Phosphorylation of *Mycobacterium tuberculosis* Ser/Thr Phosphatase by PknA and PknB

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Abstract

**Background:** The integrated functions of 11 Ser/Thr protein kinases (STPKs) and one phosphatase manipulate the phosphorylation levels of critical proteins in *Mycobacterium tuberculosis*. In this study, we show that the lone Ser/Thr phosphatase (PstP) is regulated through phosphorylation by STPKs.

**Principal Findings:** PstP is phosphorylated by PknA and PknB and phosphorylation is influenced by the presence of Zn²⁺ ions and inorganic phosphate (Pi). PstP is differentially phosphorylated on the cytosolic domain with Thr¹³⁷, Thr¹⁴¹, Thr¹⁷⁴ and Thr¹⁸⁶ being the target residues of PknB while Thr¹³⁷ and Thr¹⁷⁴ are phosphorylated by PknA. The Mn²⁺-ion binding residues Asp³⁸ and Asp²²⁸ are critical for the optimal activity of PstP and substitution of these residues affects its phosphorylation status. Native PstP and its phosphate deficient mutant PstP⁺¹³⁸⁸⁶ are phosphorylated by PknA and PknB in *E. coli* and addition of Zn²⁺/Pi in the culture conditions affects the phosphorylation level of PstP. Interestingly, the phosphorylated phosphatase is more active than its unphosphorylated equivalent.

**Conclusions and Significance:** This study establishes the novel mechanisms for regulation of mycobacterial Ser/Thr phosphatase. The results indicate that STPKs and PstP may regulate the signaling through mutually dependent mechanisms. Consequently, PstP phosphorylation may play a critical role in regulating its own activity. Since, the equilibrium between phosphorylated and non-phosphorylated states of mycobacterial proteins is still unexplained, understanding the regulation of PstP may help in deciphering the signal transduction pathways mediated by STPKs and the reversibility of the phenomena.

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Introduction

*Mycobacterium tuberculosis* has an array of proteins to ensure its existence during the course of infection. In order to thrive and maintain its homeostasis, the pathogen continuously influences its surroundings mainly through surface-located sensor proteins. Extracellular signals are communicated through the sensors to the cytosol leading to the appropriate cell responses. Apparently, a large number of pathogens employ reversible phosphorylation of proteins by kinases and phosphatases as a way of transmitting the signals from extracellular milieu which helps in their survival and pathogenicity [1–4]. Kinases carry out the phosphorylation by transferring the phosphate moiety on target proteins and phosphatases convert them back to the unphosphorylated state, either by dephosphorylating the substrate or by regulating the activity of kinases.

Apart from the well recognized two component systems targeting His/Asp residues in bacteria, Ser, Thr and Tyr residues are also the major targets for phosphorylation. *M. tuberculosis* is known to have 11 Ser/Thr protein kinases (STPKs PknA-L, except C), one tyrosine kinase (PtkA), one Ser/Thr phosphatase (PstP) and two tyrosine phosphatases (PtpA and PtpB) [5,6]. Till date a large number of mycobacterial proteins are shown to be regulated through phosphorylation by STPKs [7–11]. Some of these substrates are also known to be dephosphorylated by PstP [9,11–17]. PstP is a PP2C phosphatase (PPM family) that strictly requires Mn²⁺-ion for its activity [13]. It is a membrane localized enzyme with intracellular catalytic domain of 237 amino acids joined by a juxtamembrane region to the extracellular domain of 191 residues with a single transmembrane helix [18]. Using multidimensional anomalous diffraction studies, Pullen et al. determined the structure of the catalytic phosphatase domain of PstP [18]. PstP contains three metal-binding centers in its structure in contrast to two metal centers found in most of the PP2C phosphatases. Using atomic absorption spectroscopy and X-ray analysis, it has been shown that all the bound metal-ions are Mn²⁺. Similarities between Human Ser/Thr phosphatase PP2Cα and the mycobacterial enzyme have been explained on the basis of structural folds, metal binding and conserved residues [18]. Mutational analyses of PP2Cα have depicted the significance of
certain conserved amino acid residues [19]. The corresponding residues in PstP are involved in binding to metal-ions and catalysis in addition to managing the binding and release of phosphate moiety. These residues in PP2Cα are critical for its activity [19] and thus, they are hypothesized to be important for PstP also.

The interesting feature of *M. tuberculosis* Ser/Thr signaling molecules is that both the essential STPKs, PknB (Rv0014c) and PknA (Rv0015c) and the only Ser/Thr phosphatase PstP (Rv0018c) are located in the same genomic cluster which is conserved in several mycobacterial species [6,9,20]. Transcriptional analysis in earlier studies revealed that PknA, PknB and PstP show similar expression profiles [20] and thus, implicate that strong regulation is required for their own functions as both the classes of enzymes functionally counteract each other. In this study, we show that the activity of PstP is modulated by phosphorylation. This is the first report on the regulation of any bacterial Ser/Thr phosphatase by post-translation modification. PstP was found to be phosphorylated differentially by PknA and PknB, both in *in vitro* and in the surrogate host *Escherichia coli*. Additionally, we found that zinc ions (Zn²⁺) and inorganic phosphate (Pi) can inhibit the activity of PstP which in turn affects the phosphorylation status of both the kinases and phosphatase.

**Materials and Methods**

**Bacterial strains and growth conditions**

*E. coli* DH5α strain (Novagen) was used for cloning and BL21 (DE3) (Stratagene) was used for the expression of recombinant proteins. *E. coli* cells were grown and maintained with constant shaking (220 rpm) at 37°C in LB medium supplemented with 100 µg/ml ampicillin.

**Gene manipulation**

The genes coding for PknA (rvo0015c, representing the cytosolic region of 1-337aa) and PstP (rvo0018c, PstP: 1-514aa) were PCR amplified using *M. tuberculosis* H37Rv genomic DNA. Resulting PCR products were digested with corresponding restriction enzymes and ligated into the vectors pProEx-HTc (Invitrogen) and/or pGEX-5X-3 (GE Healthcare Bio-Sciences) previously digested with the same enzymes. Htc-PknB and Htc-PstP were obtained as described earlier [9]. pGEX-PknB was sub-cloned from Htc-PknB, using standard protocols under the same restriction sites. For cloning in dual-expression vector pETDuet-1 (Novagen), the primers and clones are provided in tables 1 and 2, respectively.

**Protein expression and purification**

Proteins were expressed and purified from *E. coli* as described previously [9]. The purified proteins were assessed by SDS-PAGE and concentrations were estimated by Bradford assay (Bio-Rad).

**In vitro kinase assays and phosphoamino acid analysis**

In *in vitro* kinase assays of PstP, or its mutants (0.5–3 µg) by PknA, (0.5–1 µg) or PknB, (1–3 µg) was carried out in kinase buffer (20 mM PIPES [pH 7.2], 5 mM MnCl₂, 5 mM MgCl₂) containing 2 µCi [γ-³²P]ATP (BRIT, Hyderabad, India) followed by incubation at 25°C for 20 min. Reactions were terminated by 5X SDS sample buffer followed by boiling at 100°C for 5 min. Proteins were separated by 12% SDS-PAGE and analyzed by SDS-PAGE and phosphorylated bands were observed and measured to calculate dephosphorylation potential. For phosphoamino acid analysis, proteins were digested with TEV protease as mentioned above, separated by SDS-PAGE and proteins were visualized by PhosphorImager (FLA 2000, Fuji). Zn²⁺ and Pi were added to the kinase reactions as per requirement of the assay.

**In vitro dephosphorylation and p-nitrophenol phosphate (pNPP) hydrolysis assays**

PknB, and PknA, were autophosphorylated by *in vitro* kinase assays using [γ-³²P]ATP. 1 µg of purified PstP/PstP<sub>D38G</sub>/PstP<sub>D229G</sub> were added in four sets of reactions and incubated at 25°C for increasing time points up to 30 min to measure the dephosphorylation potential of PstP, and its mutants. For auto-dephosphorylation assays, PknB, and PknA, cleaved with TEV protease as mentioned above, separated by SDS-PAGE and electroblotted onto Immobilon PVDF membrane (Millipore). Phosphoamino acid analysis by two-dimensional thin layer electrophoresis was performed as described earlier [9,22].

**Table 1. Primers used in the study.**

| Primer Name | Sequence Details (5'→3') ** |
|-------------|-----------------------------|
| PknB<sub>T171/173D</sub> FP | CGGCAACAGTGGTCGACGGCGATGATCG |
| PknB<sub>T171/173D</sub> RP | CGGATCCTGCTGGTTCACCGCTGTCG |
| PknA FP | TGATCGAACCGAGATGACGGCCACAGGTCG |
| PknA RP | ACGGCCGCCGCCGCCGCCGCGGCAAGAGGCG |
| PstP<sub>D38G</sub> FP | CTATTGGCCCTGGCCG |
| PstP<sub>D38G</sub> RP | GATCGCTGGATGAGGGCTG |
| PstP<sub>T5A</sub> FP | GGAGAGTGGCGCGCG |
| PstP<sub>T5A</sub> RP | GTATCGCAGGACCAGGGCCAGG |
| PstP<sub>T141E</sub> FP | CGGCAACAGCGTG |
| PstP<sub>T141E</sub> RP | GACGCCAGCTGAGCTG |
| pETDuet-PstP FP | CACC CGGGCCCCCCTATAG |
| pETDuet-PstP RP | CGGTGCTCAGGCCGCCGCGG |

**Restriction sites/stop codon/mutated sequences have been underlined.**

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Table 2. Description of the plasmids used in this study.

| Plasmid construct | Description | Reference |
|-------------------|-------------|-----------|
| pProEx-HTc        | E. coli expression vector containing N-terminal His6-tag | Invitrogen |
| pProEx-HTc-PknB6Aa | Expression of His6-PknB6Aa (cytosolic domain) | [9] |
| pProEx-HTc-PknB6Aa<sup>Thr77Thr77</sup> | pProEx-HTc-PknB6Aa with activation loop residues Thr<sup>77</sup> and Thr<sup>77</sup> mutated to Asp, phosphomimetic amino acid | This study |
| pProEx-HTc-PknA6a | Expression of His6-PknA6a<sup>Thr77Thr77</sup> (cytosolic domain) | This study |
| pProEx-HTc-PstP<sup>R20G</sup> | Expression of His6-PstP<sup>R20G</sup> (cytosolic domain) | This study |
| pProEx-HTc-PstP<sup>D38G</sup> | Expression of His6-PstP<sup>D38G</sup> with Asp<sup>38</sup> mutated to Gly | This study |
| pProEx-HTc-PstP<sup>D229G</sup> | Expression of His6-PstP<sup>D229G</sup> with Asp<sup>229</sup> mutated to Gly | This study |
| pProEx-HTc-PstP<sup>TSA</sup> | Expression of His6-PstP<sup>TSA</sup> with Thr<sup>5</sup> mutated to Ala | This study |
| pProEx-HTc-PstP<sup>T41E</sup> | Expression of His6-PstP<sup>T41E</sup> with Thr<sup>41</sup> mutated to Glu | This study |
| pGEX-5X-3         | E. coli expression vector containing N-terminal Glutathione S-Transferase tag | GE Healthcare |
| pGEX-5X-3-PknA6a  | Expression of GST-PknA6a (cytosolic domain) | This study |
| pGEX-5X-3-PknB6Aa | Expression of GST-PknB6Aa (cytosolic domain) | This study |
| pETDuet1          | E. coli dual expression vector containing N-terminal His6-tag in MCS1 and C-terminal S-tag in MCS2 | Novagen |
| pETDuet1-PstP<sup>D38G</sup>/MBP | Expression of His6-PstP<sup>D38G</sup> in MCS1 with Myelin basic protein (MBP) in MCS2 | This study, [21] |
| pETDuet1-PstP<sup>D38G</sup>/MBP-PknA | Expression of His6-PstP<sup>D38G</sup> and MBP tagged PknA in MCS2 | This study, [21] |
| pETDuet1-PstP<sup>D38G</sup>/MBP-PknB | Expression of His6-PstP<sup>D38G</sup> and MBP tagged PknB in MCS2 | This study, [21] |

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βNPP hydrolysis assay was performed as a measure of phosphatase activity. PstP<sup>c</sup> was added to a reaction mixture containing phosphatase assay buffer (50 mM Tris pH 8.0, 5 mM DTT, 4 mM MnCl<sub>2</sub>) and 10 mM βNPP in a 96-well plate and incubated at 57°C for indicated time points and absorbance was read at 405 nm (Microplate reader, Bio-Rad). To assay the relative activity of PstP<sup>c</sup> and its phosphatase-deficient variants, increasing concentrations of enzymes were added to the reaction mix and processed as above. Alkaline phosphatase (Roche) and PknB were taken as positive and negative controls, respectively, for the βNPP hydrolysis assays. Variations of PstP<sup>c</sup> activity by addition of Zn<sup>2+</sup> and Pi was assessed by adding ZnCl<sub>2</sub> or sodium phosphate [pH 7.2] to the reaction mixture as above, to achieve the indicated final concentrations. pETDuet-1 purified PstP<sup>c</sup> and PstP<sup>c D38G</sup>, co-expressed with or without kinases, were employed for βNPP-assays to measure the effect of phosphorylation on their activities.

Metabolic labeling in E. coli

The procedure described by Kumar et al. was followed for metabolic labeling [23]. E. coli (BL21-DE3) transformants harbouring either pETDuet-PstP<sup>c</sup>/PstP<sup>c D38G</sup>-mbp or pETDuet-PstP<sup>c</sup>/PstP<sup>c D38G</sup>-mbpPknA or pETDuet-PstP<sup>c</sup>/PstP<sup>c D38G</sup>-mbpPknB were grown in 5 ml LB medium containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol in O.D<sub>600</sub> of ~0.6. The cells were induced with 1 mM IPTG and further grown for 4 hr at 16°C. Cultures were harvested, washed with 5 ml of M9 medium [pH 7.0] without phosphates salts (for 1 L: Na<sub>2</sub>HPO<sub>4</sub>-1 g, NaCl-0.5 g, 20% glucose-10 ml, MgSO<sub>4</sub>-0.5 g, 7H<sub>2</sub>O-1 ml, Thiamine-HCl-1 ml, CaCl<sub>2</sub>-1 ml). The cells were resuspended in 2 ml of M9 media supplemented with 1 mM of [β<sup>32</sup>P]orthophosphoric acid (BRIT, Hyderabad, India), 100 μg/ml ampicillin and 1 mM IPTG and further grown at 16°C for 4 hr. Under specific conditions, Sodium phosphate [pH 7.2] (2 mM) or ZnCl<sub>2</sub>(4 mM) were added to M9 media and subsequent processing steps of metabolic labeling. The cells were harvested and lysed by sonication in the lysis buffer containing phosphate-buffered saline, 5% glycerol and protease inhibitor cocktail. The cell lysate was clarified and the lysates containing His6-fusion protein were incubated with lysis buffer equilibrated Ni<sup>2+</sup>-NTA affinity beads for 2 hr at 4°C. The beads were then thoroughly washed with lysis buffer containing 20 mM imidazole and resuspended in 3X SDS sample buffer followed by boiling for 15 min. The samples were resolved on SDS-PAGE followed by autoradiography.

Identification of phosphorylation sites in PstP<sup>c</sup>

PknB and PknA<sup>c</sup> were employed for in vitro kinase assay using 50 μM cold ATP and PstP<sup>c</sup> or PstP<sup>c D38G</sup>. The samples were run on 12% SDS-PAGE, stained with Coomassie Brilliant Blue and de-stained. Bands corresponding to PstP<sup>c</sup> or PstP<sup>c D38G</sup> were excised from the gel and washed with MilliQ water. The samples were processed for identification of phosphorylation sites by using Thermo-Finnagen LTQ electrospray instrument (Proteomics Core Facility, Children’s Hospital, Boston). The detailed protocol of sample processing for identification of phosphorylation sites has been provided in File S1.

Generation of polyclonal antibodies for PstP<sup>c</sup> in rabbit and immunoblotting

Polyclonal antibodies against PstP<sup>c</sup> were generated in rabbit. To confirm the presence of PstP<sup>c</sup> or PstP<sup>c D38G</sup> in Ni<sup>2+</sup>-NTA pulled-out proteins after metabolic labeling by western blot analysis, the samples were resolved by SDS-PAGE along with positive (purified PstP<sup>c</sup>) and negative controls (GST-PknB) and transferred onto nitrocellulose membrane (Bio-Rad). Standard procedure for immunoblotting was followed [9,11]. The blots were developed using SuperSignal<sup>®</sup> West Pico Chemiluminescent Substrate kit (Pierce Protein Research Products) according to manufacturer’s instructions.

Results

Identification of the residues critical for the activity of PstP

On the basis of structural data available for PstP and alignment with the residues important for Human PP2Cα activity [18], PstP,
mutants were generated using site-directed mutagenesis. These residues include the Mn²⁺-ion binding sites-Asp³⁸ and Asp²²⁹ and phosphate (Pi) binding residue-Arg²⁰ (Figure 1A). In the resulting mutants, these sites were converted to Glycine (PstPcD³⁸G, PstPcD²²⁹G and PstPcR²⁰G). The activity of these mutants was compared using chromogenic substrate pNPP. To confirm the authenticity of the assay, increasing concentrations of alkaline phosphatase were utilized as a positive control while PknB was used as negative control (Figure S1). The pNPP assay with increasing amounts of PstP-mutants showed that the mutation of Asp³⁸ and Asp²²⁹ to Gly resulted in >90% loss of the dephosphorylation activity of PstPc, while the PstPcR²⁰G mutant lost about 60% of its activity (Figure 1B and 1C). Thus, Arg²⁰, Asp³⁸ and Asp²²⁹ were identified as the residues required for optimum activity of PstP. To confirm that the loss in activity was specifically due to mutagenesis of Asp³⁸, Asp²²⁹ and Arg²⁰, irrelevant residues (Thr⁵ and Thr¹⁴¹) in PstP, were mutagenized to generate PstPcT⁵A and PstPcT¹⁴¹E. The relative activities of these mutants were compared with the native enzyme through pNPP-assay (Figure S2). There were no significant changes observed in the mutants in comparison to PstPc, thus reinforcing the importance of Arg²⁰, Asp³⁸ and Asp²²⁹ residues.

Phosphatase activity of PstPc and its mutants

The dephosphorylation potential of PstPc and its mutants was also assessed by their ability to dephosphorylate PknB, in a time-dependent dephosphorylation (Figure 2A) and pNPP hydrolysis assays (Figure S3). PstPcD³⁸G dephosphorylated the autophosphorylated PknB to some extent, whereas substantial loss of phosphatase activity was observed with PstPcD²²⁹G and PstPcR²⁰G (Figure 2A). The activity of PstPcD²²⁹G was relatively higher than that of PstPcD³⁸G as opposed to the observation in pNPP-assays (Figures 1C and S3). Similar observations have been reported earlier where the activity of an enzyme, specifically Ser/Thr phosphatases, is shown to be dependent on the nature of substrate [24–26]. pNPP is an artificial substrate while PknB is a natural substrate of PstP, which may be recognized and subsequently dephosphorylated more optimally. Additionally, in this case, the activity of the phosphatase also depends on the activity of PknB, as discussed in later sections. The assays were also performed using autophosphorylated PknA, which showed similar results (data not shown). Surprisingly, in this assay, additional phosphorylated bands corresponding to the size of PstPcD³⁸G were observed when incubated with kinase for longer time. No such bands were observed with PstPc, PstPcR²⁰G and PstPcD²²⁹G at the given concentrations.

![Figure 1. Critical residues of PstP.](image-url)

(A) Schematic representation of PstP with critical residues (Arg²⁰, Asp³⁸ and Asp²²⁹) being highlighted with upward arrows. (B) Activity profiles of PstPc and its mutants: Activity assays were performed by pNPP-hydrolysis mediated by PstPc, PstPcR²⁰G, PstPcD³⁸G and PstPcD²²⁹G. Increasing concentrations of proteins were taken with constant substrate concentration (10 mM pNPP) and incubated at 37°C for 30 mins. As shown in the graph, the mutants had lost phosphatase activity to different extents. Activity is calculated as a measure of μmoles of pNPP hydrolyzed per min. at a given enzyme concentration. (C) The relative activity of all the phosphatase variants (5 μg each, 30 min.) showed that PstPcD³⁸G and PstPcD²²⁹G had lost >90% of activity while PstPcR²⁰G lost ~60% of the activity as compared to PstPc. The error bars indicate the SD of three individual experiments.

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on the cleaved substrate protein (data not shown). Additionally, TEV-protease cleavage of the tag was performed after the kinase assay as a control. Phosphorylation was confirmed to be specifically localized to the region of the tag on PstP. Moreover, rabbit anti-PstP antibodies confirmed the metabolically labelled protein to be PstP (data not shown).

Phosphorylation of PstP<sub>C</sub><sup>D38G</sup>, PstP<sub>C</sub><sup>D229G</sup> and PstP<sub>C</sub><sup>R20G</sup>

After identification of the residues critical for PstP<sub>C</sub> activity and measuring the activity of corresponding mutants, the phosphorylation status of PstP<sub>C</sub> mutants was studied. PknA and PknB were employed for the phosphorylation assays. PstP<sub>C</sub><sup>D38G</sup> and PstP<sub>C</sub><sup>D229G</sup> were efficiently phosphorylated by both PknA and PknB (Figure 3A), whereas faint signal on PstP<sub>C</sub><sup>R20G</sup> was observed owing to its partial phosphatase activity. Phosphorylation of PstP<sub>C</sub> (at 3 μg concentration) was not observed by <em>in vitro</em> kinase assay as it completely dephosphorylated PknAc and PknBc, making them inactive (heat-inactive PstP was found to be phosphorylated-data not shown). To confirm that the observed phosphorylation is on PstP<sub>C</sub>-mutants and not on the N-terminally attached His<sub>6</sub>-tag, TEV-protease cleavage of the tag was performed after the kinase assays. Phosphorylation was confirmed to be specifically localized on the cleaved substrate protein (data not shown). Additionally, the R20G, D30G and D229G mutants were also created in full length PstP<sub>C</sub> construct and autophosphorylation assays and phosphorylation reactions were also confirmed using full length PstP and its mutants (data not shown).

Phosphoamino acid analysis and identification of phosphorylation site(s) of PknA and PknB in PstP<sub>C</sub><sup>D38G</sup>

Phosphoamino acid analysis by two-dimensional thin layer electrophoresis showed that both PknA<sub>C</sub> (Figure 3B, upper panel) and PknB<sub>C</sub> (Figure 3B, lower panel) phosphorylated PstP<sub>C</sub><sup>D38G</sup> on Thr residues while no signal was observed on the spots corresponding to pSer and pTyr. For further experiments, PstP<sub>C</sub><sup>D38G</sup> was utilized.

Validation of PstP phosphorylation in <em>E. coli</em>

To further substantiate our results, the phosphorylation status of PstP<sub>C</sub> and PstP<sub>C</sub><sup>D38G</sup> was examined specifically by PknA and PknB in <em>E. coli</em> using a dual expression system. PstP<sub>C</sub> and PstP<sub>C</sub><sup>D38G</sup> were co-expressed in <em>E. coli</em> along with MBP alone or MBP-tagged PknA or PknB. <em>E. coli</em> BL21 (DE3) cells transformed with <em>pETDuet1-PstP<sub>C</sub>/PstP<sub>C</sub><sup>D38G</sup>-MBP</em> or <em>pETDuet1-PstP<sub>C</sub>/PstP<sub>C</sub><sup>D38G</sup>-MBP-kinase</em> (kinase, PknA or PknB) were metabolically labelled with <sup>32</sup>P orthophosphoric acid. Phosphorylation of PstP<sub>C</sub> and PstP<sub>C</sub><sup>D38G</sup> could only be detected when PknA or PknB were co-expressed (Figures 4A and 4B), suggesting the phosphorylation of phosphatase by both the kinases in native conditions in <em>E. coli</em>. Western blot analysis of Ni<sup>2+</sup>-NTA purified samples using rabbit anti-PstP<sub>C</sub> antibodies confirmed the metabolically labelled protein to be PstP<sub>C</sub> (data not shown).

Activity assays of <em>pETDuet1</em>-purified PstP<sub>C</sub> and PstP<sub>C</sub><sup>D38G</sup>

The activity profiles of PstP<sub>C</sub> and PstP<sub>C</sub><sup>D38G</sup> co-expressed with and without PknA/PknB were evaluated. According to the pNPP assays, the activity of phosphorylated PstP<sub>C</sub> (co-expressed with PknA or PknB) was higher than that of unphosphorylated phosphatase (co-expressed with MBP alone) (Figure 4C). The phenomenon was also confirmed by measuring the activity of PstP<sub>C</sub><sup>D38G</sup>. As already discussed, PstP<sub>C</sub><sup>D38G</sup> had retained about 10% of the dephosphorylation activity as a result of which, it was phosphorylated efficiently by kinases. The relative activity of phosphorylated PstP<sub>C</sub><sup>D38G</sup> with PknA/PknB and unphosphorylated protein was measured for 420 min. Interestingly, the activity of phosphorylated PstP<sub>C</sub><sup>D38G</sup> was remarkably higher than that of unphosphorylated protein, thus the similar profile as that of PstP<sub>C</sub>
was observed (Figure 4D). Also, the activity of PknA phosphor-
phosphorylated phosphatase was even more than the protein phosphor-
phosphorylated by PknB. Noticeably, the increase in phosphatase activity
after phosphorylation may also account for the observed increase
in the activity of PstPc
D229G in the time-dependent dephosphor-
phosphorylation assays (Figure 2A).

The dephosphorylation of in vitro autophosphorylated PknA,
was assessed by PstPc
D38G+MBP, PstPc
D38G+MBP-PknA and
PstPc
D38G+MBP-PknB. As expected, due to higher activity of
phosphorylated PstPc
D38G, intensity of phosphorylation on PknA,
was low as compared to the reaction containing unphosphorylated
PstPc
D38G (Figures 4E and S4). Also, since PknA-phosphor-
phosphorylated PstPc
D38G was more active than PknB-phosphorylated
PstPc
D38G (Figure 4D), the extent of dephosphorylation was more
in lane 3 as compared to lane 4.

Auto-dephosphorylation of PstPc

Next, we tried to understand whether the inability of PstPc to be
effectively phosphorylated was due to the phosphatase activity
on the kinases resulting in their inactivation or it was
due to auto-dephosphorylation. Consequently, phosphomimetic
mutants of PknBc were generated for the Thr residues of activation
loop in catalytic domain [12], forming PknBc
T171/173D which
cannot be dephosphorylated by PstPc on Thr171 and Thr173. As
reported by Boitel et al., PknB does not lose phosphorylation
signals after mutagenesis of Thr171 and Thr173. Through a series of
careful analysis of single and double mutants of PknB, it has been
shown that PknB can be additionally phosphorylated on Ser166
and/or Ser169 residues [12]. Thus, we utilized PknBc and
PknBc
T171/173D, that were autophosphorylated in an in vitro kinase
assay using [γ-32P]ATP, before incubation with PstPc. Phospho-
phosphorylation of PstPc was still not observed with constitutively active
PknBc
T171/173D, as confirmed by phosphotransfer observed on
PstPc
D38G (Figure 5A). This suggests that PstPc can dephosphor-
phosphorylate itself. Additionally, PknBc
T171/173D was completely dephosphorylated in presence of PstPc, suggesting that PstP could also
dephosphorylate the surplus sites Ser166/Ser169.

Identification of the factors affecting the activity of PstP

The phosphorylation of PstP suggested that additional factors
may be involved in the cellular milieu that can regulate and control
the phosphatase activity, preceding its phosphorylation. In general,
phosphatases are known to be affected by a number of factors like
metal-cations, Pi, creatine phosphate (CP) and ATP/ADP ratio.
PstPc activity assay was carried out in the presence of selected factors. Interestingly, activity of PstPc was reduced in the presence of Zn\(^{2+}\) and Pi, as assessed by \(pNPP\) assay. Reduction of almost 50% activity was observed at 0.2 mM Zn\(^{2+}\) (Figure 5B) and 0.5 mM Pi (Figure 5C). Maximum inhibition of PstPc was observed at 1 mM Zn\(^{2+}\) and 4 mM Pi. Inhibition by Zn\(^{2+}\) at 1 mM was not calculable due to protein precipitation in the reaction mixture.

Phosphorylation of PstPc in the presence of Zn\(^{2+}\) and Pi

The inhibition of PstPc in the presence of Zn\(^{2+}\) and Pi provided a condition that could favour the phosphorylation of PstPc by STPKs. PstPc was indeed phosphorylated by PknA, and PknB, in presence of 0.2 mM Zn\(^{2+}\) or 0.5 mM Pi (Figure 5D), under in vitro conditions. Since the phosphorylated bands of His\(_6\)-tagged PknA and PknB were not able to resolve on SDS-PAGE (Figure S5), the assay was performed with GST-tagged kinases and similar results were obtained. To further assess the effects of Zn\(^{2+}\) and Pi, metabolic labeling of PstPc by co-expressed kinases PknA and PknB was performed in E. coli in the presence of Zn\(^{2+}\) (4 mM) and Pi (2 mM) (Figure 5E). Phosphorylation of PstPc was indeed enhanced in the presence of Zn\(^{2+}\) by ~40%-50%. The enhancement in phosphorylation in the presence of Pi was not as prominent (~10%-20%), possibly due to competition of phosphate ions with \(^{32}\)Porthophosphoric acid. Nevertheless, as

Figure 4. Co-expression analysis of STPKs and PstPc/PstPc\(_{D38G}\). (A) Metabolic labeling of PstPc/PstPc co-expressed with MBP-PknA (lane 2) or MBP-PknB (lane 3) gets phosphorylated in E. coli under native conditions while PstPc co-expressed with MBP alone (lane 1) was not phosphorylated. (B) Metabolic labeling of PstPc\(_{D38G}\)/PstPc\(_{D38G}\) co-expressed with MBP-PknA (lane 2) or MBP-PknB (lane 3) gets phosphorylated in E. coli while PstPc\(_{D38G}\) co-expressed with MBP alone (lane 1) was not phosphorylated. As expected, the intensity of phosphorylation on PstPc\(_{D38G}\) was comparatively higher than that of PstPc. (C) Relative activity profile of pETDuet1 purified PstPc and (D) PstPc\(_{D38G}\), \(pNPP\) assays were performed with PstPc and PstPc\(_{D38G}\) (1 µg each) purified from pETDuet1 co-expressing MBP or MBP-PknA/PknB. The dephosphorylation potential of phosphorylated PstPc and PstPc\(_{D38G}\) (co-expressed with either kinase) is higher than that of unphosphorylated protein. For PstPc\(_{D38G}\), activity was evaluated over long time points due to its low dephosphorylation activity. Activity is calculated as a measure of umoles of pNPP hydrolyzed per µg of protein at a given time. The error bars indicate the SD of the three individual experiments. (E) Relative dephosphorylation of PknA\(_c\) by pETDuet-1 purified PstPc\(_{D38G}\). Autophosphorylated PknA\(_c\) was incubated for 30 mins with unphosphorylated and phosphorylated PstPc\(_{D38G}\) and the extent of dephosphorylation was assessed by in vitro dephosphorylation assays. The image obtained after autoradiography was analyzed by ImageGauge software (Fujifilm) and relative intensity of phosphorylation was measured: (1) PknA\(_c\) alone, (2) PknA\(_c\)+MBP-PstPc\(_{D38G}\), (3) PknA\(_c\)+PstPc\(_{D38G}\) phosphorylated by PknA and (4) PknA\(_c\)+PstPc\(_{D38G}\) phosphorylated by PknB. As shown, the PknA-phosphorylated PstPc\(_{D38G}\) dephosphorylated the kinase to a greater extent in comparison to the unphosphorylated PstPc\(_{D38G}\). The error bars represent the SD of the three individual experiments. The corresponding autoradiogram is shown in Figure S4.
a proof of principle, Zn$^{2+}$ and Pi were identified as the novel regulators which can inhibit the activity of PstP, and facilitate its phosphorylation.

**Discussion**

The coordinated regulation of Ser/Thr protein kinases and phosphatases is essential for maintaining the appropriate equilibrium of protein phosphorylation. Membrane associated kinases and phosphatases are known or hypothesized to be regulated by external stimulus. It is of great relevance to decipher the regulatory mechanisms especially in the systems like *M. tuberculosis* where one Ser/Thr phosphatase PstP is accountable for the effects caused by 11 STPKs. In general, the processes involved in regulating the phosphatases include some external signals, variation in pH [27], cellular concentrations of ATP, ADP, Pi (or their ratios) [28,29], cytosolic cations like Mn$^{2+}$, Zn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$ [13,27,29–31] and post-translation modifications (phosphorylation, methylation) [28,30,32–39]. Present study demonstrates an example of PknA and PknB mediated regulation of PstP through inter-dependent phosphorylation-dephosphorylation reactions. Regulation of phosphatases by phosphorylation is a critical step for cell signaling pathways. It is also associated with feedback phenomena in case where phosphatases are phosphorylated by the kinases that are in turn dephosphorylated by the same phosphatase. Certain examples illustrate the phosphorylation of PP2C phosphatases such as rat Mg$^{2+}$-dependent protein phosphatase α (MPPα) by casein kinase II [39], Soybean kinase associated protein phosphatase (Soybean KAPP) [37], *Oryza sativa* KAPP [40], but these have not been detailed in terms of feedback regulation.

Figure 5. Factors affecting PstP activity. (A) Auto-dephosphorylation of PstPc: Autoradiogram showing phosphorylation by PknBc, PstPc, and PstPc$^{D38G}$ (3 µg each) were used for in vitro phosphorylation assay by PknBc and PknBc$^{T171/173D}$ (2 µg each). Since PknBc$^{T171/173D}$ cannot be dephosphorylated by PstPc, lack of signal signifies auto-dephosphorylation of phosphatase. PstPc$^{D38G}$ was used as positive control to show that PknBc cannot be phosphorylated by PstPc. Regulation of PstPc activity: pNPP assay showing the effect on activity of PstPc (1 µg) by (B) Zn$^{2+}$ and (C) Pi. pNPP assay was carried out for 30 mins and activity was calculated as a measure of µmoles of pNPP hydrolyzed per min per µg of protein. The error bars show SD of three independent experiments. (D) Phosphorylation of PstPc: Autoradiogram showing the phosphorylation of PstPc (1 µg) by GST-PknAc, (left panel) and GST-PknBc (right panel) in presence of 0.2 mM Zn$^{2+}$ and 0.5 mM Pi. Since His$_6$-tagged STPKs were not resolved properly from PstPc on SDS-PAGE (Figure S5), the assay was also performed with GST-tagged kinases having higher molecular weights. (E) Metabolic labeling of PstPc by PknA and PknB in E. coli in presence of Zn$^{2+}$ and Pi. Phosphorylation level of PstPc was observed to be increased when Zn$^{2+}$ (4 mM) and Pi (2 mM) were added during the culture conditions and subsequent processing steps. The autoradiograms obtained after SDS-PAGE were analyzed by ImageGauge software and intensity of the band corresponding to PstPc phosphorylation without any added factor was taken as 100%. Relative phosphorylation is depicted in the bar graph.

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PstP has conserved domain architecture of PP2C-phosphatases (PPM family). PPM family phosphatases play an imperative role in a number of systems described earlier [41–46]. Except a few PP2C-phosphatases like Human PP2Ca [49] and Arabidopsis KAPP [50], not much is known about other members of this family. For PstP, we have previously shown that PknA and PknB are the targets for dephosphorylation by PstP and detailed the basic biochemical requirements of this enzyme along with its membrane localization [13]. In a later study, Pullen et al. resolved the crystal structure of PstP catalytic domain and described the most important features of this molecule having characteristic PP2C-fold along with three-metal binding centers that associate with Mn$^{2+}$ [18]. The discovery of third-metal centre was a unique feature of PstP as other PP2C phosphatases were found to have two metal-binding centres. In the recent studies, the PP2C-phosphatases of Streptococcus agalactiae and Thermosynechococcus elongatus have been shown to have a similar third-metal binding centre [51,52]. The third metal ion center in PstP is proposed to be involved in structural perturbations leading to altered phosphoprotein recognition profiles.

In this study, three conserved residues were selected for generation of site-directed mutants in PstPc, on the basis of similarity with Human phosphatase PP2Ca [18], Arg20 (PP2Ca Arg20) is responsible for hydrolysis of phosphate moiety from pSer/pThr residues in target proteins. Asp38 (PP2Ca Asp36) and Asp229 (PP2Ca Asp232) constitute a part of Mn$^{2+}$-metal centers and coordinate with the two critical Mn$^{2+}$. Mutations of Asp38 and Asp229 affected the activity of PstP rendering it active to minimal level, though R20G mutant retained about 40% activity. Thus, the residues that are involved in Mn$^{2+}$-ion binding and hydrolysis of phosphate are deemed critical for its activity. Accordingly, the extent of phosphorylation of each mutant was dependent on the remaining dephosphorylation activity, so that PstPc$^{D38G}$ and PstPc$^{D229G}$ were efficiently phosphorylated by PknA and PknB.

Association with metals is crucial for PP2C phosphatases and any perturbation with inherently associated metals may lead to altered functional profile. The minimum requirement for PstPc activity is the presence of Mn$^{2+}$ [13]. For PP2C-class of phosphatases, divalent ions other than Mn$^{2+}$/Mg$^{2+}$ can inhibit their activity by competitively replacing the Mn$^{2+}$ in the core enzyme structure [27] and Zn$^{2+}$ are the most potent regulators, having comparable ionic radii with that of Mn$^{2+}$. PstPc was partially inactive in the presence of 0.2 mM ZnCl$_2$ and displayed lower activity on increasing the Zn$^{2+}$-ion concentration up to 2 mM, as observed by pNPP assays. In vitro kinase assays with PknA, and PknB, in presence of Zn$^{2+}$ resulted in phosphorylation of PstPc. Also, there was increase in phosphorylation of PstPc during metabolic labeling by PknA and PknB in the presence of Zn$^{2+}$ added in the E. coli culture. These results indicate that in mycobacterial cell, if cytosolic Zn$^{2+}$ concentration increases, it may inhibit PstPc perhaps leading to its phosphorylation. In an elaborative elemental analysis, Wagner et al. have reported that during infection, intravacular Zn$^{2+}$-ion concentration increases from 0.037 mM to 0.46 mM in macrophages infected with M. tuberculosis [53]. Although there is no report of concomitant increase in mycobacterial Zn$^{2+}$-ion concentration, it can only be speculated that if these changes in vacuolar ion concentrations alter the mycobacterial ionic profile, a condition may develop where the enzymes that respond to Zn$^{2+}$ (like PstP) can be activated or deactivated.

End-product inhibition of enzymes is a well-established phenomenon to prevent the accumulation of a particular metabolite. In case of reversible reactions, end-product accumulation can change the direction of the reaction. Similarly, Pi is known to inhibit a number of phosphatases [27,42,49] and in present study, PstPc mediated pNPP hydrolysis is inhibited by Pi. To confirm that this effect is not limited to pNPP, in vitro kinase assays and metabolic labeling in E. coli led to the specific phosphorylation of PstPc, and PstPc$^{D38G}$. Co-expression in pETDuet-1 has previously been utilized extensively to assess the interaction of mycobacterial STPKs with their cognate substrates in the surrogate host E. coli [21,23]. Such dual-expression systems are increasingly becoming useful for analysis of protein-protein interactions specifically for challenging systems like mycobacteria [54]. Activity assays of the pETDuet-1 purified PstPc and PstPc$^{D38G}$ revealed the higher activity of PknA-phosphorylated phosphatase as compared to the PknB-phosphorylated protein. Prominent variations in the activity of phosphorylated and unphosphorylated PstPc$^{D38G}$ were observed with phosphorylated protein being proficient to hydrolyze pNPP to a greater extent (~15-fold higher) in contrast to the unphosphorylated protein. The difference in the activities of phosphorylated and unphosphorylated PstPc, was not as prominent as that of PstPc$^{D38G}$ (~2–3 fold higher). These differences may be attributed to the fact that PstPc may get auto-dephosphorylated to a greater extent than PstPc$^{D38G}$ during expression and purification procedures. Higher activity of phosphorylated phosphatase is suggestive of reverse regulation of signaling cascade emanating from the kinases. In the constitutively active state, STPKs perform their regular functions and phosphorylate the target substrates following the stimulus. This may ultimately lead to the phosphorylation of PstP. The resulting increase in the activity of phosphatase may itself act as a control mechanism for kinases, eventually impeding the continued effect of that particular stimulus. The overall process has to be dynamic due to auto-dephosphorylation of PstP, eventually ceasing the effect of signaling cascade. In the conditions of high Zn$^{2+}$ or high Pi content of the cell, PstP may not be active and will allow the kinase to work at its maximal activity. The proposed phosphorylation of PstPc in such conditions may act as a mechanism to overcome the inhibition of PstPc, hence balancing the cellular signaling pathways.

Supporting Information

Figure S1 pNPP-assay. To confirm the authenticity of pNPP assay, increasing amounts of alkaline phosphatase (0–100 ng) was used a positive control and PknBc (0–5 µg) was used as a negative control. The assay was performed for 30 mins at 37°C and the activity is calculated as pmoles of pNPP hydrolyzed per min at a given amount of enzyme used. As clearly evident, alkaline phosphatase showed very high activity while no such activity was detected in PknBc.

(TIF)

Figure S2 Effect of mutations on the activity of PstPc. To show that the loss in activity of PstPc was specifically due to mutations of Arg20, Asp38 and Asp229, PstPc was mutagenized on irrelevant residues Thr and Thr$^{141}$ to Ala and Glu, respectively.
and PNP hydrolysis was performed for 30 mins at 37°C. Activity of PstP was taken as 100% and relative activity was calculated. As evident from the bar graph, there were no significant changes in the activity of the mutants PstP<sup>R20G</sup>, PstP<sup>D38G</sup>, and PstP<sup>D38G,R20G</sup> variants (2 μg each) at 37°C. Alkaline phosphatase (2 μg) was used as a positive control and PknB<sub>c</sub> (5 μg) was used as a negative control. Activity was calculated as nmoles of pNP hydrolyzed per μg of enzyme used at a given time and depicted in logarithmic scale. Nevertheless, the results are essentially similar as that of time-dependent dephosphorylation of PknB<sub>c</sub> (Figure 2A). (TIF)

Figure S4 In vitro dephosphorylation activity of pET-Duet-1 purified PstP<sub>D38G</sub>. Autophosphorylated PknB<sub>c</sub> was incubated with unphosphorylated and phosphorylated PstP<sub>D38G</sub>. As shown in the autoradiogram, the PknA-phosphorylated PstP<sub>D38G</sub> dephosphorylated the kinase to a greater extent in comparison to the unphosphorylated PstP<sub>D38G</sub>. The image was also analyzed by ImageGauge software and corresponding values are depicted by bar-graph (Figure 4E). (TIF)

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Figure S5 Phosphorylation of PstP<sub>c</sub>. Autoradiogram showing the phosphorylation of PstP<sub>c</sub> (1 μg) by His<sub>6</sub>-tagged STPKs PknA<sub>c</sub> (upper panel) and PknB<sub>c</sub> (lower panel) in presence of 0.2 mM Zn<sup>2+</sup> and 0.5 mM Fe. Due to overlapping molecular weights of PknA<sub>c</sub> and PknB<sub>c</sub> with PstP<sub>c</sub>, the bands were not separated properly. Still, the phosphotransfer on PstP<sub>c</sub> was evident in presence of Zn<sup>2+</sup> and P<sub>c</sub> by both the kinases. The reaction was also performed with GST-tagged STPKs to clearly depict the reaction (Figure 5D). (TIF)

File S1 Detailed protocol of sample processing for identification of phosphorylation sites. (DOC)

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Author Contributions
Conceived and designed the experiments: AS GA. Performed the experiments: AS GA MG SU. Analyzed the data: AS GA VKN YS. Contributed reagents/materials/analysis tools: VKN YS. Wrote the paper: AS GA MG.
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