Ability of PknA, a mycobacterial eukaryotic-type serine/threonine kinase, to transphosphorylate MurD, a ligase involved in the process of peptidoglycan biosynthesis

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INTRODUCTION

Eukaryotic-type serine/threonine protein kinases in bacteria have been implicated in controlling a host of cellular activities. PknA is one of eleven such protein kinases from Mycobacterium tuberculosis which regulates morphological changes associated with cell division. In the present study we provide the evidence for the ability of PknA to transphosphorylate mMurD (mycobacterial UDP-N-acetylmuramoyl-L-alanine:D-glutamate-ligase), the enzyme involved in peptidoglycan biosynthesis. Its co-expression in Escherichia coli along with PknA resulted in phosphorylation of mMurD. Consistent with these observations, results of the solid-phase binding assays revealed a high-affinity in vitro binding between the two proteins. Furthermore, overexpression of m-murD in Mycobacterium smegmatis yielded a phosphorylated protein. The results of the present study therefore point towards the possibility of mMurD being a substrate of PknA.

Key words: bacterial two-hybrid system, mycobacterium, PknA, pulldown assay, serine/threonine kinase, solid-phase binding, transphosphorylation, UDP-N-acetylmuramoyl-L-alanine:D-glutamate-ligase (MurD).

MATERIALS AND METHODS

Plasmid construction

Genomic DNA isolation from M. tuberculosis strain H37Rva or E. coli strain K12 has been described previously [9,10]. Interacting protein(s) of PknA was identified in the present study through a bacterial two-hybrid approach. To do this the BacterioMatch II two-hybrid system vector kit (Stratagene) was used according to the manufacturer’s protocol. An E. coli genomic DNA library (fragment size between 1.5 and 3.5 kb obtained by Sau3A1 partial digestion) was constructed in a pTRG vector (cloned at the BamHI site) following standard procedures [13], and was used as the target (fusion protein with the N-terminal domain of the α-subunit of E. coli RNA polymerase) in our experiments. The pknA ORF (open reading frame; Rv0015c) was excised from pUC19-PknA [9] by restriction

Abbreviations used: GST, glutathione transferase; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactoside; MBP, myelin basic protein; mMurD, mycobacterial UDP-N-acetylmuramoyl-L-alanine:D-glutamate-ligase; Ni-NTA, Ni2+-nitrilotriacetate; ORF, open reading frame.

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digestion (EcoRI/BamHI) and cloned in pBT (fused to full-length bacteriophage λ repressor protein) for its use as bait. The bait and target plasmids were co-transformed in the reporter strain harbouring a HIS3-aadA reporter cassette. Clones obtained on His drop-out medium in the presence of 3-AT (a competitive inhibitor of the HIS3 enzyme) were further selected on streptomycin to monitor expression of the aadA gene product. These were sequenced and identified through a BLAST search.

*M. tuberculosis* genomic DNA was used as the template for PCR amplification of the *murD* gene (*m-murD*; Rv2155c). Primers (CM74, 5′-CATATGGCTGGACCCTTGG GGC-3′ and CM75, 5′-CTACCCGGATCACCAGCGG-3′) designed for this purpose were based on the *M. tuberculosis* genome sequence [8]. ‘CATA’ in primer CM74 does not correspond to the genome sequence but was incorporated to introduce Ndel at the 5′ end of the PCR-amplified product and also to change the initiator codon from GTG to ATG to facilitate expression in *E. coli*. PCR was carried out using the GC-rich PCR system (mixture of Taq and Tgo DNA polymerases; Roche) following the manufacturer’s protocol. The amplified fragment (*m-murD*) through an intermediate subcloning in pUC19 (pUC-mMurD) was finally cloned either in pGEX-KG at SacI/HindIII sites [GST (glutathione transferase)-mMurD] or in a mycobacterial expression vector, pVV {N-terminal histidine-tagged, under hsp60 (heat-shock protein 60) promoter; [14]} at Ndel/HindIII sites (pVV-mMurD). The sequencing of the *m-murD* revealed absolute identity at the nucleotide level with the same gene from the *M. tuberculosis* pathogenic strain H37Rv. In the present study, we used the cytosolic region of PknA (spanning amino acid residues 1–338; hereafter referred to as PknA) that has the ability to exhibit auto-/trans-phosphorylation (spanning amino acid residues 1–338; hereafter referred to as PknA).

### Protein expression and purification

*E. coli* BL21(DE3) cells harbouring the GST–*mMurD* were grown (*A_{sof} 0.6) and induced with 0.4 mM IPTG (isopropyl β-D-thiogalactoside) for 3 h for monitoring the expression of protein. Cells were harvested, suspended in lysis buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM PMSF, 1 μg/ml pepstatin and 1 μg/ml leupeptin] and sonicated. The supernatant fraction was purified on a glutathione–Sepharose column, eluted in elution buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl and 10 mM glutathione] and was used in subsequent studies. Methods used for the expression and purification of other proteins used in the present study have been described elsewhere [9,10,15].

The pVV-mMurD was transformed in *Mycobacterium smegmatis* cells and grown to mid-log phase at 37°C. In initial experiments, one sample from each culture was incubated at 37°C and two other samples were subjected to stress either by the adjustment to 0.002% H₂O₂ or incubation at 45°C for 4 h. Cells were collected, washed once with 0.9% saline solution and disrupted by sonication on ice (10 cycles of 60 s on and 90 s off) in lysis buffer containing 50 mM Tris/HCl (pH 7.5), 10 mM MgCl₂, 5 mM 2-mercaptoethanol and protease inhibitors. The resulting suspension was centrifuged (15 000 g at 4°C for 15 min) to separate supernatant and pellet fractions. Supernatant fractions (25 μg of protein/slot) were resolved on SDS/PAGE to analyse the overexpression of protein. Western blotting with monoclonal anti-His primary and anti-mouse IgG–HRP (horseradish peroxidase) secondary antibodies was performed to monitor the expression of histidine-tagged protein. For large-scale protein purification, *M. smegmatis* cells harbouring pVV or pVV-mMurD were grown at 37°C to mid-log phase, induced with 0.002% H₂O₂ for 4 h and processed to obtain the soluble fraction of the cell lysate. The supernatant fraction obtained was loaded on Ni-NTA (Ni²⁺-nitrilotriacetate) resin equilibrated with lysis buffer [50 mM Tris/HCl (pH 7.5), 10 mM MgCl₂, 5 mM 2-mercaptoethanol and protease inhibitors], washed (ten bed volumes of lysis buffer containing 20 mM imidazole) and finally eluted with 250 mM imidazole.

### Pulldown assay

Cells expressing pMAL-PknA were sonicated and the lysate obtained following centrifugation (17 600 g for 15 min at 4°C) was pre-cleaned using glutathione resin. The lysate was incubated with GST–*mMurD*/GST and 25 μl of glutathione beads for 2 h at 4°C in binding buffer [50 mM Tris/HCl (pH 8) and 150 mM NaCl] with gentle end-to-end mixing. For monitoring the interaction using purified proteins, 100 μg each of GST–*mMurD*/GST and MBP–PknA/MBP–βgal (where MBP is myelin basic protein) were incubated with the beads. The beads were thoroughly washed five times with binding buffer containing 0.1% Nonidet P40 (1 ml/wash). The proteins bound to the beads were extracted with SDS sample buffer by heating at 90°C for 5 min. Following centrifugation (15 700 g for 10 min at 25°C), 10 μl of the supernatant was loaded on to SDS/PAGE followed by Western blotting using an anti-GST–HRP conjugate or polyclonal anti-MBP (for crude lysates)/anti-MBP-PknA (for purified protein) as primary antibodies and anti-rabbit IgG–HRP as the secondary antibody.

### Solid-phase interaction assay

The solid-phase interaction assay was performed as described previously [10,15]. Briefly, recombinant *mMurD (1 μg/well)* was immobilized on a microtitre plate and, following blocking, was incubated with increasing concentrations of biotinylated MBP–PknA (0–5 μg/ml). After thorough washing with PBS containing 0.5% Tween 20, the bound protein was detected at *A_{sof}*, using a streptavidin–HRP conjugate and TMB (3,3′,5,5′-tetramethylbenzidine) substrate. Displacement of biotinylated protein was assessed by incubating biotin-labelled MBP–PknA with immobilized GST–*mMurD* in the presence of an increasing concentration of non-biotinylated proteins (MBP–PknA, GST–*mMurD*, MBP–βgal).

### Kinase assay

MBP–PknA (1 μg/K42N (5 μg) were incubated with GST–*mMurD (10 μg) in kinase buffer [50 mM Tris/HCl (pH 7.5), 50 mM NaCl and 10 mM MnCl₂] and 2 μCi [γ-³²P]ATP (specific activity 3000–5000 Ci/mmol; Jolimadi Laboratories) at 25°C for 20 min. The reaction was stopped by the addition of 5 × SDS sample buffer, heated at 90°C for 5 min and resolved on SDS/PAGE (8–10% gels). Gels were stained with Coomassie Brilliant Blue, dried in a gel drier (Bio-Rad) at 70°C for 2 h and finally analysed on a phosphorimaging device.

### Western blotting

Plasmids (p19Kpro and pGEX-KG) harbouring different ORFs (pknA, K42N and *m-murD*) were co-transformed in *E. coli* strain...
BL21(DE3). After dual selection on ampicillin (75 μg/ml) and hygromycin (200 μg/ml) plates, the clones obtained were cultured in LB (Luria–Bertani) broth in the presence of both of the antibiotics and induced with 0.2 mM IPTG (37 °C for 3 h). Cell extracts (25 μg of supernatant/slot) or purified proteins (GST/His-tagged; 2 μg/slot) were resolved on SDS/PAGE and processed for Western blotting using different primary (anti-MBP–PknA, anti-GST–HRP conjugate and anti-phosphothreonine) and secondary (anti-rabbit IgG–HRP conjugate) antibodies. Finally, blots were developed with ECL® (enhanced chemiluminescence) following the manufacturer’s (GE Healthcare) protocol.

RESULTS AND DISCUSSION

PknA has been implicated in regulating cell shape and cell division in mycobacteria through reversible phosphorylation of cellular proteins [11]. Alteration in cell shape precedes the process of cell division in bacteria and at the same time peptidoglycan synthesis also occurs. These two events, although distinct from each other, have often been correlated. Interestingly, in both of the processes, ordered assembly of fairly conserved proteins have been noticed throughout bacteria [12]. Since PknA has been shown to interact with mFtsZ [10], it would really be fascinating to elucidate whether PknA has any role to play in peptidoglycan synthesis. Although unravelling such a complex signalling network of PknA is an arduous task, we decided to employ a bacterial two-hybrid screening system for identifying such interacting proteins [16,17]. The choice of target library was supported by the observation of an elongation phenotype in E. coli cells as a result of constitutive expression of PknA, thereby suggesting the involvement of protein(s) conserved in bacteria as the target of this kinase [9]. Furthermore, PknA equally interacted with FtsZs from E. coli and M. tuberculosis. Among several interactive sequences identified through a BLAST search [18], we noticed that a gene encoding the cytoplasmic enzyme MurD has been reported to be involved in peptidoglycan biosynthesis by catalysing the addition of D-glutamate to the nucleotide precursor UDP-N-acetylmuramoyl-L-alanine [19,20]. Since MurD does not show similarity to any of the host (human) proteins, it has the potential to be considered as a drug target [21]. This led us to locate murD in mycobacteria and further evaluate its interaction with PknA.

Analysis of the M. tuberculosis genome revealed that murD is located in a cluster near the other cell division genes such as ftsW (Figure 1A). Alignment of sequences, using ClustalX [22], revealed ∼ 64% homology (30% identity) between MurD of E. coli and M. tuberculosis (Figure 1B). To evaluate the interaction between PknA and mMurD, a pulldown assay using cell lysates was performed. Figure 2(A) shows that GST–mMurD was able...
Figure 2  *In vitro* interactions of PknA and mMurD

(A) Bacterial expression pulldown assay. Precleaved MBP–PknA cell lysate was incubated with GST–mMurD or GST and glutathione beads. The proteins extracted from the beads after washing were probed with either anti-MBP (upper panel) or anti-GST (lower panel) antibodies. Arrowheads indicate MBP–PknA (upper panel) or GST–mMurD (lower panel). (B) Pulldown assay. Purified GST–mMurD or GST was incubated with MBP–PknA or MBP–βgal and glutathione beads. The proteins extracted from the beads after washing were probed with either anti-GST (upper panel) or anti-MBP–PknA (lower panel) antibodies. Arrowheads indicate GST–mMurD or GST protein (upper panel), or MBP–PknA (lower panel). The purified MBP–PknA sample used in the present study is loaded in lane 1. Lane numbers are indicated at the bottom. (C) Solid-phase interaction assay. Recombinant GST–mMurD (1 μg/well) was coated on a microtitre plate and, following blocking, was incubated with increasing concentrations (0–5 μg/ml) of biotinylated MBP–PknA (bPknA). The bound protein was detected by monitoring the HRP activity as described in the text. (D) Displacement of biotinylated protein was assessed by incubating biotin-labelled MBP–PknA with immobilized GST–mMurD in the presence of an increasing concentration of non-biotinylated proteins (MBP–PknA, GST–mMurD, MBP–βgal).

to pulldown MBP–PknA from the lysate (compare lanes 2 and 3). The binding was further confirmed by pulldown using purified proteins. As shown in Figure 2(B), Western blotting of the samples eluted from the beads with anti-MBP–PknA antibody highlighted the MBP–PknA (lower panel) and an anti-GST antibody recognized the GST–mMurD and GST (upper panel). However, no band corresponding to MBP–PknA could be detected on incubating the protein with glutathione resin in the presence of GST, indicating the specificity of the interaction (Figure 2B, lower panel, compare lanes 2 and 4). The absence of signal corresponding to MBP–βgal on incubation either with GST–mMurD or GST (compare lanes 3 and 5) further ruled out the involvement of the tag in the interaction and confirmed the specificity of binding between the two proteins. These results thus revealed that PknA could interact with mMurD. To find out the affinity of interaction between the two proteins, we performed a solid-phase binding assay [10]. The enzyme activity increased with increasing concentrations of biotinylated PknA with a half maximal binding of 0.62 ± 0.07 μg/ml and a dissociation constant of 1.12 ± 0.35 μM (Figure 2C). Compared with different kinase-substrate reactions in eukaryotes, the affinity of the PknA–mMurD interaction seems to be within a physiologically relevant range [23]. The specificity of the interaction was determined by displacing the biotin-labelled PknA with the non-biotinylated proteins. As shown in Figure 2(D), increasing the concentration of non-biotinylated mMurD as well as PknA displaced the binding of the biotinylated protein. All of these results, therefore, pointed towards a specific interaction between PknA and mMurD.

We have already reported the *in vitro* autophosphorylating activity of PknA (see also Figure 3A, lane 1), its transphosphorylating ability for known exogenous proteins (histone and MBP) as well as FtsZs of *E. coli* and *M. tuberculosis* [9,10]. To investigate whether mMurD could be a substrate of PknA, a kinase assay was performed. As shown in Figure 3(A) (left-hand panel), whereas mMurD along with MBP–βgal (pMAL-c2X) was unable to show any phosphorylation (lane 4), on incubation with MBP–PknA it was phosphorylated (lane 2). No phospho-signal for mMurD (Figure 3A, left-hand panel, lane 3) was noticed when it was added in the reaction after heating (90°C for 5 min). To confirm that transphosphorylation is PknA specific, we used a kinase-dead mutant (K42N, where the lysine residue at amino acid position 42 was mutated to an asparagine residue). As expected, no γ-32P incorporation of mMurD could be detected when the reaction was carried out with K42N (Figure 3A, left-hand panel, lane 5). To rule out the possibility of the involvement of the GST- or MBP-fusion tag in the transphosphorylation reaction,
PknA-mediated transphosphorylation of MurD

Figure 3  Transphosphorylation of mMurD by PknA

(A) MBP–PknA/MBP–K42N/MBP–βgal (5 μg/reaction) was incubated with or without mMurD (left-hand panel) and GST (right-hand upper panel) in kinase buffer (10 μg/reaction). The reaction was stopped by adding 5 × SDS sample buffer, resolved on SDS/PAGE (8–10 % gels) and finally analysed on a phosphorimaging device as described in the Materials and methods section. Right-hand panel: samples (MBP–PknA, MBP–βgal+GST and MBP–PknA+GST), following incubation with [γ-32P]ATP, were resolved on SDS/PAGE (10 % gel), stained with Coomassie Brilliant Blue (lower panel) and processed for autoradiography (upper panel). Arrowheads indicate the position of different proteins. (B) A kinase assay was performed with different concentrations of mMurD (0–20 μg) in the presence of PknA. (C) MBP–PknA was incubated initially in kinase buffer in the presence of [γ-32P]-labelled PknA for 20 min at 25°C. This was followed by the addition of mMurD (10 μg/reaction) and further incubation for the indicated time periods (5–60 min). Samples were further processed as described in the Materials and methods section. The molecular mass in kDa is indicated.

we incubated MBP–PknA/MBP–βgal+GST with [γ-32P]ATP. As expected, we did not see any phospho-signal for GST or MBP–βgal (Figure 3A, right-hand upper panel, lanes 2 and 3) and the loading of MBP–βgal and GST samples were ensured following staining of the gel with Coomassie Brilliant Blue (Figure 3A, right-hand lower panel). This observation further confirmed our previous report that fusion tags (MBP and GST) do not contribute to the auto-/trans-phosphorylation reactions [10]. The transphosphorylation reaction depended on the mMurD concentration (Figure 3B) as well as on the time of incubation with [γ-32P]-labelled PknA (Figure 3C). Thus all of these lines of evidence established that mMurD, which alone does not exhibit any phosphorylation, could be phosphorylated in the presence of PknA.

In order to discover whether mMurD could be a substrate of PknA in vivo, we utilized an E. coli-based system to co-express both of the proteins. For this purpose, we co-transformed both p19Kpro-PknA/p19Kpro-K42N and pGEX-mMurD in the E. coli strain BL21(DE3). GST–mMurD protein was purified through glutathione resin. Interestingly, although mMurD protein purified in the presence of PknA yielded a strong phospho-signal with an anti-phosphothreonine antibody, no signal could be detected on purifying the protein either in the presence of a kinase-dead mutant of PknA (Figure 4A, left-hand panel, compare lanes 1 and 2) or glutathione resin. Interestingly, although mMurD protein purified in the presence of PknA yielded a strong phospho-signal with an anti-phosphothreonine antibody, no signal could be detected on purifying the protein either in the presence of a kinase-dead mutant of PknA (Figure 4A, left-hand panel, compare lanes 1 and 2) or
in the absence of kinase (Figure 4A, left-hand panel, lane 3). This observation implies that mMurD was specifically phosphorylated by PknA. Additionally, we also observed that the phosphorylated protein was labile and degraded in a time-dependent manner on storage at 4 °C (Figure 4A, right-hand panel).

In investigating physiologic target(s) of M. tuberculosis eukaryotic-type serine/threonine kinases in vivo, a second copy of a putative substrate gene under the control of an inducible promoter has often been introduced in a non-pathogenic saprophytic mycobacterium M. smegmatis and its phosphorylation status monitored [11,24–26]. Comparison of the genome sequences indicated the presence of both PknA and MurD in M. smegmatis (http://www.tigr.org) and they are very conserved (between PknAs ~90% homology; between MurDs ~88% homology at the amino acid level between M. smegmatis and M. tuberculosis). We transformed m-mult vector in M. smegmatis strain mc2155 and monitored its expression following induction with either 0.002% H2O2 or incubation at 45 °C for 4 h (uninduced control at 37 °C). The expression of mMurD was evident on SDS/PAGE when induced with H2O2 (Figure 4B, upper panel, lane 4) and was confirmed by immunoblotting with an anti-histidine antibody (Figure 4B, lower panel). Western blotting of purified mMurD (obtained through purification of M. smegmatis lysate using Ni-NTA resin) using anti-phosphotyrosine antibody revealed it as a phosphorylated protein (Figure 4C, upper panel, lane 2) and its identity was confirmed with an anti-histidine antibody (Figure 4C, lower panel, lane 2). Lysate prepared similarly from M. smegmatis cells transformed with pVV vector and purified through Ni-NTA resin was used as a control (Figure 4C, upper panel, lane 1). Furthermore, phosphorylation of mMurD at threonine residues was specific for mycobacteria, since the protein expressed in E. coli was not in the phosphorylated state (Figure 4A, lane 3).

Among eleven eukaryotic-type serine/threonine kinases in the M. tuberculosis genome, two of them (PknA and PknB) are clustered with the SEDS (shape, elongation, division and sporulation) family of proteins (PBPA and RodA) and a phosphatase [27]. The organization of ORFs spanning this region is typical of most of the mycobacterial species. PknB has been shown to phosphorylate penicillin-binding protein, PBPA [28], an FHA-domain-containing protein, GarA [29], and a cell division protein orthologue, Wag31 [11]. On the other hand, we and others have identified PknA as a potential regulator of morphological changes associated with cell division [9,11]. Our recent study further establishes PknA-mediated phosphorylation as well as regulation of the functionality of FtsZ, a protein central to bacterial cell septum formation [10]. Although alteration in cell morphology and its septum formation in bacteria are known to be mutually exclusive processes, there should be co-ordination between them [30,31]. In fact, peptidoglycan-synthesizing and cell-division genes are usually found in DCW (cell wall–cell division) clusters in bacteria [32], including mycobacteria (Figure 1A). Although, it is necessary to unravel the role of PknA in the process of mMurD-mediated peptidoglycan biosynthesis [19,20], one can speculate about its involvement in penicillin-insensitive peptidoglycan synthesizing activity during initiation of constriction in bacterial cell division [12]. Nonetheless, the fact that mMurD could be a substrate of PknA is a significant observation and the kinase might be involved in the regulation of a switch between the peptidoglycan biosynthetic pathway as well as cell division in mycobacteria.

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