of macrophage-like cells. RNA was extracted from cells growing exponentially in liquid culture at the time the cells were used to infect THP-1 macrophages that had been activated as described in Methods. Abundance of *sigA* mRNA, which parallels colony-forming units, was determined at various times using PCR and molecular beacons (see Methods) and was confirmed to increase exponentially during growth in broth and in macrophages (not shown). Abundance of mRNA of *M. tuberculosis pknI* (A), *pknA* (B), and *pknB* (C), growing in broth and in THP-1 macrophage-like cells, was normalized to *sigA* mRNA to obtain a relative copy number. Copy numbers for *sigA* were 2.8×10^4 , 1.4×10^2 , 3.2×10^2 , 1.4×10^4 , and 6.3×10^4 for broth, 2, 24, 48, and 72 h post-infection, respectively. Each value represents an average determination for three independent RNA preparations.

find that the expression pattern of *pknI* differs from that of other kinases, as exemplified by comparison with *pknA* and *pknB*: *pknI* decreases significantly over the course of infection of macrophages, while expression of *pknA* and *pknB* increases (Fig. 2). A third distinguishing feature of PknI is the asparagine (N₁₃₇) in the conserved catalytic loop (hundreds of other serine/threonine kinases analyzed have a lysine at that position).

In summary, the present work adds a third class to the serine/threonine protein kinases of *M. tuberculosis* that is represented by PknI. The

intracellular localization of this kinase suggests that it is unlikely to participate in modification of host proteins during infection, and its decreasing abundance during infection argues against a positive effect on DNA replication (the data do not rule out the possibility that PknI normally acts as a negative regulator that must be removed for active intracellular growth). Experiments are in progress to determine whether *pknI* is an essential gene in *M. tuberculosis*.

Acknowledgments

We thank Marila Gennaro for critical comments on the manuscript. Eugenie Dubnau, Issar Smith, Salvatore Marras, and Savita Prabhakar provided valuable technical advice. The work was supported by NIH grant AI35257.

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