Serine/threonine protein kinases PknF and PknG of *Mycobacterium tuberculosis*: characterization and localization

Anil Koul,1,3,5 Axel Choidas,2 Anil K. Tyagi,3 Karl Drlica,4 Yogendra Singh1 and Axel Ullrich5

Author for correspondence: Yogendra Singh. Tel.: +91 11 766 6156. Fax: +91 11 766 7471. e-mail: ysingh@cbt.res.in

1 Centre for Biochemical Technology, Mall Road, Delhi-110 007, India
2 Axxima Pharmaceuticals AG, Am Klopferspitz 19, 82152 Martinsried, Germany
3 Department of Biochemistry, University of Delhi South Campus, N. Delhi, India
4 Public Health Research Institute, 455 First Avenue, NY, USA
5 Department of Molecular Biology, Max-Planck-Institut für Biochemie, Am Klopferspitz 18A, 82152 Martinsried, Germany

Pathogenesis of *Mycobacterium tuberculosis* is closely connected to its survival and replication within the host. Some pathogenic bacteria employ protein kinases that interfere with the cellular signalling network of host cells and promote bacterial survival. In this study, the *pknF* and *pknG* genes, which encode two putative protein kinases of *M. tuberculosis* H37Rv, protein kinase F (PknF) and protein kinase G (PknG), respectively, were cloned and expressed in *Escherichia coli*. Purified PknF phosphorylated the peptide substrate myelin basic protein (MBP) at serine and threonine residues, while purified PknG phosphorylated only at serine residues. The activity of the two kinases was abrogated by mutation of the codon for the predicted ATP-binding-site lysine residue. Southern blot analysis revealed that homologues of the genes encoding the two kinases are present in *M. tuberculosis* H37Ra and *Mycobacterium bovis* BCG, but not in *Mycobacterium smegmatis*. Immunoblot analysis of various cellular fractions of *M. tuberculosis* H37Rv revealed that PknF is a transmembrane protein and that PknG is predominantly a cytosolic enzyme. The present study should aid in elucidating the role of these protein kinases in the pathogenesis of mycobacteria.

Keywords: tuberculosis, protein phosphorylation, glutathione S-transferase, transmembrane, mycobacteria

INTRODUCTION

*Mycobacterium tuberculosis*, the causative agent of tuberculosis, employs several strategies for survival in host cells. Among these are inhibition of phagosome–lysosome fusion (Armstrong & Hart, 1975), inhibition of acidification of phagosomes (Sturgill-Koszycki et al., 1994), resistance to killing by reactive oxygen and nitrogen intermediates (Lowrie, 1983), and recruitment and retention of tryptophan-aspartate-containing coat protein (TACO) on phagosomes to prevent their delivery to lysosomes (Ferrari et al., 1999). Bacterial and host factors signalling and controlling these events are poorly understood.

The regulation of eukaryotic signal transduction pathways by phosphorylation–dephosphorylation of serine, threonine and tyrosine residues has been known for many years (Yarden & Ullrich, 1988). Recently, protein kinases have also been found to coordinate stress responses, development processes and pathogenicity in several micro-organisms (Av-Gay & Everett, 2000). For example, Ser/Thr kinase (YpKA) is involved in the expression of virulence in *Yersinia pseudotuberculosis* (Galyov et al., 1993) by disrupting the eukaryotic cytoskeleton and by reprogramming the host signalling network (Hakansson et al., 1996).

The presence of functional Ser/Thr kinases (Peirs et al., 1997) and phosphorylated protein (Chow et al., 1994) in mycobacteria was reported prior to release of the complete genome sequence of *M. tuberculosis*. The genomic sequence then suggested the presence of 11 putative protein kinases and 4 protein phosphatases (Cole et al., 1998). Although the role of these mycobacterial kinases is not yet clear, they may be regulators of various metabolic processes, growth, development and interaction with host cells. Recently, inhibitors of protein kinases have been shown to prevent uptake of *Mycobacterium leprae* by peritoneal macrophages of mice (Prabhakaran et al., 2000).

Abbreviations: GST, glutathione S-transferase; MBP, myelin basic protein.
Recently, two putative protein Tyr phosphatases (Koul et al., 2000) and two functional Ser/Thr kinases, PknD (Peirs et al., 1997) and PknB (Av-Gay et al., 1999), from M. tuberculosis have been cloned and characterized. The present work describes the cloning, expression and characterization of PknF and PknG, two more Ser/Thr protein kinases of M. tuberculosis. The data show that pknF and pknG code for functional kinases, that PknF is a transmembrane protein, and that PknG is a cytosolic enzyme.

**METHODS**

**Bacterial strains and plasmids.** Proteins from M. tuberculosis H₃₇Rv cell wall, cell membrane and cytosolic subcellular fraction, and cell lysates of M. tuberculosis H₃₇Ra and H₃₇Rv were provided by John T. Belisle under the Tuberculosis Research Material and Vaccine Testing Program of the National Institute of Allergy and Infectious Diseases, National Institute of Health, CO, USA (contract no. AI-75320). Genomic DNA was isolated from M. tuberculosis H₃₇Rv, M. tuberculosis H₃₇Ra, Mycobacterium bovis BCG and Mycobacterium smegmatis mc²155. M. tuberculosis H₃₇Rv, M. tuberculosis H₃₇Ra and M. bovis BCG were grown in 7H9 medium containing 10% albumin dextrose complex (ADC) at 37 °C for 3–4 weeks with shaking at 220 r.p.m. M. smegmatis was grown in Middlebrook 7H9 medium (Difco) supplemented with 0.2% glucose at 37 °C for 2 d. The expression vectors pGEX-5X-3 and pCDNA3 were obtained from Pharmacia Biotech and Invitrogen, respectively. Enhanced Chemiluminescence (ECL) reagent was obtained from NEN Research Products.

**Plasmid construction and mutagenesis.** M. tuberculosis H₃₇Rv genomic DNA was used as a template for amplification of two putative protein kinase genes, pknF and pknG, by PCR. The sequences of the two PCR primers for cloning pknF were 5′-GAATACTTCCATGGCTCGGGAAGGTTCAACGTGAAAGCGTCAGAGACCGAAC-3′ and 5′-GGCCAAAGCGTCAGAGACCGAAC-3′ for the 5′ end (carrying an EcoRI site) and 5′-CCGCTGAGTCAAGCCGACGCTTCTGCG-3′ for the 3′ end (carrying an XhoI site). For cloning pknG, the sequences of the two primers were 5′-GAATATTCCATGGCTCGGGAAGGTTCAACGTGAAAGCGTCAGAGACCGAAC-3′ for the 5′ end (carrying an EcoRI site) and 5′-CCGCTGAGTCAAGCCGACGCTTCTGCG-3′ for the 3′ end (carrying an XhoI site). The amplified products of pknF and pknG were then electroblotted to a PVDF membrane. The protein components were separated by 15% SDS-PAGE. The gels were electroblotted to nitrocellulose membranes and autoradiographed to determine the phosphorylation of MBP.

**Analysis of phosphorylated residues of MBP.** Analysis of phosphoamino residues of MBP phosphorylated by PknF or PknG was performed as described by Vincent et al. (1999). In brief, MBP was phosphorylated by either PknF or PknG, and the phosphorylated components were separated by 15% SDS-PAGE. They were then electroblotted to a PVDF membrane. The bands corresponding to the phosphorylated MBP were excised from the membrane and hydrolysed in 6 M HCl for 90 min at 110 °C. The acid-stable phosphoamino acids liberated by hydrolysis were separated by two-dimensional TLC and detected by autoradiography.

**Expression of PknF in A549 cell line.** The human lung epithelial cell line A549 was maintained in Ham’s F12 medium (Gibco-BRL) supplemented with 10% fetal calf serum. Cells were transfected with pCDNA3 alone or the pCDNA3-pknF construct by the calcium phosphate method (Chen & Okayama, 1987) and lysed as described previously (Zwick et al., 1999).

**Southern blot analysis.** Analysis of the presence of pknF and pknG homologues in various species of Mycobacteria was performed using Southern blot analysis.
Protein kinases of *M. tuberculosis*
carried out as described by Reyrat *et al.* (1995). In brief, genomic DNA samples (3 µg each) from *M. tuberculosis* H₃₇Rv, *M. tuberculosis* H₃₇Ra, *M. bovis* BCG and *M. smegmatis* were digested with *Bam*HI/ *Nru*I (for hybridization with *pknF*) or with *Sma*I (for hybridization with *pknG*). Digested products were separated by electrophoresis in a 1% agarose gel at 25–30 V for 16 h and transferred to nitrocellulose membranes. Hybridization was performed at 66 °C using 6 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7–2) with a ³²P-labelled *pknF* probe or with a ³²P-labelled *pknG* probe. After washing with 0/1 × SSC and 0/1 × SDS for 20 min at 68 °C, hybrids were detected by autoradiography.

**RESULTS**

Expression and purification of PknF and PknG

Genome sequence data of *M. tuberculosis* revealed the presence of 11 putative protein kinases (Cole *et al.*, 1998). Two genes with sequence homologies to protein kinases, *pknF* (Rv 1746) and *pknG* (Rv 0410c) were amplified by PCR from genomic DNA of *M. tuberculosis* H₃₇Rv using oligonucleotide primers deduced from the genome sequence of *M. tuberculosis* (Cole *et al.*, 1998). The amplified products were cloned in pGEX-5X-3. The resulting plasmids, designated pGEX-*pknF* or pGEX-*pknG*, were used to transform *E. coli*. The expressed GST-chimeric proteins (GST-PknF and GST-PknG) were purified using glutathione-Sepharose 4B matrix. The GST-PknF and GST-PknG chimeric proteins migrated in 10% SDS-PAGE as a 93 kDa protein (Fig. 1a, lane 2) and a 111 kDa protein (Fig. 1b, lane 2), respectively. The predicted size of GST-PknF was 80 kDa (51 kDa for the PknF protein and 29 kDa for the attached GST protein). The size of the GST-PknG chimeric protein was consistent with the predicted molecular mass of the PknG protein (82 kDa) plus the attached GST protein (29 kDa). The mutated chimeric proteins, GST-PknF-K41M (Fig. 1a, lane 3) or GST-PknG-K181M (Fig. 1b, lane 3), were purified by the same procedure.

Protein kinase assay of PknF and PknG

The ability of PknF and PknG to phosphorylate MBP was examined. Purified GST-PknF, GST-PknF-K41M, GST-PknG or GST-PknG-K181M were added to a reaction mixture containing MBP and [γ⁻³²P]ATP. After incubation, the products were separated by 15% SDS-PAGE and the phosphorylated proteins were identified by autoradiography. Purified GST-PknF protein phosphorylated MBP, while mutant GST-PknF-K41M did not (Fig. 2a, lanes 3 and 2, respectively). Similarly, GST-PknG was able to phosphorylate MBP, while mutant GST-PknG-K181M had no effect (Fig. 2b, lanes 3 and 2, respectively). GST-PknF (Fig. 2a, lane 4) and GST-PknG (Fig. 2b, lane 4) were also auto-phosphorylated. GST-PknG exhibited less auto-phosphorylating ability than GST-PknF (Fig. 2b, lane 4 and Fig. 2a, lane 4, respectively). However, in the presence of MBP, autophosphorylation by PknF was markedly reduced whereas that of PknG was enhanced. It is well known that addition of proteins or peptides may enhance the activity of protein kinases (Hubler

![Fig. 1. Electrophoretic analysis of recombinant PknF (a) and PknG (b). Affinity-purified PknF and PknG were separated by 10% SDS-PAGE and stained with Coomassie blue. (a) Lanes: 1, GST alone; 2, GST-PknF chimeric protein; 3, GST-PknF-K41M chimeric protein. (b) Lanes: 1, GST alone; 2, GST-PknG chimeric protein; 3, GST-PknG-K181M chimeric protein. Positions of molecular mass markers are indicated on the left.](image-url)
et al., 1992). To identify the residues of MBP phosphorylated by PknF or PknG, phosphorylated MBP was acid-hydrolysed and analysed by two-dimensional TLC. Incubation of MBP with GST-PknF phosphorylated serine and threonine residues of MBP (Fig. 3a), while GST-PknG phosphorylated MBP only at serine residues (Fig. 3b).

Localization of PknF and PknG in mycobacterial cells

Polyclonal antibodies raised against PknF and PknG were used to analyse the expression and localization of PknF in mycobacterial cells. Equal amounts of protein from cell wall, cell membrane and cytoplasmic subcellular fractions of M. tuberculosis H$_{37}$Rv, and whole-cell lysates from M. smegmatis, M. tuberculosis H$_{37}$Rv and M. tuberculosis H$_{37}$Ra were separated by 10% SDS-PAGE. The proteins were electroblotted onto a nitrocellulose membrane and incubated with anti-PknF serum or anti-PknG serum. The membranes were developed using an Enhanced Chemiluminescence kit. After development, PknF was seen as a doublet protein with an apparent molecular mass of 67–70 kDa when recovered from cell wall (Fig. 4a, lane 1) or cell membrane (Fig. 4a, lane 2) fractions of M. tuberculosis H$_{37}$Rv. PknF was absent from cytoplasmic subcellular fractions (Fig. 4a, lane 3) of M. tuberculosis H$_{37}$Rv. However, the cytoplasmic fraction showed a band corresponding to 50 kDa, which may represent the unphosphorylated form of the protein. These results suggested that PknF is predominantly localized in the cell envelope of M. tuberculosis H$_{37}$Rv. PknF was also detected as a doublet protein of 67–70 kDa in the whole-cell lysates of M. tuberculosis H$_{37}$Rv (Fig. 4a, lane 4) and M. tuberculosis H$_{37}$Ra (Fig. 4a, lane 5), but not in those of M. smegmatis (Fig. 4a, lane 6).

The calculated molecular mass of PknF is 50–6 kDa, but during 10% SDS-PAGE it migrated as a doublet protein of 67–70 kDa. To check the specificity of anti-PknF serum, human lung epithelial cell line A549 was transfected with the pCDNA3-pknF construct, which encoded a translational product of 50–6 kDa. Equal amounts of cell lysates from mock (pCDNA3 alone)- and pCDNA3-pknF-transfected A549 cell line were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane and incubated with anti-PknF serum. PknF was then seen as a double band of 67–70 kDa after PAGE of protein extracts from pCDNA3-pknF transfected A549 cells (Fig. 4a, lane 8). This result was similar to that found with whole-cell lysates of M. tuberculosis H$_{37}$Rv and M. tuberculosis H$_{37}$Ra (Fig. 4a, lanes 4 and 5). The aberrant migration of PknF during SDS-PAGE may be due to post-translational modifications such as phosphorylation. Such an effect has been shown for many Ser/Thr protein kinases (Hanlon et al., 1997; Zhang, 1996; Motley & Lory, 1999). A549 cells transfected with pCDNA3 vector alone (Fig. 4a, lane 7) failed to show bands that reacted with anti-PknF serum.

PknG was detected as an 82 kDa protein predominantly in the cytoplasmic fraction of M. tuberculosis H$_{37}$Rv.
onto nitrocellulose membranes. Hybridizations were separated by agarose gel electrophoresis and blotted hybridization with pknF (for hybridization with M tuberculosis).

(Fig. 4b, lane 6). Thus, the two proteins differ with respect to subcellular localization. PknG was also associated with cell membrane proteins (Fig. 4b, lane 2) of M. tuberculosis H37Rv and a faint band corresponding to PknG was detected in the cell wall fraction (Fig. 4b, lane 3). PknF was associated with cell membrane proteins (Fig. 4b, lane 2) of M. smegmatis and not in whole-cell lysates from M. tuberculosis H37Rv, M. tuberculosis H37Ra (lanes 5) and M. smegmatis (lanes 6) were loaded onto a 10% SDS-polyacrylamide gel and electroblotted. The blots were probed either with anti-PknF antibodies (a) or anti-PknG antibodies (b) and developed using Enhanced Chemiluminescence reagents. Cell lysates from A549 cell lines transfected with pCDNA3 alone (a, lane 7) or with pCDNA3-pknF construct (a, lane 8) were used to compare the molecular mass of the pknF gene translational product and PknF expressed protein in whole-cell lysates of M. tuberculosis H37Rv and M. tuberculosis H37Ra. Positions of molecular mass markers are indicated on the left.

**Analysis of pknF and pknG in other mycobacterial strains**

The PCR products of pknF or pknG were used as probes in Southern hybridization experiments to determine the presence of pknF or pknG homologues among different species of mycobacteria. Genomic DNA from M. smegmatis, M. bovis BCG, M. tuberculosis H37Rv and M. tuberculosis H37Ra was digested with BamHI/NruI (for hybridization with pknF) or with Smal (for hybridization with pknG). The digested fragments were separated by agarose gel electrophoresis and blotted onto nitrocellulose membranes. Hybridizations were performed with a 32P-labelled 1350 bp pknF fragment or 32P-labelled 1747 bp pknG fragment, derived from PCR amplification and restriction digestion of the pknF (BamHI/NruI) or pknG (Smal) genes, respectively. Hybridization results revealed that genes homologous to pknF (Fig. 5a) and pknG (Fig. 5b) were present in members of the M. tuberculosis complex analysed in this study, but they were absent from M. smegmatis, a non-pathogenic mycobacterium. Interestingly, pknF hybridization also revealed one or two additional hybridization fragments of 2-2 kb and 5-3 kb in the M. tuberculosis H37Rv and H37Ra lanes, respectively, consistent with the finding that PknF belongs to a family of.
related eukaryotic-like Ser/Thr protein kinases (Cole et al., 1998).

DISCUSSION

Phosphorylation of proteins at specific amino acid residues is a general mechanism by which both eukaryotes and prokaryotes regulate cell function. In prokaryotes, a two-component system consisting of a histidine kinase and its associated response regulator protein are used in sensing extracellular signals and coordinating intracellular events (Stock et al., 1989). In a number of prokaryotes Ser/Thr protein kinases also regulate development, response to stress conditions and pathogenicity (Av-Gay & Davies, 1997). Examples include regulation of antibiotic production in Streptomyces coelicolor (Matsumoto et al., 1994), spore production in Myxococcus xanthus (Munoz-Dorado et al., 1991) and transduction of environmental signals in Bacillus subtilis (Yang et al., 1996). In these cases, the intracellular Ser/Thr kinases phosphorylate their target proteins within the bacterial cytoplasm. In contrast, an extracellular autophosphorylating protein kinase (YpkA) secreted by Yersinia species is translocated into the host cell cytoplasm where it phosphorylates host proteins and alters host cell function (Hakansson et al., 1996). Yersinia mutants deficient in YpkA are avirulent (Galyov et al., 1993).

Although phosphorylated proteins (Chow et al., 1994) and protein kinases (Av-Gay & Everett, 2000; Peirs et al., 1997) have been found in mycobacteria, their role in mycobacterial pathogenesis is poorly understood. It has been well documented that M. tuberculosis has unique properties that allow it to remain in a dormant state within the host (Parrish et al., 1998). Protein kinases may play an important role in metabolic processes of M. tuberculosis. Therefore, we expressed and characterized two putative protein kinases, protein kinase F (PknF) and protein kinase G (PknG) of M. tuberculosis H₃₇Rv.

PknF and PknG have a characteristic protein kinase amino acid sequence signature, including all 11 conserved domains of Ser/Thr kinases (Hanks & Quinn, 1991). The two enzymes phosphorylated MBP, an in vitro substrate for Ser/Thr kinases (Hanks & Quinn, 1991). The two enzymes phosphorylated MBP, an in vitro substrate for Ser/Thr kinases, except for PknI, possess a lysine in the ATP-binding site (Av-Gay & Everett, 2000). When lysine at position 41 (K41) of PknF or lysine at position 181 (K181) of PknG were substituted with methionine, the mutant proteins failed to phosphorylate MBP. These results suggest that PknF and PknG are functional kinases and that K41 of PknF or K181 of PknG are required for enzymic activity, consistent with their having the same catalytic properties as other Ser/Thr kinases.

Immunoblot analysis of various cellular fractions of M. tuberculosis H₃₇Rv with anti-PknF serum revealed that PknF is localized mainly in the cell envelope. Also, predicted structural analysis of PknF from M. tuberculosis revealed that it has a transmembrane domain (Av-
Gay & Everett, 2000). These results suggest that PknF, like PknB, is a transmembrane kinase and that it may serve as a receptor for environmental signals, as is known to be true for transmembrane kinases from Streptomyces and Myxobacteria (Nadvorinik et al., 1999). It has also been postulated that since the pknF gene is located in the ATP-binding cassette transporter operon, it may be involved in phosphate transport (Av-Gay & Everett, 2000).

The pknF ORF encoded a translational product of 50.6 kDa, but Western blot analysis of whole-cell lysates of M. tuberculosis H37Rv and M. tuberculosis H37Ra revealed that PknF migrates during 10% SDS-PAGE as a 67–70 kDa doublet. Similar results were obtained with A549 cells transfected with pCDNA3-pknF. No deleterious effects were observed with transfected A549 cells. However, this does not rule out the possible involvement of PknF in virulence. The aberrant migration of PknF during SDS-PAGE may be due to phosphorylation, as is observed in Ser/Thr kinase Pkn9 (Hanlon et al., 1997) and Pkn6 (Zhang, 1996) from Myxococcus xanthus and Ser/Thr kinase PpkA from Pseudomonas aeruginosa (Motley & Lory, 1999). The migration of PknF as a doublet protein may reflect PknF molecules having different levels of phosphorylation.

In contrast to the transmembrane kinase PknF, PknG is predominantly a cytoplasmic protein in M. tuberculosis H37Rv. A cytoplasmic localization for PknG suggests that it is a soluble protein kinase. The secreted kinase (YopO) of Yersinia and PknG have similar structures (Av-Gay & Everett, 2000), suggesting that PknG may have a role in the pathogenesis of mycobacteria. It has also been suggested from the genetic localization of pknG within the glutamate-binding operon that PknG may be involved in virulence by regulation of glutamate metabolism (Av-Gay & Everett, 2000).

The genes encoding PknF and PknG, which were present in M. bovis BCG as well as in M. tuberculosis H37Rv and M. tuberculosis H37Ra, were absent from the avirulent strain M. smegmatis. This observation is consistent with our Western blot data in which we failed to detect the expression of PknG or PknF in M. smegmatis culture lysates. The absence of pknF and pknG in non-pathogenic M. smegmatis suggests possible roles in the processes specific to pathogenic mycobacteria. More experimental evidence will be necessary to demonstrate a critical function of PknF or PknG in the pathology of tuberculosis, which could open new opportunities for the development of target-specific therapies.

ACKNOWLEDGEMENTS

We would like to express our gratitude to Dr John T. Belsle (Colorado, USA) for providing cell wall, cell membrane and cytosolic fraction proteins of M. tuberculosis H37Rv and whole-cell lysates of M. tuberculosis H37Rv and M. tuberculosis H37Ra, and Y. Dong for purification of M. tuberculosis DNA. Sincere thanks go to Norbert Prenzel, Johannes Bange, Reimar Abraham and Hemant Khanna for valuable discussions. A.K. was supported by the Council of Scientific and Industrial Research (India) and DAAD (Germany).

REFERENCES


Downloaded from www.microbiologyresearch.org by 
IP: 54.70.40.11
On: Thu, 16 May 2019 18:50:26
secondary metabolism in *Streptomyces* species by a eukaryotic-type protein kinase. *Gene* 146, 47–56.


