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Phosphorylation-dependent interaction between a serine/threonine kinase PknA and a putative cell division protein Wag31 in *Mycobacterium tuberculosis*

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SUMMARY

Mycobacterium tuberculosis genome contains eleven serine/threonine protein kinases (STPKs). Among these STPKs, PknA is a key component of signal transduction pathway that regulates cell shape and possibly cell division in *M. tuberculosis* via reversible phosphorylation of intracellular proteins. The *in vitro* peptide library screen showed that Wag31, a putative cell division protein, was a new substrate phosphorylated by PknA. The signal transduction pathway involving Wag31 and PknA plays a unique role in *M. tuberculosis* growth regulation that may participate in the pathogenesis of tuberculosis. In this study, genes of PknA, wild-type Wag31 (Wag31WT), phosphoablative Wag31T73A, and phosphomimetic Wag31T73E were cloned and expressed. Far-western analyses were performed using partial purified PknA and completely purified Wag31 proteins (Wag31WT, Wag31T73A, and Wag31T73E). Far-western analysis data revealed that the direct interaction between PknA and Wag31 is dependent on the phosphorylation state of Wag31, which can represent a novel target for the development of new anti-tuberculosis drugs.

KEY WORDS: Mycobacterium tuberculosis, PknA, Wag31, Phosphorylation-dependent, Protein-protein interaction, Far-western blotting.

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INTRODUCTION

Tuberculosis (TB) is a major health problem with a high mortality worldwide (WHO, 2012). Most infected persons never develop active disease. However, in approximately 10% of infected subjects, reactivation of latent infection results in active tuberculosis (Flynn and Ernst, 2000). *Mycobacterium tuberculosis* is a resilient organism that can adapt to a wide array of environmental conditions, making it a successful human pathogen (Wayne and Hayes, 1996; Manabe and Bishai, 2000). Protein phosphorylation is the principal mechanism by which extracellular signals are transmitted to cause changes in gene expression. The main phosphorylation sites known in eukaryotes are on tyrosine and serine/threonine residues (Hanks *et al*., 1998). The first eukaryotic-like protein serine/threonine protein kinase (STPK) identified in a prokaryote was discovered in *Myxococcus xanthus* (Munoz-Dorado *et al*., 1991). The completion of the *M. tuberculosis* genome sequencing project showed that this pathogen contains eleven STPK-encoding genes, including *pknA* and *pknB*

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(Cole *et al*., 1998; Av-Gay and Everett, 2000). We previously showed that PknA and PknB are key components of a signal transduction pathway regulating cell morphology (Kang *et al*., 2005). One substrate of PknA we identified is Wag31, a homolog of the cell-division protein DivIVA in other Gram-positive bacteria (Cha and Stewart, 1997; Flardh, 2003; Kang *et al*., 2005; Kang *et al*., 2011).

We demonstrated that Wag31 is localized to the cell poles. We showed that *wag31* is an essential gene and that depletion of its product causes a dramatic morphological change in which one end of the cell becomes round rather than rod-shaped. These results indicate that Wag31 regulates cell shape and cell wall synthesis in *M. tuberculosis* through a molecular mechanism by which the activity of Wag31 can be modulated in response to environmental signals (Kang *et al*., 2008). We showed that the differential growth caused by the expression of different *wag31* alleles (wild-type, phosphoablative, and phosphomimetic) is due to dissimilar nascent peptidoglycan biosynthesis. We further showed that the phosphorylation state of Wag31 is important for protein-protein interaction between the Wag31 molecules, and thus, for its polar localization (Jani *et al*., 2010). However, it is unknown whether the phosphorylation of Wag31 changes its interaction with PknA.

Here, we report the cloning, expression, and purification of *pknA* and three different *wag31* alleles as a fusion with maltose-binding protein (MBP) gene. Furthermore, Far-western blotting determined whether the phosphorylation state of Wag31 affects the direct interaction between PknA and Wag31.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *Escherichia coli* TOP10 and plasmid-containing *E. coli* strains were grown in Luria-Bertani medium (Difco, Detroit, MI, USA). *E. coli* strains were grown at 37°C. Media were solidified with 1.5% (wt/vol) agar as needed. Antibiotics were added as required at the following final concentrations: ampicillin, 100 μg/mL, kanamycin, 50 μg/mL.

Plasmid construction of pET-MBP vector

Maltose-binding protein (MBP) gene without His-tag was amplified by polymerase-chain reaction (PCR) using pMCSG19 (Table 1) as template and the NdeI-MBP-F and BamHI-MBP-R primers.

All primer sequences are shown in Table 1. The PCR products were cleaved with *Nde*I and *BamH*I, and then ligated with the *Nde*I/*BamH*I backbone of pET30a(+) (Novagen, Madison, WI, USA) to construct the corresponding MBP fusion vector. The sequence of clones was confirmed by DNA sequencing.

Recombinant protein expression and purification

To express the kinase domain of PknA as N-terminal MBP fusion protein, the pGEX-PknA (Table 1) was double-digested with *BamH*I and *Not*I, then *pknA* gene was purified using agarose gel electrophoresis and QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA). Purified *pknA* gene was inserted into the expression vector, pET-MBP digested with the same DNA restriction enzymes, to produce the pET-MBP-PknA plasmid. After verifying the DNA sequence, the plasmid DNA was transformed into *E. coli* strain BL21(DE3) for the overexpression of MBP-PknA.

The transformants were grown in LB medium containing 50 μg/mL kanamycin to an OD_{600} of 0.5 at 37°C. The expression of MBP-PknA was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 16 h at 20°C. Cells were harvested by centrifugation at 5,000 *g* (Hanil, Seoul, Korea) for 10 min at 4°C. The resulting cell pellet was resuspended with ice-cold 10 mM Tris-HCl buffer (pH 7.0) and homogenized with a sonicator (Sibata, Saitama, Japan). The crude lysate was centrifuged at 20,000 *g* (Hanil) for 30 min at 4°C and the clarified supernatant was loaded onto an MBPTrap™ HP column (GE Healthcare, Piscataway, NJ, USA) equilibrated with binding buffer (20 mM Tris-HCl, 200 mM NaCl, and 1 mM EDTA pH 7.4). MBP-PknA was eluted with the same buffer containing 10 mM maltose. Eluted fractions of

MBP-PknA were pooled and concentrated to a volume of approximately 1 mL using a VIVA 20 (Sartorius, Goettingen, Germany).

Wag31WT, Wag31T73A, and Wag31T73E were amplified by PCR using primers (LIC-Wag31-F and LIC-Wag31-R) and templates (pET-Wag31WT, pET-Wag31T73A, and pET-Wag31T73E; Table 1), and PCR products were cloned into pMCSG19 according to the ligation-independent procedure (Stols *et al*., 2002; Doneelly *et al*., 2006). Then the produced recombinant plasmids (pMCSG19-Wag31WT, pMCSG19-Wag31T73A, and pMCSG19-Wag31T73E) were transformed into the *E. coli* strain BL21(DE3) containing the plasmid pRK1037 (Table 1). Transformants were isolated on LB plates containing ampicillin and kanamycin. Isolated transformants were grown at 37°C in LB media containing ampicillin and kanamycin to an OD_{600} of 0.5 at which time the temperature was dropped to 16°C and protein synthesis was induced by addition of 0.5 mM IPTG. The cells were incubated at 16 h, harvested and resuspended with ice-cold 10 mM Tris-HCl buffer (pH 7.0), and homogenized with sonication (Sibata). The insoluble cellular materials were removed by centrifugation at 20,000 *g* (Hanil) for 30 min at 4°C. The His_6-Wag31WT, His_6-Wag31T73A, and His_6-Wag31T73E were purified using a His-Bind column (Novagen) and a gel permeation column (HiLoad 16/60 Superdex™ 200 prep grade, GE Healthcare). Eluted fractions of His_6-Wag31WT, His_6-Wag31T73A, and His_6-Wag31T73E were pooled and concentrated to a volume of approximately 1 mL by using a VIVA 20. The homogeneity of the purified proteins was analyzed via SDS-PAGE (Sambrook *et al*., 1989).

TABLE 1 - *Strains, plasmids, and primers used in this study.*

Strains, plasmids, or primers	*Phenotype, genotype, and/or characteristics*	*Reference*
Strains		
E. coli TOP10	F⁻ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *recA1* *araD139* Δ(ara- *leu)7697 gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
E. coli BL21(DE3)	F⁻ *ompT* $hsdS_B$ ($r_B^-m_B^-$) *gal dcm* (DE3)	Invitrogen
Plasmids		
pET30a(+)	Expression vector, $kanamycin^R$	Novagen
pMCSG19	Expression vector, $ampicillin^R$	Doneelly *et al*. (2004)
pRK1037	Vector producing tobacco vein mottling virus (TVMV) protease gene, $kanamycin^R$	Nallamsetty *et al*. (2004)
pGEX-PknA	pGEX-4T-3 containing *pknA* from *Mycobacterium tuberculosis* H37Rv	Kang *et al*. (2005)
pET-Wag31WT	pET28a(+) containing *wag31WT* from *Mycobacterium tuberculosis* H37Rv	Kang *et al*. (2005)
pET-Wag31T73A	pET28a(+) containing *wag31T73A* mutated from pET-Wag31WT	Kang *et al*. (2005)
pET-Wag31T73E	pET28a(+) containing *wag31T73E* mutated from pET-Wag31WT	Kang *et al*. (2005)
pET-MBP	MBP fusion vector, $kanamycin^R$	This study
pET-MBP-PknA	pET-MBP containing *pknA* from pGEX-PknA	This study
pMCSG19-Wag31WT	pMCSG19 containing *wag31WT* from pET-Wag31WT	This study
pMCSG19-Wag31T73A	pMCSG19 containing *wag31T73A* from pET-Wag31T73A	This study
pMCSG19-Wag31T73E	pMCSG19 containing *wag31T73E* from pET-Wag31T73E	This study
Primers		
NdeI-MBP-F	5'– TCATATGAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAA CGGCGAT–3'	This study
BamHI-MBP-R	5'–CATTGTACTGGATCCTTCCGAGCCTGCTTTTTTGTACA–3'	This study
LIC-Wag31-F	5'–TACTTCCAATCCAATGCGATGCCGCTTACACCTGCC– 3'	This study
LIC-Wag31-R	5'–TTATCCACTTCCAATGCTAGTTTTTGCCCCGGTTGA–3'	This study

R: resistant.

Western blotting analyses

Standard western blotting analyses were performed using 15% SDS-PAGE gels and 1 µg each of MBP-PknA (prey protein), BSA, MBP, His_6-Wag31WT, His_6-Wag31T73A, and His_6-Wag31T73E. Samples were immobilized on a nitrocellulose membrane (Whatman, Dassel, Germany). Standard western blots were developed using anti-His_6 monoclonal antibody (Clontech, Mountain view, CA, USA). Goat anti-mouse IgG horseradish peroxidase (HRP) was used as secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To know the phosphorylation state of purified His_6-Wag31WT, standard western blots immobilized with purified proteins were developed using an anti-phospho-(S/T)Q antibody (Cell Signaling Technology, Boston, MA, USA).

To detect phosphorylation-dependent interaction between PknA and different type(s) of Wag31 (Wag31WT, Wag31T73A, or Wag31T73E) *in vitro*, Far-western blotting analyses (Wu *et al*., 2007) were performed. MBP-PknA was used as a "prey" protein, whereas purified His_6-Wag31WT, His_6-Wag31T73A, and His_6-Wag31T73E were the "bait" proteins for Far-western analyses. Far-western analyses were performed using 15% SDS-PAGE gels and 1 µg each of MBP-PknA (prey protein), BSA, and MBP. Samples were immobilized on nitrocellulose membrane (Whatman, Dassel, Germany). Far-western analyses were performed according to the method of Wu *et al*. (2007). Briefly, 5 µg of the bait proteins (His_6-Wag31WT, His_6-Wag31T73A, and His_6-Wag31T73E) were incubated with the nitrocellulose membrane immobilized with the prey protein (MBP-PknA). And then Far-western blot was incubated with anti-His_6 monoclonal antibody and the secondary antibody to detect MBP-PknA bound to His_6-Wag31WT, His_6-Wag31T73A, or/and His_6-Wag31T73E.

RESULTS

In our study, *in vitro* kinase assays were performed using wild-type Wag31WT and variant forms in which the T73 residue of the TQ motif was altered to A or S. Immunoblotting performed with the phospho-(S/T)Q antibody

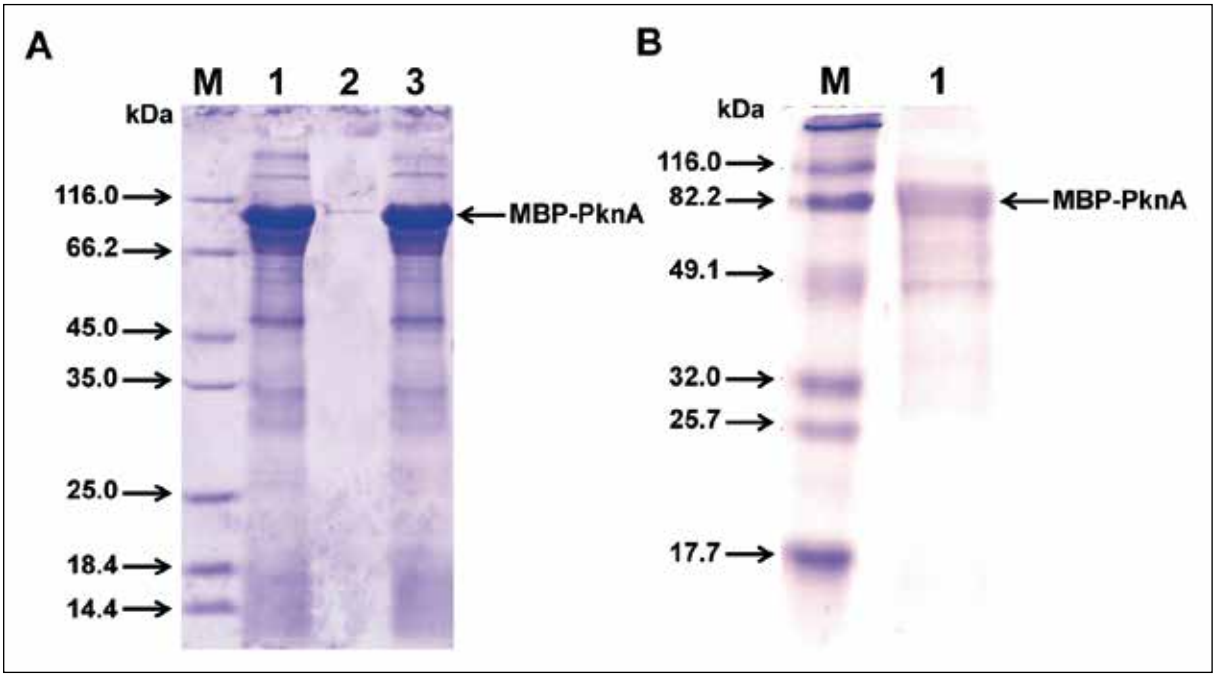


FIGURE 1 - *Soluble expression (A) and partial purification (B) of MBP-PknA. A: Lane 1, cell lysate; lane 2, pellet; and lane 3, supernatant fractions after sonication. B: Lane 1, MBP-PknA eluted from an MBPTrap™ HP column. Lane M, molecular mass standards.*

showed that PknA catalysed the TQ-specific phosphorylation of Wag31WT, but not of the two altered forms (Kang *et al*., 2005). As a next step, we wanted to know if phosphorylation of Wag31 changes its interaction with PknA.

First, MBP (maltose-binding protein) tag was used to obtain the soluble form of PknA. MBP tag is often used to improve protein solubility as reported previously (Kapust and Waugh, 1999; Braun *et al*., 2002; Fox and Waugh, 2003; Dayson *et al*., 2004; Jeon *et al*., 2005). Insertion of the gene encoding MBP, amplified by PCR from the pMCSG19 (Table 1), into the leader sequence encoding region of pET30a(+) gave pET-MBP (without His-tag). We expressed the MBP-PknA in *E. coli* as a fusion protein with MBP at its N-terminus. Elution of the fusion protein from MBPTrap™ HP column followed by SDS-PAGE and staining with Coomassie brilliant blue revealed a protein at approximately 79 kDa (Figures 1A and 1B). The prey protein (such as MBP-PknA) in Far-western analyses do not need to be purified as a single band. Generally, the prey proteins are proteins in a cell lysate (Wu *et al*., 2007).

A single band of Wag31WT, Wag31T73A, and Wag31T73E (expressed in *E. coli* BL21(DE3)) was obtained using His_6-tag (left panel of Figure 2), confirmed by standard western blotting using anti-His_6 monoclonal antibody (right panel of Figure 2).

To test if phosphorylation of Wag31 changes its interaction with PknA, Far-western blotting analysis was performed. First, SDS-PAGE analysis was performed to identify the position of the prey protein (MBP-PknA), MBP, and BSA (Figure 3). Second, standard western blotting was performed on the same set of SDS-PAGE, using anti-His_6 monoclonal antibody (mAb). Standard western blotting analyses showed that

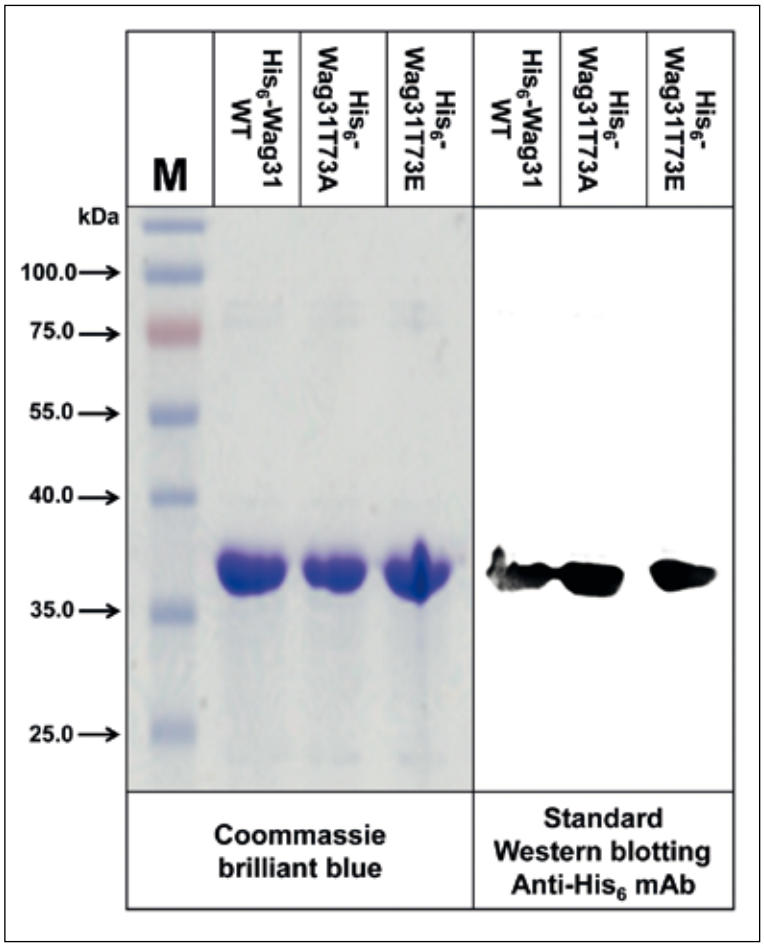
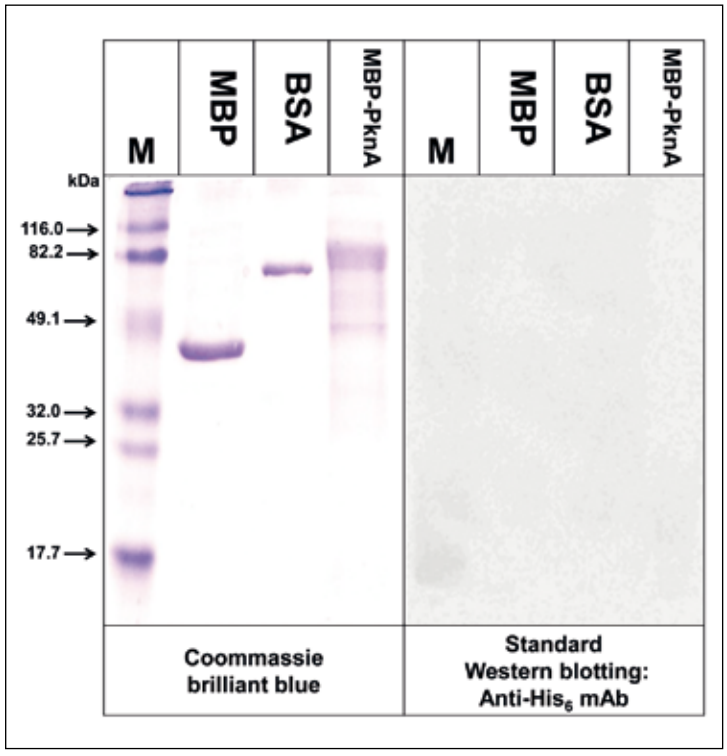
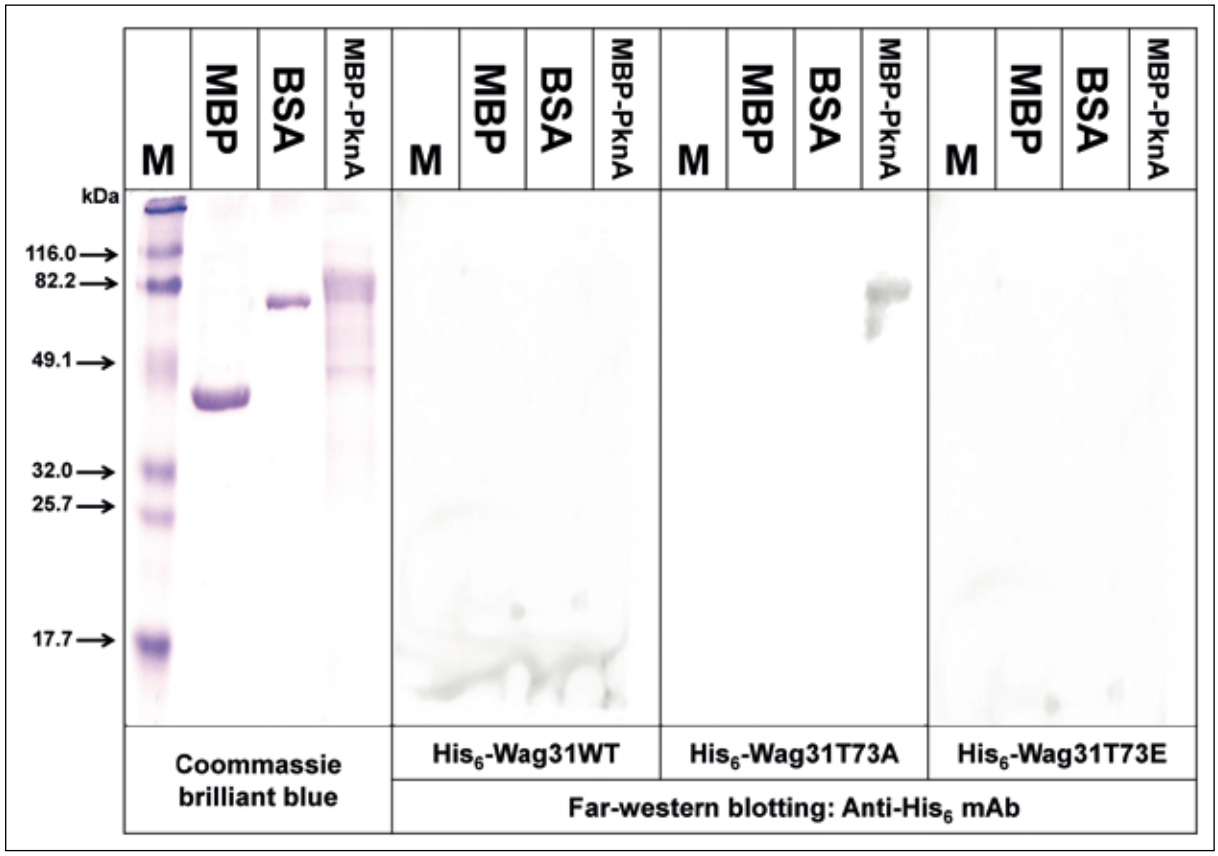


FIGURE 2 - *SDS-PAGE (left panel) and standard western blotting analyses (right panel) of purified His_6-Wag31WT, His_6-Wag31T73A, and His_6-Wag31T73E. Three proteins purified from soluble fractions were separated on 15% SDS-PAGE. The gel was transferred onto a nitrocellulose membrane and probed with an anti-His_6 monoclonal antibody (mAb). Lane M, molecular mass standards.*



◀ FIGURE 3 - *SDS-PAGE (left panel) and standard western blotting analyses (right panel) of maltose-binding protein (MBP) and MBP-PknA. Each protein was subjected to standard western blotting with an anti-His_6 mAb. BSA (bovine serum albumin) serves as a negative control. Lane M, molecular mass standards.*

FIGURE 4 - *Far-western blotting analyses. MBP, MBP-PknA, and BSA were separated by SDS-PAGE (the first panel) and transferred onto nitrocellulose membranes. Proteins were denatured, renatured and then incubated with purified His_6-Wag31WT (the second panel), His_6-Wag31T73A (the third panel), and His_6-Wag31T73E (the fourth panel). After washing, bound proteins were detected with an anti-His_6 mAb. MBP and BSA serve as negative controls. Lane M, molecular mass standards.* ▼



there were no bands for any proteins, eliminating the possibility of nonspecific anti-His_6 mAb interactions (Figure 3). Finally, membranes containing MBP, BSA, and MBP-PknA were incubated with purified bait proteins (His_6-Wag31WT, His_6-Wag31T73A, and His_6-Wag31T73E) and then immunoblotted with anti-His_6 mAb.
As shown in Figure 4, the anti-His_6 mAb bound at the same position as MBP-PknA in case of His_6-Wag31T73A lacking phosphorylation of Wag31. Importantly, no bands were detected from the case of His_6-Wag31T73E mimicking the phosphorylation state (Figure 4). To examine the reason for no band in the case of His_6-Wag31WT, immunoblotting was performed with purified His_6-Wag31WT and a phospho-(S/T)Q antibody, which showed that the purified His_6-Wag31WT was phosphorylated (data not shown).

DISCUSSION

Among eukaryotic-like protein serine/threonine protein kinases (STPKs), PknA is a key component of signal transduction pathway regulating cell shape and possibly cell division in *M. tuberculosis* via reversible phosphorylation of intracellular proteins (Chaba *et al*., 2002). In this work we determined that the phosphorylation state of Wag31 affects the direct interaction between PknA and Wag31.
The study of protein-protein interactions is vital to understand how proteins function within a cell. There are numerous *in vitro* approaches, such as tandem affinity purification and mass spectrometry, surface plasmon resonance (SPR), protein microarray, dot blotting, co-immunoprecipitation (co-IP) using cultured cells or tissues and pull-down assays using GST-(glutathione-S-transferase), His-, or FLAG (DYKDDDDK)-tags (Rigaut *et al*., 1999; Smith *et al*., 2003; Jones *et al*., 2006; Hall, 2004; Vikis *et al*., 2004). Far-western blotting was originally developed to screen protein expression library ^{32}P-labeled GST-fusion proteins (Blackwood *et al*., 1991; Kaelin *et al*., 1992). The technique has now been used to study protein-protein interactions, for example, receptor-ligand interactions, and to screen interacting partners in a library (Kadio *et al*., 2007; Korch *et al*., 2009; Garbrielsen *et al*., 2012). To understand the direct interaction between PknA and Wag31, Far-western blotting was performed. This result showed that the His_6-Wag31T73A lacking phosphorylation of Wag31 was bound at MBP-PknA but His_6-Wag31T73E mimicking the phosphorylation state was not bound. These results suggest a direct interaction between PknA and unphosphorylated Wag31. Thus, the phosphorylation of Wag31 changes in its interaction with PknA and release from PknA.
Interestingly, wild-type His_6-Wag31WT was not bound at MBP-PknA. In previous reports (Zheng *et al*., 2007; Jeong *et al*., 2009), *E. coli* BL21(DE3) had two STPKs (YihE and RdoA). Bioinformatic analysis revealed that PknA has an N-terminal catalytic, juxtamembrane, transmembrane, and C-terminal extracellular domains, like known STPKs from other bacteria (Thakur *et al*., 2008). Brenner's motif (H-X-D-X_4-N; the core catalytic domain) was present in three STPKs (PknA, YihE, and RdoA). Therefore, it is possible that purified His_6-Wag31WT can be phosphorylated by STPKs from *E. coli*, as previously described (Chaba *et al*., 2002).
In conclusion, this study revealed the following facts: (1) Far-western blotting showed well that PknA can directly interact with Wag31; (2) the interaction between PknA and Wag31 is dependent on the phosphorylation state of Wag31 (binding: unphosphorylated state; unbinding: phosphorylated state); and (3) this binding mechanism can play an important role in developing new anti-tuberculosis drugs (new inhibitors to the direct interaction) to overcome public health problems caused by the emergence of extensively (or multi-) drug-resistant *M. tuberculosis*.

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