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GTPase Activity of Mycobacterial FtsZ Is Impaired Due to Its Transphosphorylation by the Eukaryotic-type Ser/Thr Kinase, PknA*

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FtsZ, a homolog of eukaryotic tubulin, is involved in the process of cell division, particularly in septum formation in bacteria. The primary amino acid sequences of this protein are fairly conserved in prokaryotes. We observed that a eukaryotic-type Ser/Thr protein kinase, PknA from *Mycobacterium tuberculosis*, when expressed in *Escherichia coli* exhibited cell elongation due to a defect in septum formation. We found that FtsZ either from *Escherichia coli* (eFtsZ) or from *M. tuberculosis* (mFtsZ) was phosphorylated on co-expression with PknA. Consistent with these observations, solid phase binding and *in vitro* kinase assays revealed the ability of PknA to interact with mFtsZ protein and also to phosphorylate it. We, therefore, ascertained mFtsZ as a substrate of PknA. Furthermore, the phosphorylated mFtsZ exhibited impairment in its GTP hydrolysis and polymerization abilities. Thus, our results highlighted the ability of PknA to phosphorylate as well as to regulate the functionality of FtsZ, the protein central to cell division throughout the bacterial lineage.

Bacterial cell division is a tightly regulated process. It comprises a DNA cycle and a division cycle. The DNA cycle involves DNA replication and chromosome segregation. During the division cycle, the cell identifies the midpoint and differentiates the site in preparation for septum formation as well as cytokinesis. The septum formation initiates with the development of a contractile Z-ring at mid-cell, which sets up a scaffold for the recruitment of additional proteins. These proteins congregate at the Z-ring in a highly orchestrated fashion, causing an orderly assembly of the septal ring. The septal ring during the progression of the event constricts, culminating in complete disappearance, and finally, the division septum forms that lead toward cell separation (1).

The ring-like structure is primarily composed of FtsZ protein, which is the key molecule responsible for initiating the earliest event in the process of septation. In several bacterial species, the FtsZ-binding proteins involved in the process of cytokinesis have been identified (2). In fact, many, albeit not all, proteins involved in the division cycle are known, and their interactions have already been well characterized in several bacteria. In *Escherichia coli*, at least 15 different proteins have been localized at the division site in a sequential manner (3). On the other hand, in *Bacillus subtilis*, the Z-ring has been shown to recruit at least 10 cell division proteins directly or indirectly (4). Interestingly, the majority of proteins involved in septum formation are well conserved among bacteria, indicating common division machinery.

The bacterial FtsZ is known to be a homolog of eukaryotic tubulin (5, 6). Like tubulin, FtsZ is also a GTPase and has been reported to have the ability to polymerize into dynamic polymers in a GTP-regulated manner (7–9). In this context, we have focused on FtsZ from a slow growing pathogen *Mycobacterium tuberculosis*, the causative agent of the dreadful disease tuberculosis. The genome sequence of *M. tuberculosis* has revealed the presence of the FtsZ homolog, which has been purified, characterized, and recently crystallized (10–12). However, except for FtsW, identifiable analogs of putative FtsZ-interacting proteins are lacking (13, 14). It is also not clear how the FtsZ activities are regulated in this pathogen.

In earlier reports, we and others have indicated the involvement of PknA, a Ser/Thr protein kinase, in the process of cell division (15, 16). However, the precise regulation of activities by this kinase has not yet been elucidated. In the present study, we have concentrated on this aspect and identified FtsZ as an interacting partner of PknA. *In vitro* assays with PknA showed its ability to phosphorylate *M. tuberculosis* FtsZ (mFtsZ)[3] protein. In concordance with this observation, our results also indicated that mFtsZ was phosphorylated on co-expression with PknA in *E. coli* cells. Interestingly, we found that the phosphorylation of mFtsZ affected its GTPase activity as well as polymerization ability. Thus, our results, for the first time, established the association of PknA in regulating the functionality of FtsZ, the protein involved in the process of cytokinesis throughout the bacterial lineage.

EXPERIMENTAL PROCEDURES

Materials—Restriction/modifying enzymes were obtained from New England Biolabs. All other fine chemicals including 3,3′,5,5′-tetramethylbenzidine, GTP, glutathione, biotin *N*-hydroxysuccinimide ester, streptavidin-HRP (Sigma), and gluta-

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[3] The abbreviations used are: mFtsZ, *M. tuberculosis* FtsZ; eFtsZ, *E. coli* FtsZ; DAPI, 4′,6-diamino-2-phenylindole; GST, glutathione *S*-transferase; HRP, horseradish peroxidase; MBP, maltose-binding protein; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid.

thione-Sepharose (GE Healthcare) were commercially available. Oligonucleotides used in this study were custom-synthesized (Biobasic Inc. or IDT). [γ-^{32}P]ATP (3000–5000 Ci/mmol) was purchased from Jonaki Laboratories, Board of Radiation and Isotope Technology, Hyderabad, India.

PCR Amplification, Construction of Recombinant Plasmids—Genomic DNA isolated from *E. coli* strain K12 (17) or *M. tuberculosis* strain H37Ra (15) was used for PCR amplification of *ftsZ*. Primers (*E. coli*: CM1, 5′-CATATGTTTGAACCAATGGAACTT-3′ and CM3, 5′-TTAATCAGCTTGCTTACGCAGGA-3′; *M. tuberculosis*: CM5, 5′-CGATGCTCTCAGCGGCGCATGAAG-3′ and CM7, 5′-CATAGACCCCCCCGCACAACTACC-3′) used for this purpose were designed based on the published genome sequences of *E. coli* (b0095; *eftsZ*; 1151 bp; Ref. 18) and *M. tuberculosis* (Rv 2150c; *mftsZ*; 1140 bp; Ref. 19). The *ftsZ* open reading frames were PCR-amplified using the Expand long template PCR system (mixture of *Pwo* and TaqDNA polymerases; Roche Applied Science) following the manufacturer's recommended protocol. Both of the amplified fragments were initially cloned in pUC19 vector (pUC-eFtsZ and pUC-mFtsZ), and nucleic acid sequences were determined using an automated sequencer (Applied Biosystems). These constructs (pUC-eFtsZ/pUC-mFtsZ) were digested with SacI/HindIII, ligated to corresponding sites of pGEX-KG (pGEX-eFtsZ/pGEX-mFtsZ) and subsequently transformed *in E. coli* strain DH5α. Clones containing the genes of interest were confirmed by restriction analysis. The methods adopted for PCR amplification, site-directed mutagenesis, and subsequent cloning in expression vectors of other genes (pMAL-PknA, p19Kpro-PknA, pMAL-K42N, p19Kpro-K42N, and pMAL-PPP) used in this study either were described elsewhere or will be provided on request (15).

Expression and Purification of Recombinant Proteins—The pGEX-eFtsZ/pGEX-mFtsZ were transformed into *E. coli* strain BL21(DE3) for monitoring overexpression and subsequent purification of proteins. Overnight cultures (~15 h at 37 °C in LB broth containing 100 μg/ml ampicillin) were reinoculated and grown to an A_{600} of ~0.6. Cells were then induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside, harvested after 3 h, and suspended in lysis buffer (50 mM Tris, pH 8, containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, and 1 μg/ml leupeptin). Cells were sonicated, and the supernatant fraction was collected after centrifugation (17,600 $\times$ *g* for 15 min at 4 °C). This fraction was loaded onto a glutathione-Sepharose affinity column (GE Healthcare) and eluted with elution buffer (5 mM glutathione, 50 mM Tris, pH 8) following the manufacturer's recommended protocol. The procedure adopted for purification of other proteins used in this study was mentioned earlier (15).

Biotinylation and Solid Phase Protein-Protein Interaction Studies—Purified mFtsZ after dialysis against PBS was incubated (2 h at 25 °C with gentle mixing) with 10-fold molar excess of biotin *N*-hydroxysuccinimide ester. The mixture was again dialyzed (overnight with four changes of buffer) against PBS to remove traces of free biotin and used in interaction studies following estimation of protein (20). In protein-protein interaction studies, MBP-PknA (1 μg/well) was coated in enzyme-linked immunosorbent assay plates and kept for 3 h at 37 °C. After washing with PBS containing 0.5% Tween, blocking buffer (1% bovine serum albumin in PBS containing 0.5% Tween) was added to the wells of the plates and incubated for 1 h at 37 °C. This was followed by washing and incubation (1 h at 37 °C) with different concentrations of biotinylated mFtsZ in PBS. The interaction of mFtsZ with PknA was finally assessed by the addition of streptavidin-HRP (1 μg/ml for 30 min at 37 °C) and detection of the enzyme activity in each well with 3,3′,5,5′-tetramethylbenzidine. Binding of biotinylated mFtsZ with immobilized PknA was assessed in the presence of different concentrations of non-biotinylated mFtsZ or PknA to determine the specificity of interaction. In each case, for controls instead of PknA, the equivalent amount of bovine serum albumin was adsorbed to the wells of microtiter plates.

Kinase Assay—The auto-/transphosphorylation ability of PknA was determined in an *in vitro* kinase assay mentioned elsewhere (15). Briefly, aliquots of PknA (1 μg) with or without mFtsZ were mixed with kinase buffer (50 mM Tris/HCl, pH 7.5, 50 mM NaCl, 10 mM $MnCl_2$), and the reaction was initiated by adding 2 μCi of [γ-^{32}P]ATP. Following incubation at 25 °C for 20 min, 5$\times$ SDS sample buffer was added to stop the reaction. Samples were heated at 90 °C for 5 min and resolved using 8–10% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue, dried in a gel drier (Bio-Rad) at 70 °C for 2 h, and finally analyzed on a phosphorimaging device (Bio. Rad). For monitoring the effect of PPP, the above reaction was incubated at 37 °C for 30 min after the addition of purified MBP-PPP fusion protein. The reaction was terminated by the addition of 5$\times$ sample buffer and subjected to SDS-PAGE.

GTPase Assay—The GTPase activity of the protein (mFtsZ or its phosphorylated form) was assessed in a microtiter plate-based colorimetric assay by determining the ability of the enzyme to liberate inorganic phosphate (P_i) from GTP (21, 22). Briefly, mFtsZ or its phosphorylated form (5 μM) was incubated with increasing concentration of GTP (0–1 mM) in reaction buffer (50 mM Tris, 5 mM $MgCl_2$) at 37 °C for 15 min. The enzymatic reaction was terminated by the addition of an acidic solution (200 μl) of malachite green, ammonium molybdate, and polyvinyl alcohol. The liberated P_i formed phosphomolybdate-malachite complex, and this was detected at 650 nm. The values obtained were corrected by subtracting the blank readings obtained for non-enzymatic release of P_i. A standard curve with sodium phosphate monobasic was run concurrently with each experiment, and GTPase activity (mean $\pm$ S.D.) is expressed as nanomoles of P_i liberated/min/mg of protein.

Western Blotting—Plasmids (p19Kpro and pGEX-KG) harboring different open reading frames (*pknA*, *K42N*, and *ftsZs* from *E. coli*/*M. tuberculosis*) were co-transformed in *E. coli* (strain DH5α for PknA/eFtsZ and BL21(DE3) for PknA/mFtsZ). After dual selection on ampicillin (75 μg/ml) and hygromycin (200 μg/ml) plates, the clones obtained were cultured in LB broth in the presence of both of the antibiotics and induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (37 °C/3 h). Cell extracts obtained were resolved in 10% SDS-PAGE and processed for Western blotting using different primary (anti-PknA, anti-GST-HRP conjugate, and anti-phosphothreonine) and secondary (anti-rabbit IgG-HRP conjugate) antibodies. Finally, blots were developed with ECL detection

system following the manufacturer's (GE Healthcare) recommended protocol.

Light Scattering—The mFtsZ polymerization was measured by light scattering in a fluorescence reader (Molecular Devices, model Gemini EM) with both the excitation and the emission wavelengths set at 600 nm (23). mFtsZ or its phosphorylated form was added to a final concentration of 5 μM to the well of 96-well FLUOTRAC (Greiner) plate, which was then placed (30 °C for 10 min) into the chamber of fluorescence reader to establish a base-line. Following the addition of 1 mM GTP (reaction volume = 100 μl), data were collected for an additional 50 min. The net change in light scattering was determined by subtracting the base line from each reading and plotted as a function of time.

Protein Sedimentation—Purified mFtsZ or its phosphorylated form (5 μM protein/reaction) was incubated with or without 1 mM GTP at 25 °C for 10 min (reaction volume = 100 μl) in polymerization buffer (50 mM sodium MES, pH 6.5, 50 mM KCl, 5 mM $MgCl_2$). This was followed by high speed centrifugation (Beckman TL100 ultracentrifuge) as described elsewhere (9) to separate the supernatant and pellet fractions. Pellets were resuspended (volume = 100 μl), and samples (both supernatant and pellet fractions) were resolved on 10% SDS-PAGE and visualized following staining with Coomassie Brilliant Blue.

Fluorescence Microscopy—Overnight grown *E. coli* DH5α cells transformed with p19Kpro-PknA or p19Kpro were harvested by centrifugation. After washing with PBS, cells were suspended in 1 ml of 70% ethanol for fixation. The fixed cells were collected by centrifugation, washed with PBS, and spread on a glass slide pretreated with poly-lysine. Cells were stained with DAPI solution (1 mg/ml in 50% glycerol) and examined in a fluorescence microscope (Carl Zeiss).

RESULTS

PknA Expression in E. coli Exhibited Elongated Cells with Segregated Nucleoids—We previously reported that constitutive expression of the *pknA* open reading frame of *M. tuberculosis* in *E. coli* resulted in a remarkable elongation of cells (15). There could be two possible arguments in respect to the filamentous morphology of these *E. coli* cells. Firstly, the morphological changes could be a consequence of defect in nuclei replication and segregation, which is the first step in cell division. Secondly, the elongated morphology might be the result of an imperfection in septum formation (cytokinesis), which is the later stage in the process of cell division. To investigate these aspects, we stained the cells with DAPI and visualized them under a confocal microscope. Interestingly, the *E. coli* cells harboring p19Kpro-PknA showed a beaded structure with no sign of constriction (Fig. 1*A*, *right panel*), indicating that the nucleoids were segregating from each other in the filamentous cells. However, cells transformed with p19Kpro vector (Fig. 1*A*, *left panel*) were either mono- or bi-nucleoidal. This led us to believe that the elongated cell morphology, as a result of expression of PknA in *E. coli*, was due to defect in septum formation. Therefore, we hypothesized that PknA might be interacting with proteins involved in the process.

Available literature indicated that FtsZ is the key cell division protein involved in septum formation of bacteria (2). To have an insight on its association with PknA, we co-transformed

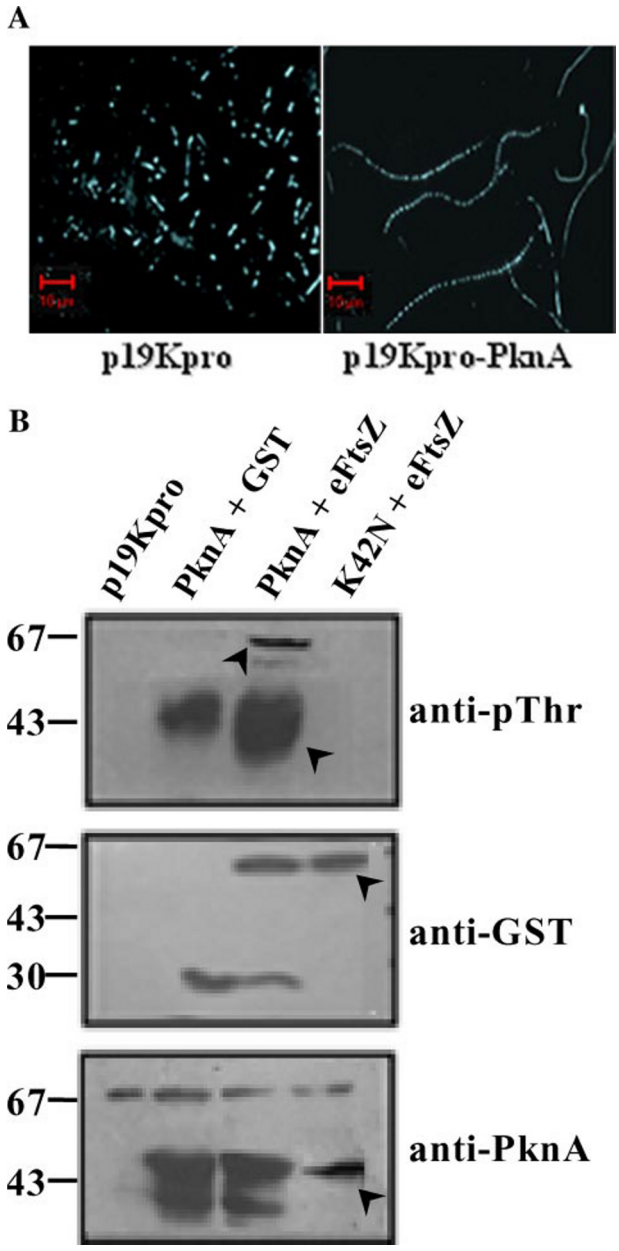

FIGURE 1. **Constitutive expression of *M. tuberculosis* PknA in *E. coli* (*A*) and detection of phosphorylation status of eFtsZ overexpressed in the presence of this kinase (*B*).** *A*, DAPI-stained confocal micrographs of *E. coli* DH5α cells harboring plasmids p19Kpro (*left panel*) and p19Kpro-PknA (*right panel*). The *bar* in each panel indicates magnification. *B*, *in vivo* phosphorylation of eFtsZ by PknA. Immunoblots of cell lysates from *E. coli* cells co-expressing PknA or K42N and eFtsZ, with anti-phosphothreonine (*anti-pThr*; *upper panel*), anti-GST (*middle panel*), and anti-MBP-PknA (*anti-PknA*; *lower panel*) antibodies. *Numbers* indicate position of molecular mass makers in kDa. Expressed proteins (PknA, K42N, and eFtsZ) are denoted with *arrowheads*.

both p19kpro-PknA and pGEX-eFtsZ in *E. coli* strain DH5α. Colonies grown in the presence of both ampicillin and hygromycin were screened. One such colony harboring both of the expression plasmids was selected, cultured in LB broth in the presence of both of the antibiotics, and induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside. Lysates from these cells exhibited co-expression of both PknA and eFtsZ in Western blotting using anti-phosphothreonine (Fig. 1*B*, *upper panel*), anti-GST (Fig. 1*B*, *middle panel*), and anti-PknA (Fig. 1*B*, *lower panel*) antibodies. It is worth mentioning here that both of the

proteins co-expressed (although at different levels) despite using two incompatible plasmids carrying the same replicon, and this was consistent with earlier reports (24).

To visualize the phosphosignal generated by expressed proteins in *E. coli*, we utilized the anti-phosphothreonine antibody, which detects phosphothreonine residues in a protein or peptide largely independent of surrounding amino acids. As expected, the absence of any Ser/Thr protein kinases in *E. coli*, the antibody did not recognize any protein in the lysates prepared from the cells transformed with p19Kpro vector alone (Fig. 1*B*, *upper panel*, *lane 1*). On the other hand, phosphosignals corresponding to PknA but not for GST could be detected in the lysates prepared from cells transformed with p19Kpro-PknA and pGEX-KG (Fig. 1*B*, *upper panel*, *lane 2*). Interestingly, lysates prepared from cells co-expressing both PknA and eFtsZ showed phosphosignal corresponding to both of the proteins (Fig. 1*B*, *upper panel*, *lane 3*). Furthermore, co-expression of K42N, a kinase-dead mutant of PknA (15) with eFtsZ, did not yield any phosphosignal (Fig. 1*B*, *upper panel*, *lane 4*). Expression of all the proteins was confirmed subsequently by using anti-GST or anti-PknA antibodies (Fig. 1*B*, *middle* and *lower panels*). It is noteworthy that in Western blotting using anti-GST antibody, besides recognizing only the GST fusion protein, samples co-expressing PknA and GST-eftsZ exhibited an additional band corresponding to the molecular weight of GST (Fig. 1*B*, *middle panel*, *lane 3*). This, we presume, could be cross-contamination of sample from the adjacent lane (Fig. 1*B*, *middle panel*, *lanes 2* and *3*) and did not seem to be unusual. Additionally, as evident in the Western blot with anti-PknA polyclonal antisera, the expression profiles of PknA and K42N mutant proteins were slightly different (see multiple bands for wild-type protein, whereas a single band was visible for the K42N mutant protein; Fig. 1*B*, *lower panel*, compare *lanes 2* and *3* with *lane 4*). This was noticed in spite of the use of same vector (p19Kpro) for expression of both of the proteins. Such an observation perhaps points toward the difference in the stability of the PknA and K42N mutant proteins. In fact, similar behavior of kinase-dead mutants of other Ser/Thr protein kinases has already been reported (25). We also observed a ~70-kDa band recognized by the anti-PknA antibody in all the samples. We interpret this band as a result of nonspecific recognition of some *E. coli* protein by the anti-PknA antibody. This is certainly not unusual since the antiserum we used in our experiment was not purified. However, such nonspecific recognition of host proteins did not affect the interpretation of our results. Thus, all these lines of evidence established that the PknA phosphorylated the eFtsZ *in vivo*.

mftsZ Is a Substrate of PknA—Our findings with eFtsZ tempted us to envisage the relationship between mFtsZ and PknA. Besides this, analysis of nucleotide-derived amino acid sequences through the Clustal X 1.81 program (26) revealed significant homology (~78.6%) between eFtsZ and mFtsZ. Therefore, it is very likely that both of the proteins would behave similarly. To test this hypothesis, we expressed mFtsZ as a fusion protein with GST in *E. coli* strain BL21(DE3), purified by affinity chromatography (data not shown), and subsequently monitored its binding with purified MBP-PknA (15) *in vitro* (see "Experimental Procedures"). Fig. 2*A* clearly indicates that binding of biotinylated mFtsZ and PknA exhibited a saturation kinetics (half-maximal binding = ~1.2 μg/ml; dissociation constant = 41 ± 8 nM). On the other hand, no detectable binding of mFtsZ could be observed when bovine serum albumin replaced PknA. Both unlabeled PknA and unlabeled mFtsZ effectively inhibited the binding of biotinylated mFtsZ to PknA (Fig. 2*B*). The 50% inhibition of binding was achieved at ~50 and ~80 μg/ml PknA and mFtsZ, respectively. However, no significant inhibition of binding could be detected with MBP-β-galactosidase, further confirming the specificity of the *in vitro* interaction between PknA and mFtsZ.

We have already reported the autophosphorylating activity of PknA as well as its transphosphorylating ability for known exogenous substrates (histone and myelin basic protein) *in vitro* (15). To examine whether mFtsZ could be a substrate of PknA, we performed an *in vitro* kinase assay. Although mFtsZ alone was unable to show any phosphorylation (Fig. 2*C*, *left panel*, *lane 8*), on incubating with MBP-PknA, it was phosphorylated (Fig. 2*C*, *left panel*, *lane 3*). A weak phosphosignal for mFtsZ (Fig. 2*C*, *left panel*, *lane 5*) was noticed when it was added in the reaction after heating (90 °C for 5 min). The transphosphorylation was PknA-specific since no γ-^{32}P incorporation of mFtsZ could be detected when the reaction was carried out with the kinase-dead mutant K42N (Fig. 2*C*, *left panel*, *lane 6*). To rule out the possibility of the involvement of GST fusion tag in the transphosphorylation reaction, we incubated MBP-PknA + GST with [γ-^{32}P]ATP. As expected, we did not see any phosphosignal for GST (Fig. 2*C*, *right panel*, *lane 1*). Furthermore, incubation of MBP + GST with [γ-^{32}P]ATP did not yield any phosphosignal, confirming no contribution of fusion tags in auto-/transphosphorylation reactions (Fig. 2*C*, *right panel*, *lane 2*). It is interesting to mention here that the transphosphorylation of tubulin, the eukaryotic homolog of FtsZ, by cyclin-dependent kinase, Cdk1, in regulating microtubule dynamics during mitosis has recently been documented (27).

Available reports with PPP (renamed as Pstp or Mstp), the only Ser/Thr protein phosphatase in *M. tuberculosis*, indicated its ability to dephosphorylate PknA as well as artificial substrates phosphorylated by this kinase (28, 29). Interestingly, incubation with PPP dephosphorylated the PknA mediated transphosphorylation of mFtsZ (Fig. 2*C*, *left panel*, *lane 4*). However, it needs to be mentioned here that we do not have any evidence that PPP *per se* dephosphorylated mFtsZ. Based on the available report it seems very likely that PPP dephosphorylated PknA, which in turn prevented the transphosphorylation of mFtsZ (29).

At this juncture, it was intriguing to know whether mFtsZ could be a substrate of PknA *in vivo*, and for this, we utilized an *E. coli*-based expression system to co-express both of the proteins. mFtsZ on co-expression with PknA yielded a phosphosignal in Western blotting with anti-phosphothreonine antibody (data not shown), and this result was analogous to that obtained with eFtsZ (Fig. 1*B*). We further purified mFtsZ by affinity chromatography utilizing glutathione affinity resin from the *E. coli* cells harboring both pGEX-mFtsZ and p19Kpro-PknA. As evidenced by the Western blotting using anti-phosphothreonine antibody, the purified protein was phosphorylated (Fig. 2*D*, *upper panel*). In contrast, the mFtsZ protein purified from *E. coli* cells

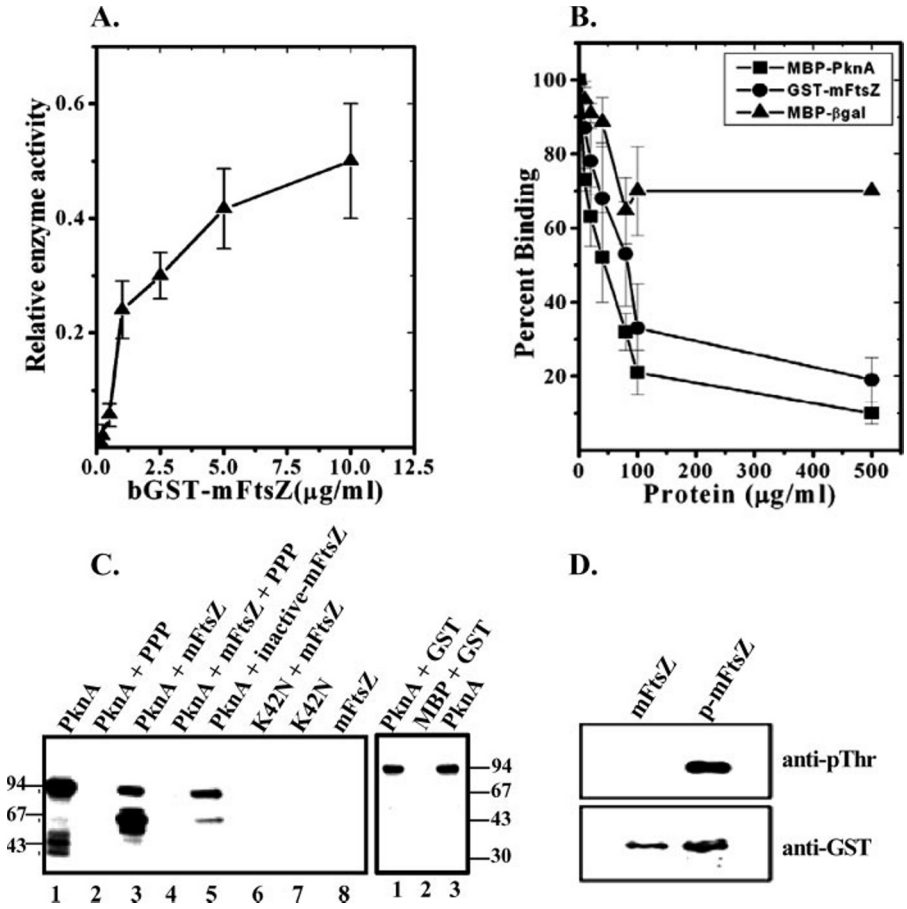

FIGURE 2. **Interaction of mFtsZ and PknA.** *A*, binding of mFtsZ with PknA. Recombinant MBP-PknA (1 μg/well) was coated on enzyme-linked immunosorbent assay plates, washed, and incubated in the presence of the indicated concentrations of biotinylated mFtsZ (bGST-mFtsZ) as described under "Experimental Procedures." Following the addition of streptavidin-HRP, enzyme activity of each well was monitored to quantitate binding. The relative enzyme activity was calculated after subtracting the blank (well coated with 1 μg of bovine serum albumin) and expressed as mean ± S.D. from three independent experiments. *B*, competition of PknA-mFtsZ binding. The binding of biotinylated mFtsZ (1 μg/well) with immobilized PknA was determined. The HRP activity in the absence of any competitor was taken as 100%, and the results (mean ± S.D., $n = 3$) are expressed as the percentage of biotinylated mFtsZ bound to PknA. *C*, *in vitro* phosphorylation of mFtsZ by PknA. *Left panel*, GST-mFtsZ was incubated (25 °C for 20 min) with MBP-PknA or K42N in kinase buffer along with [γ-^{32}P]ATP. The reaction was stopped by adding 5× SDS buffer, and samples were resolved in 8% SDS-PAGE and processed for autoradiography as described under "Experimental Procedures." For monitoring the dephosphorylating ability of PPP, it was added to the above reaction and incubated further at 37 °C for 30 min and then processed for autoradiography. *Right panel*, GST + PknA or GST + MBP following incubation (25 °C for 20 min) with [γ-^{32}P]ATP were resolved in 10% SDS-PAGE and processed for autoradiography as mentioned above. The position of molecular mass markers (in kDa) is indicated. The *lane numbers* are shown at the *bottom*. *D*, *in vivo* phosphorylation of mFtsZ by PknA. Purified mFtsZ protein and its phosphorylated form (*p-mFtsZ*) were immunoblotted as described under "Experimental Procedures" using anti-phosphothreonine (*anti-pThr*; *upper panel*) and anti-GST (*lower panel*) antibodies.

transformed with only pGEX-mFtsZ did not show any phosphorylation (Fig. 2*D*, *upper panel*). As expected, mFtsZ and its phosphorylated form were recognized by anti-GST antibody (Fig. 2*D*, *lower panel*). Thus, our results unambiguously established the interaction between these two proteins and argued in favor of mFtsZ being one of the substrates of PknA.

Phosphorylation of mFtsZ Affected Its Functionality—FtsZ protein is known to possess GTPase activity (7). To know the effect of phosphorylation on the GTP-hydrolyzing ability of mFtsZ, it was incubated with increasing concentrations of substrate (0–1 mM). Although the mFtsZ exhibited a typical Michaelis-Menten curve ($K_m = 42 \pm 9$ μM; $V_{max} = 7 \pm 1$ nmol/min/mg of protein, $K_{cat}/K_m = 193 \pm 25$/M/sec; see also Ref. 30), the phosphorylated protein showed hardly any activity (Fig. 3*A*). When compared with the mFtsZ, phosphorylated protein did not exhibit any detectable phosphate-liberating ability even at increasing protein concentrations.

It is well known that mFtsZ has the ability to polymerize in GTP-dependent manner (8). We further examined the polymerization of phosphorylated mFtsZ in the presence of GTP. Interestingly, in a situation when the mFtsZ showed an increase in light scattering on the addition of GTP, the phosphorylated protein did not show any significant changes (Fig. 3*B*). We further confirmed the GTP-dependent polymerization behavior of mFtsZ and its phosphorylated form by sedimentation at high speed to separate large polymers from soluble protein. Following high speed centrifugation, proteins (mFtsZ and its phosphorylated form) were visualized in both the supernatant and the pellet fractions by Coomassie Brilliant Blue staining (Fig. 3*C*), and we confirmed the identity by immunoblotting with anti-GST antibody (data not shown). Interestingly, mFtsZ was localized in the pellet fraction after the addition of GTP. On the other hand, there was no difference in the partitioning of the phosphorylated form of mFtsZ protein in the presence or absence of GTP (Fig. 3*C*). Thus, our results argued that phosphorylation of mFtsZ impaired its functionality.

DISCUSSION

FtsZ is a crucial molecule in bacterial cell division. It initiates Z-ring formation and congregates several other proteins to form a septosomal complex. The interacting proteins and the sequence of events that occur during the differentiation of cell division apparatus have already been identified in different bacteria (31). Despite uniformity, subtle differences do exist in the mechanism of regulation, presumably to match the diversity in bacterial cell shapes and life cycles. As a matter of fact, several FtsZ-interacting proteins have been reported to be unique to the particular bacterial species (32). Studies on the interacting partners of FtsZ in mycobacteria are still in their infancy. Except for the direct association as well as co-localization of FtsW, no other report is available to date in mycobacteria (16, 17). In the present study, we, therefore, focused on FtsZ from *M. tubercu-*

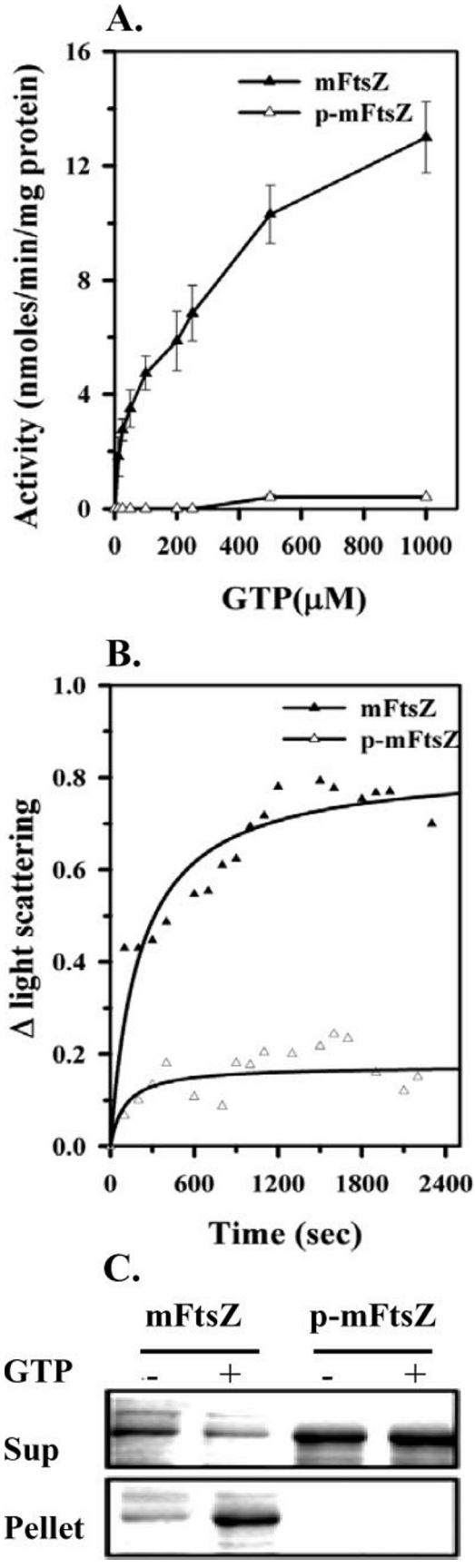

FIGURE 3. **Functionality of mFtsZ and its phosphorylated form.** *A*, the GTPase activities of mFtsZ and its phosphorylated form (*p-mFtsZ*) were assayed (5 μM protein/reaction) at 37 °C with the indicated amounts of substrate. The specific activity in nmol of P_i liberated/min/mg of protein (mean ± S.D., $n = 3$) is plotted against the substrate concentration. *B*, comparison of the polymerization abilities of mFtsZ and p-mFtsZ. Purified mFtsZ and p-mFtsZ proteins were incubated in 50 mM MES-NaOH, pH 6.5, 5 mM $MgCl_2$ in fluorescence plate at 30 °C. The light scattering of samples was monitored at 600 nm as described under "Experimental Procedures" prior (to obtain a base line) and after the addition of 1 mM GTP. The net change in light scattering is plotted against time of polymerization. *C*, sedimentation profile of both mFtsZ and p-mFtsZ proteins after polymerization in the presence and absence of GTP. Samples, both supernatant (*Sup*) and pellet, obtained following high speed centrifugation were subjected to SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

losis and its interaction with a signaling protein, PknA, a eukaryotic-type Ser/Thr kinase in mycobacteria, which has been reported to regulate morphological changes associated with cell division process (15, 16).

We observed that constitutive expression of PknA in *E. coli* led to a significant elongation of cells (Fig. 1*A*) (Ref. 15). The presence of multiple nucleoids in these filamentous cells with no signs of cellular constriction, as visualized by DAPI staining, argued that the defect was not in the DNA replication or segregation; indeed, the morphology seems to be a consequence of aberrant septum formation (Fig. 1*A*). A similar filamentous nature of *E. coli* cells has been reported during under- or overproduction of FtsZ, indicating that a critical concentration of FtsZ was necessary for perfect septum formation (33). We found eFtsZ was phosphorylated *in vivo* in the presence of PknA (Fig. 1*B*); therefore, it was logical to contemplate the association of eFtsZ with PknA. One can definitely argue about the specificity or relevance of such an interaction. However, the alteration of cell shape, which is the initial event in bacterial cell division, involves ordered assembly of proteins that are fairly conserved among prokaryotes (4). In fact, eFtsZ exhibited more than 78% amino acid sequence homology with mFtsZ. Additionally, eFtsZ is not a basic protein (predicted pI = 4.5), so PknA would nonspecifically phosphorylate it (15). These findings led us to investigate the association of PknA and mFtsZ. Our results with the *in vitro* interaction studies convincingly established that PknA interacted with mFtsZ and phosphorylated the latter (Fig. 2, *A–C*).

The *FtsZ* has been shown to be an essential gene in *M. tuberculosis* and *Mycobacterium smegmatis* (34, 35). An analogous condition was observed with PknA in mycobacteria, resulting in a difficulty in obtaining a null background to study *in vivo* interactions between these two proteins. Furthermore, the presence of the Ser/Thr phosphatase, PPP, in mycobacteria might obstruct the identification of *in vivo* phosphorylation of FtsZ (28, 29). In such a scenario, to study the interaction and *in vivo* phosphorylation of these two proteins (PknA and mFtsZ), we tried to utilize an *E. coli* expression system where intrinsic PknA and PPP are absent. In Western blotting using anti-phosphothreonine antibody as a probe, we found a strong phosphosignal corresponding to mFtsZ when co-expressed with PknA (Fig. 2*D*). Thus, this observation together with our *in vitro* studies certainly validated mFtsZ as a substrate of PknA. Recently, a cell division protein, Wag31, has been identified as a substrate of PknA (16). Since kinases could have multiple substrates (36), the identification of mFtsZ as another substrate perhaps indicated multiplicity in the function of PknA.

We purified phosphorylated mFtsZ and characterized the protein. Surprisingly, when compared with the mFtsZ, its phosphorylated form showed negligible GTPase activity (Fig. 3*A*) and significantly reduced polymerization ability (Fig. 3*B*). This might have happened due to alteration in the conformation of the mFtsZ as a result of phosphorylation (37). Conversely, the phosphorylation of mFtsZ could have affected the functionality of the nucleotide-binding loop of the protein, and as a result, GTP was not able to bind (38).

PknA, when overexpressed in *M. smegmatis* or *Mycobacterium bovis*, formed long, broad, and in some cases, branched bacilli, which appeared to be a consequence of incomplete septation (16). Intriguingly, our studies in *E. coli* also point toward a similar function of PknA (Fig. 1). Thus, phosphorylation and interaction of PknA with mFtsZ provide a possible explanation for such an alteration. It is very likely that the overexpression of PknA might have phosphorylated mFtsZ, which in turn inactivated the protein, and thus, affected septum formation, leading to alteration in the morphology of the bacilli. However, our observation of dephosphorylation of PknA by PPP, and as a result, inhibition of transphosphorylation of mFtsZ also needs to be mentioned here. It seems that PPP could play a role in maintaining PknA, and in turn, mFtsZ in correct phosphorylated form. Thus, the phosphorylation of mFtsZ by PknA may provide an insight into the regulation of septum formation in *M. tuberculosis* in a situation where no known regulators of septation are identified as yet (39).

In conclusion, our results provide, for the first time, evidence for the phosphorylation of FtsZ in any bacteria and involvement of a signaling protein in the process of septum formation in *M. tuberculosis*. In fact, demonstration of the direct interaction and phosphorylation of FtsZ by PknA of *M. tuberculosis* marks the beginning. Further work needs to be carried out to understand the exact mechanism.

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REFERENCES

1. Rothfield, L. I., and Justice, S. S. (1997) *Cell* **88,** 581–584
2. Weiss, D. S. (2004) *Mol. Microbiol.* **54,** 588–597
3. Vicente, M., Rico, A. I., Martinez-Arteaga, R., and Mingorance, J. (2006) *J. Bacteriol.* **188,** 19–27
4. Errington, J., Daniel, R. A., and Scheffers, D. (2003) *Microbiol. Mol. Biol. Rev.* **67,** 52–65
5. Bi, E. F., and Lutkenhaus, J. (1991) *Nature* **354,** 161–164
6. Erickson, H. P. (1997) *Trends Cell Biol.* **7,** 362–367
7. RayChaudhuri, D., and Park, J. T. (1992) *Nature* **359,** 251–254
8. Romberg, L., Simon, M., and Erickson, H. P. (2001) *J. Biol. Chem.* **276,** 11743–11753
9. Mukherjee, A., and Lutkenhaus, J. (1998) *EMBO J.* **17,** 462–469
10. White, E. L., Ross, L. J., Reynolds, R. C., Seitz, L. E., Moore, G. D., and Borhani, D.W. (2000) *J. Bacteriol.* **182,** 4028–4034
11. Leung, A. K. W., White, E. L., Ross, L. J., Reynolds, R. C., DeVito, J. A., and Borhani, D. W. (2004) *J. Mol. Biol.* **342,** 953–970
12. Leung, A. K., White, E. L., Ross, L. J., and Borhani, D. W. (2000) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **56,** 1634–1637
13. Datta, P., Dasgupta, A., Bhakta, S., and Basu, J. (2002) *J. Biol. Chem.* **277,** 24983–24987
14. Rajagopalan, M., Maloney, E., Dziadek, J., Poplawska, M., Lofton, H., Chauhan, A., and Madiraju, M. V. (2005) *FEMS Microbiol. Lett.* **250,** 9–17
15. Chaba, R., Raje, M., and Chakraborti, P. K. (2002) *Eur. J. Biochem.* **269,** 1078–1085
16. Kang, C. M., Abbott, D. W., Park, S. T., Dascher, C. C., Cantley, L. C., and Husson, R. N. (2005) *Genes Dev.* **19,** 1692–1704
17. Sambrook, J., Fritsch, E. F., and Manitis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
18. Blattner, F. R., Plunkett, G., Bloch, C. A., Perna, N. T., Burland, D., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrik, H. A., Goeden, M. A., Rose, D. J., Mau, B., and Shao, Y. (1997) *Science* **277,** 1453–1474
19. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., III, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) *Nature* **393,** 537–544
20. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
21. Sarin, J., Aggarwal, S., Chaba, R., Varshney, G. C., and Chakraborti, P. K. (2001) *J. Biol. Chem.* **276,** 44590–44597
22. Henkel, R., Vandeberg, J., and Walsh, A. (1988) *Anal. Biochem.* **169,** 312–318
23. Mukherjee, A., and Lutkenhaus, J. (1999) *J. Bacteriol.* **181,** 823–832
24. Yang, W., Zhang, L., Lu, Z., Tao, W., and Zhai, Z. (2001) *Protein Expression Purif.* **22,** 472–478
25. Beilina, A., Brug, M. V. D., Ahmad, R., Kesavapany, S., Miller, D. W., Petsko, G. A., and Cookson, M. R. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 5703–5708
26. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res.* **25,** 4876–4882
27. Fourest-Lieuvin, A., Peris, L., Gache, V., Garcia-Saez, I., Juillan-Binard, C., Lantez, V., and Job, D. (2006) *Mol. Biol. Cell* **17,** 1041–1050
28. Boitel, B., Ortiz-Lombardia, M., Duran, R., Pompeo, F., Cole, S. T., Cervenansky, C., and Alzari, P. M. (2003) *Mol. Microbiol.* **49,** 1493–1508
29. Chopra, P., Singh, B., Singh, R., Vohra, R., Koul, A., Meena, L. S., Koduri, H., Ghildiyal, M., Deol, P., Das, T. K., Tyagi, A. K., and Singh, Y. (2003) *Biochem. Biophys. Res. Commun.* **11,** 112–120
30. Rajagopalan, M., Atkinson, M. A. L., Lofton, H., Chauhan, A., and Madiraju, M. V. (2005) *Biochem. Biophys. Res. Commun.* **331,** 1171–1177
31. Liu, Z., Mukherjee, A., and Lutkenhaus, J. (1999) *Mol. Microbiol.* **31,** 1853–1856
32. Ishikawa, S., Kawai, Y., Hiramatsu, K., Kuwano, M., and Ogasawara, N. (2006) *Mol. Microbiol.* **60,** 1364–1380
33. Addinall, S. G., Bi, E., and Lutkenhaus, J. (1996) *J. Bacteriol.* **178,** 3877–3884
34. Dziadek, J., Madiraju, M. V., Rutherford, S. A., Atkinson, M. A., and Rajagopalan, M. (2002) *Microbiology* (*Reading*) **148,** 961–971
35. Dziadek, J., Rutherford, S. A., Madiraju, M. V., Atkinson, M. A., and Rajagopalan, M. (2003) *Microbiology* (*Reading*) **149,** 1593–1603
36. Yoon, S., and Seger, R. (2006) *Growth Factors* **24,** 21–44
37. Margalit, D. N., Romberg, L., Mets, R. B., Hebert, A. M., Mitchison, T. J., Kirschner, M. W., and RayChaudhuri, D. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 11821–11826
38. Cordell, S. C., Robinson, E. J., and Lowe, J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 7889–7894
39. Hu, Z., and Lutkenhaus, J. (2000) *J. Bacteriol.* **182,** 3965–3971