

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* H₃₇Rv and H₃₇Ra 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'-GGAATTCCATGCCGCTCGCGGAAGGTTCAACGTTCC-3' for the 5' end (carrying an *EcoRI* site) and 5'-CCGCTCGAGTCACGGCCAGCCGTTGCTTCTGCG-3' for the 3' end (carrying an *XhoI* site). For cloning *pknG*, the sequences of the two primers were 5'-GGAATTCCATGGCCAAAGCGTCAGAGACCGAAC-3' for the 5' end (carrying an *EcoRI* site) and 5'-CCGCTCGAGTTAGAAGGTGCTGGTGGGCCGGAC-3' for the 3' end (carrying a *XhoI* site). The amplified products of *pknF* and *pknG* were digested with *EcoRI* and *XhoI*, and the resulting fragments were inserted into pGEX-5X-3, which was previously digested with the same restriction enzymes. The resulting plasmids were designated pGEX-*pknF* and pGEX-*pknG*.

Site-directed mutagenesis of lysine-41 of PknF and lysine-181 of PknG to methionine was carried out as described by Kunkel *et al.* (1991). The oligonucleotide used for mutagenesis of *pknF* was 5'-GGCCCGCAGTACCATGAGCGCGTCCTG-3', and the oligonucleotide for mutagenesis of *pknG* was 5'-ATGCACCAGGCCCATGAGCACCACCGG-3' (underlined bases indicate the change from lysine to methionine). The plasmids carrying the mutant genes were designated pGEX-*pknF*-K41M and pGEX-*pknG*-K181M. The *pknF* gene was also cloned into a eukaryotic expression vector, pCDNA3, and the resulting plasmid was designated pCDNA3-*pknF*. The nucleotide sequences were confirmed by sequencing using the dideoxynucleotide method (Sanger *et al.*, 1977).

Expression and purification of PknF and PknG. *Escherichia coli* strain BL21 was separately transformed with plasmids pGEX-*pknF*, pGEX-*pknF*-K41M, pGEX-*pknG* and pGEX-

pknG-K181M. Transformants were grown in 2YT medium (Difco) containing 100 µg ampicillin ml⁻¹ at 37 °C until the OD₆₀₀ reached 0.5. IPTG was then added to a final concentration of 0.5 mM, and cultures were grown for an additional 5 h at 37 °C with shaking. Cells were harvested by centrifugation at 5000 g for 15 min and resuspended in 20 ml sonication buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1 mM PMSF and 10 µg aprotinin ml⁻¹). The cells were then sonicated on ice for 2 min, and the sonicate was supplemented with Triton X-100 to a final concentration of 1% before centrifugation at 30 000 g for 30 min at 4 °C. The supernatant fluid was incubated overnight at 4 °C with glutathione-Sepharose 4B matrix (Pharmacia Biotech). The resin bound to protein was packed into a column and washed with 5 bed vols PBS (1.44 g disodium hydrogen phosphate, 0.24 g potassium dihydrogen orthophosphate, 0.2 g potassium chloride, 8.0 g sodium chloride in 1 litre distilled water, pH 7.2). Proteins were eluted with 50 mM Tris/HCl pH 8.0 containing 1 mM DTT, 5 mM MgCl₂ and 15 mM glutathione. Fractions were analysed by 10% SDS-PAGE (Laemmli, 1970). Fractions containing purified chimeric proteins were pooled and dialysed against PBS containing 20% glycerol and stored at -20 °C.

PknF and PknG kinase activities. The kinase assay is based on the phosphorylation of myelin basic protein (MBP). The activities of wild-type and mutated PknF and PknG were determined by incubating 0.5 µg glutathione S-transferase (GST)-PknF, GST-PknF-K41M, GST-PknG or GST-PknG-K181M with MBP (1 µg) for 30 min at 37 °C in a kinase buffer (25 mM Tris/HCl pH 7.4 containing 5 mM MgCl₂, 2 mM MnCl₂, 1 mM DTT, 200 µM orthovanadate and 3.7 × 10⁵ Bq [γ -³²P]ATP). The reactions were terminated by the addition of SDS sample buffer and proteins 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 carried out as described by Vincent *et al.* (1999). In brief, MBP was phosphorylated by either PknF or PknG, and the protein 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 acid-hydrolysed in 5.7 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.

Production of polyclonal antiserum to PknF and PknG. Purified GST-PknF or GST-PknG chimeric proteins (150 µg) were dissolved in 1 ml Freund's incomplete adjuvant and injected into rabbits. Subsequently, injections of 100 µg each of GST-PknF or GST-PknG in 1 ml Freund's incomplete adjuvant were given three times at 14 d intervals. Ten days after the final injection, animals were bled, and titres of anti GST-PknF were determined by ELISA as described by Harlow & Lane (1988).

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

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

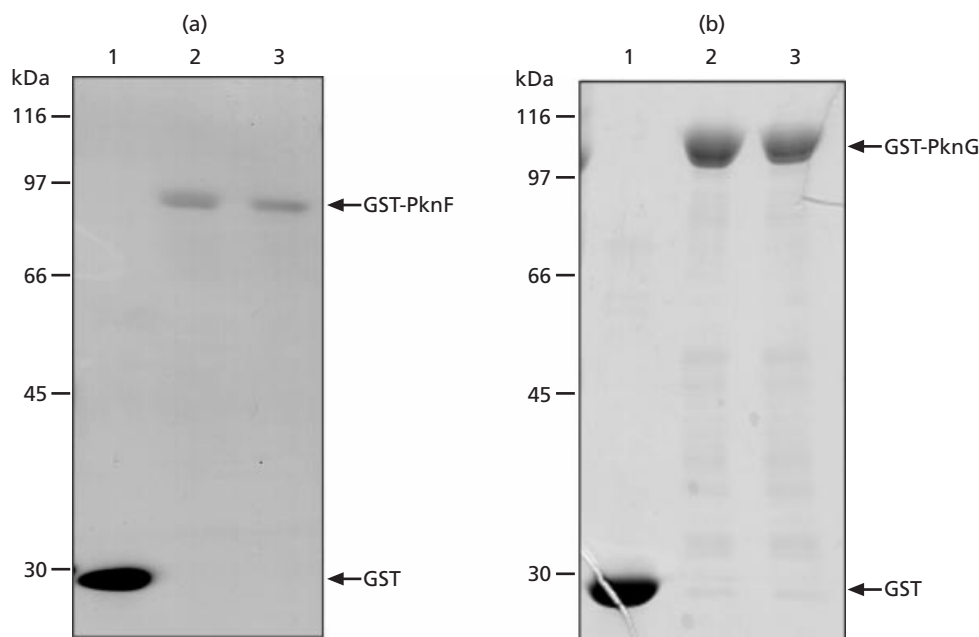


Fig. 1. Electrophoretic analysis of recombinant PknF (a) and PknG (b). Affinity-purified PknF and PknG were separated by 10% SDS-PAGE and stained with Coomassie blue. (a) Lanes: 1, GST alone; 2, GST-PknF chimeric protein; 3, GST-PknF-K41M chimeric protein. (b) Lanes: 1, GST alone; 2, GST-PknG chimeric protein; 3, GST-PknG-K181M chimeric protein. Positions of molecular mass markers are indicated on the left.

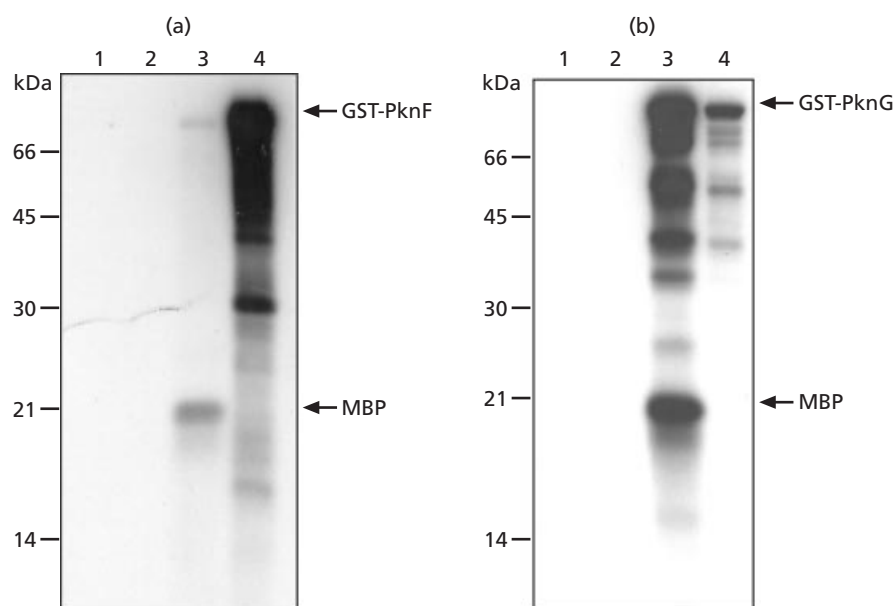


Fig. 2. Protein kinase activity of PknF (a) and PknG (b). Purified wild-type and mutant GST-PknF or GST-PknG (0.5 µg) were incubated with MBP (1 µg) in kinase buffer for 30 min at 37 °C. Proteins were separated by 15% SDS-PAGE, electroblotted and autoradiographed to determine phosphorylation of MBP. (a) Lanes: 1, MBP alone; 2, MBP plus mutant GST-PknF-K41M; 3, MBP plus wild-type GST-PknF; 4, GST-PknF alone. (b) Lanes: 1, MBP alone; 2, MBP plus mutant GST-PknG-K181M; 3, MBP plus wild-type GST-PknG; 4, GST-PknG alone. Positions of molecular mass markers are indicated on the left.

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 sub-cellular fractions of *M. tuberculosis* H₃₇Rv, and whole-cell lysates from *M. smegmatis*, *M. tuberculosis* H₃₇Rv and *M. tuberculosis* H₃₇Ra 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* H₃₇Rv. PknF was absent from cytoplasmic subcellular fractions (Fig. 4a, lane 3) of *M. tuberculosis* H₃₇Rv. 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* H₃₇Rv. PknF was also detected as a doublet protein of 67–70 kDa in the whole-cell lysates of *M. tuberculosis* H₃₇Rv (Fig. 4a, lane 4) and *M. tuberculosis* H₃₇Ra (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 doublet 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* H₃₇Rv and *M. tuberculosis* H₃₇Ra (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* H₃₇Rv

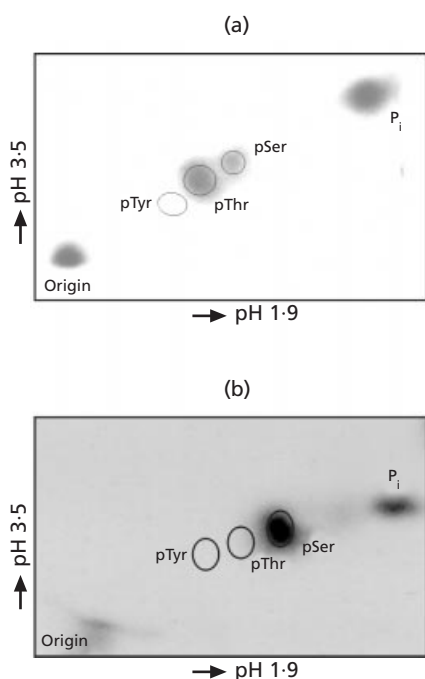


Fig. 3. Analysis of PknF-phosphorylated (a) and PknG-phosphorylated (b) residues of MBP. Phosphorylated MBP residues were separated by 15% SDS-PAGE and electroblotted onto a PVDF membrane. Bands containing proteins were excised and hydrolysed in acid. The acid-stable phosphoamino acids liberated by hydrolysis were separated by two-dimensional TLC and autoradiographed. $^{32}\text{P}_i$ was produced by partial hydrolysis of labelled amino acids. Samples of non-radioactive phosphotyrosine, phosphoserine and phosphothreonine were run in parallel and visualized by ninhydrin staining. PknF phosphorylates MBP on serine and threonine residues (a). PknG phosphorylates MBP on serine residues only (b).

(Fig. 4b, lane 3). 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 1). PknG expression could be detected in whole-cell lysates of *M. tuberculosis* H₃₇Rv (Fig. 4b, lane 4) and H₃₇Ra (Fig. 4b, lane 5), but not in whole-cell lysates from *M. smegmatis* (Fig. 4b, lane 6). Thus, the two proteins differ with respect to subcellular localization.

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 *Bam*HI/*Nru*I (for hybridization with *pknF*) or with *Sma*I (for hybridization with *pknG*). The digested fragments were separated by agarose gel electrophoresis and blotted onto nitrocellulose membranes. Hybridizations were

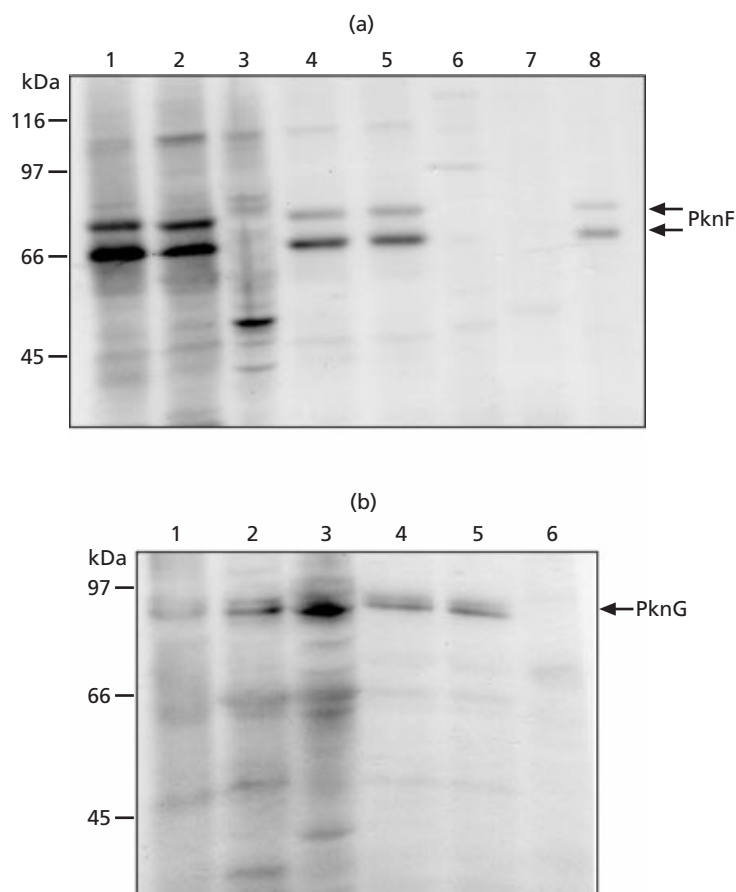


Fig. 4. Expression and localization of PknF (a) and PknG (b) in mycobacteria. Equal amounts of protein (40 µg each) of cell wall (lanes 1), cell membrane (lanes 2) and cytoplasmic fraction (lanes 3) from *M. tuberculosis* H₃₇Rv, and whole-cell lysates from *M. tuberculosis* H₃₇Rv (lanes 4), *M. tuberculosis* 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.

performed with a ^{32}P -labelled 1350 bp *pknF* fragment or ^{32}P -labelled 1747 bp *pknG* fragment, derived from PCR amplification and restriction digestion of the *pknF* (*Bam*HI/*Nru*I) or *pknG* (*Sma*I) 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

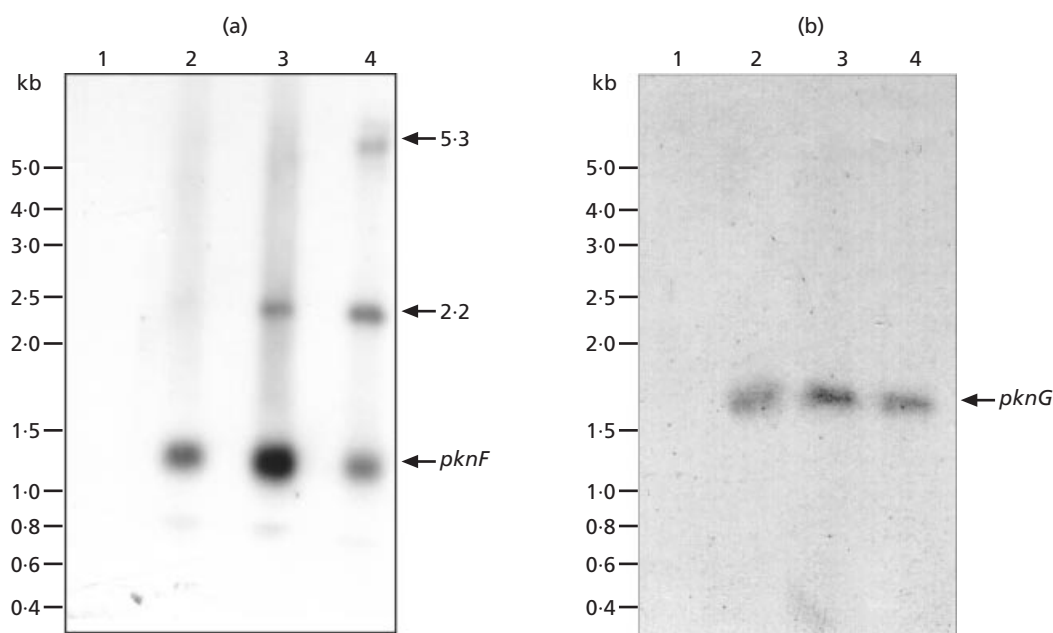


Fig. 5. Presence of *pknF* (a) and *pknG* (b) in other mycobacteria. Genomic DNA samples (3 µg each) from mycobacterial species were digested with *Bam*HI/*Nru*I (for hybridization with *pknF*) or with *Sma*I (for hybridization with *pknG*) and the fragments were separated by 1% agarose gel electrophoresis for 16 h at 25–30 V and transferred to nitrocellulose membranes. Hybridization was performed with ³²P-labelled *pknF* probe (a) or with ³²P-labelled *pknG* probe (b) and autoradiographed. Lanes: 1, *M. smegmatis*; 2, *M. bovis* BCG; 3, *M. tuberculosis* H₃₇Rv; 4, *M. tuberculosis* H₃₇Ra. Positions of size markers are shown on the left.

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. All putative Ser/Thr kinases of mycobacteria, except for PknI, possess a lysine in the ATP-binding site (Av-Gay & Everett, 2000). This feature is typical of Ser/Thr kinases (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* (Nadvornik *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.

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