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Phosphorylation control of protein tyrosine phosphatase A activity in *Mycobacterium tuberculosis*

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ABSTRACT

Protein tyrosine phosphatase A (PtpA) has been shown to play a key role in human macrophage infection by *Mycobacterium tuberculosis* (*Mtb*). Protein tyrosine kinase A (PtkA) was the first protein tyrosine kinase shown to phosphorylate PtpA. Here, we found that PtkA-mediated phosphorylation of PtPA on Tyr-128 and Tyr-129 enhances the PtPA phosphatase activity. Moreover, ex-vivo protein–protein interaction assays showed that PtpA can be phosphorylated by several eukaryotic-like Ser/Thr protein kinases, such as protein kinase A (PknA). PknA was found to regulate PtpA phosphatase activity through Thr-45 phosphorylation. These results indicate that members of two independent families of protein kinases tune PtpA activity in *Mtb*.

1. Introduction

Tuberculosis (TB), one of the most notorious lung infections is caused by *Mycobacterium tuberculosis* (*Mtb*) and remains even today a serious public health threat. According to the World Health Organization, in 2012, 8.6 million people developed active TB with 1.3 million succumbing to the disease [1]. In recent years, the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mtb* and HIV co-infection continue to determine TB as a serious threat to mankind [2].

The success of *Mtb* as a pathogen partially depends on its ability to grow and replicated inside the phagocytic vacuole of the macrophage [3,4]. Upon infection, macrophages normally engulf invading microorganisms and through a process of phagosome maturation-elicit anti-microbial process, which results in proteolytic digestion and presentation of antigens on the surface of the macrophage for immune recognition [5,6]. *Mtb* is able to circumvent the macrophage killing machinery through inhibition of phagosome maturation and prevention of the phagolysosome fusion processes [4,7]. As a result of *Mtb* induced phagosome maturation arrest, macrophages are not able to process and present antigens on the surface of the macrophage [8]. Macrophages harboring *Mtb* also show reduced responsiveness to IFNγ [9], reduced production of cytokines, lower levels of reactive oxygen and nitrogen intermediates [10] and suppression of host cell apoptosis [11]. The effect of *Mtb* on these biological functions of the macrophage is attributed to an array of *Mtb* effectors composed of proteins and lipids [4,7].

The *Mtb* protein tyrosine phosphatase A (PtpA) has been shown to play a key role in the inhibition of both phagosome acidification and maturation processes [12,13]. PtpA is secreted from *Mtb* into the host macrophage cytosol and disrupts key components of the endocytic pathway, resulting in the arrest of phagosome maturation [12–14]. In the macrophage, PtpA interferes with the formation of the homotypic fusion and protein-sorting complex (HOPS), which regulates membrane trafficking throughout the endocytic pathway. It does so by dephosphorylating vacuolar protein sorting 33B (VPS33B) at the phagosome-lysosome interface. This is a two step process which involves initial binding to the macrophage H^+-ATPase (V-ATPase) through its subunit H [13].

Abbreviations: Mtb, *Mycobacterium tuberculosis*; TB, tuberculosis; PtkA, protein tyrosine kinase A; PtpA, protein tyrosine phosphatase A; PknA, protein kinase A; VPS33B, vacuolar protein sorting 33B; V-ATPase, vacuolar H^+-ATPase; SDM, site-directed mutagenesis

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We have previously shown that PtpA serves as a substrate for the odd tyrosine kinase, protein tyrosine kinase A (PtkA) [15], the first identified protein tyrosine kinase in *Mtb* [16]. PtkA phosphorylates PtpA on Tyr^{128} and Tyr^{129} residues, which reside in close proximity to one of its catalytically active site amino acid, Asp^{126}. However, the effect of PtpA's phosphorylation on its phosphatases activity is not fully clear. In this study, we show that PtkA enhances the phosphatase activity of PtpA *in vitro*. Furthermore, we demonstrate that both phosphorylation sites Tyr^{128} and Tyr^{129} residues together are essential for its phosphatase activity. Our studies also revealed that several eukaryotic-like Ser/Thr protein kinases interact with and phosphorylate PtpA, indicating potential regulation of PtpA activity prior to its secretion. Among these STPKs, we found that protein kinase A (PknA) enhances its phosphatase activity through Thr^{45} phosphorylation. These results show that representatives of two independent protein kinases families control PtpA phosphatase activity, which may suggest multiple regulatory pathways for this key pathogenesis phosphatase of *Mtb*.

2. Materials and methods

2.1. Strains and growth conditions

Growth and transformation of *Mycobacterium smegmatis* mc^2155 was performed as described [17]. When necessary, Middlebrook medium was supplemented with kanamycin (25 μg/mL), hygromycin B (50 μg/mL), or trimethoprim (10–30 μg/mL). *Escherichia coli* DH5α and *E. coli* BL21 (DE3) were grown in Luria-Bertani (LB) broth or on LB agar. When necessary, ampicillin was added at a final concentration of 100 μg/mL.

2.2. Site-directed mutagenesis (SDM)

The parental plasmid pGEX4T3-*ptpA* was used as a template to produce the mutated protein $PtpA^{Y128EY129E}$ by site-directed mutagenesis using the oligonucleotide-overlapping method [18]. The parental plasmid pET-22b-*ptpA* was used as a template to produce the mutated protein $PtpA^{T8A}$, $PtpA^{T12A}$, $PtpA^{T41A}$, $PtpA^{T45A}$, $PtpA^{T69A}$, $PtpA^{T78A}$ and $PtpA^{T119A}$. The primers used for mutagenesis are listed in Table S1. Mutations were confirmed by DNA sequencing. Plasmid used for expressing the mutant protein $PtpA^{Y128AY129A}$ was constructed as previously described [16] and sub-cloned into pGEX4T3 to get plasmid pGEX4T3-*ptpA*^{Y128AY129A}.

2.3. Protein expression and purification

Recombinant plasmids (Table S1) were transformed into *E. coli* BL21 (DE3) for protein expression. The overnight culture was diluted 1:100, and the cells were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when OD_{600} reached 0.6–0.8. His-tagged proteins were purified by Ni-NTA (Ni^{2+}-nitrilotriacetate) resin affinity chromatography (Qiagen) and GST (Glutathione transferase)-tagged proteins were purified by Glutathione HiCap Matrix resin (Qiagen) according to the manufacturer's protocol.

2.4. PtpA phosphatase activity assay

PtpA phosphatase activity was determined using *p*-nitrophenyl phosphate (pNPP) as chromogenic substrate according to published protocols [14,19]. 4, 4.5 or 6 μM of purified wild type PtpA or mutant PtpA recombinant protein was added to the reaction buffer (20 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM DTT). When necessary, 0.55–1.65 μM of purified wild type PtkA, mutant PtkA or 0.4–1.2 μM of purified PknA recombinant protein was added to estimate its effect on PtpA phosphatase activity. Adenosine Triphosphate (ATP) and pNPP were added to a final concentration of 0.33 mM and 1 mM, respectively, to start the reaction. Absorbance at 405 nm was monitored over time using a BioTek™ Epoch™ Microplate spectrophotometer. Each reaction was performed in triplicates and the data was read at 20 min to compare their phosphatase activity.

2.5. In vitro kinase assay

In vitro kinase assay was performed according to published protocols [16,20]. Briefly, purified recombinant PtpA was incubated with [γ-32P] ATP (Perkin Elmer) in reaction buffer: 20 mM Tris–HCl, pH 7.5, 5 mM $MnCl_2$, 5 mM $MgCl_2$ and 1 mM DTT. The reactions were started by the addition of 1 μCi of [γ-32P] ATP, and the incubation was performed at room temperature (25 °C). At the end of the incubation period, reactions were stopped by the addition of SDS-sample loading buffer and heated at 95 °C for 10 min. Samples were resolved by 12% SDS–PAGE containing 8 M urea, and the gels were silver stained and dried. The ^{32}P-radioactively labeled protein bands were detected using a PhosphorImager SI (Molecular Dynamics). Bands corresponding to the phosphorylated proteins were excised and subjected to scintillation count when appropriate (Beckman Coulter LS 6500). The data was then used for enzyme kinetic calculation.

2.6. Phosphoamino acid analysis

PknA phosphorylated PtpA was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto 0.45 μm PVDF membranes. Phosphoamino acid analysis was performed as described [12], using cellulose plates, resolved in one dimension with isobutyric acid/0.5 M NH_4OH (5:3 v/v).

2.7. Mycobacterial protein–protein interaction assay

Mycobacterial protein fragment complementation assay was used to detect protein–protein interactions in *M. smegmatis* as previously described [21]. Genes of interest were PCR-amplified and cloned into pUAB100 (expressing mDHFR fragment F1, 2) or pUAB200 (expressing mDHFR fragment F3). *M. smegmatis* was co-transformed with both plasmids, and the co-transformants were selected on 7H11 agar plates with 25 μg/mL kanamycin and 50 μg/mL hygromycin B and tested for growth over 3–4 days on 7H11 kanamycin/hygromycin plates supplemented with 10 μg/mL and 30 μg/mL trimethoprim.

3. Results

3.1. PtkA enhances PtpA activity in vitro

To monitor the effect of PtpA phosphorylation by PtkA, we measured PtpA phosphatase activity in the presence of either recombinant PtkA or the auto-phosphorylation deficient mutant, $PtkA^{Y262A}$ [16]. We found that PtkA is capable of enhancing PtpA phosphatase activity in a dose-dependent manner with possible saturation effect at higher concentrations (Fig. 1A). Incubation of PtpA with $PtkA^{Y262A}$ failed to activate PtpA phosphatase activity (Fig. 1B), compared to incubation with intact PtkA (Fig. 1A).

3.2. Both Tyr^{128} and Tyr^{129} together are essential for PtpA phosphatase activity

To assess the PtpA tyrosine phosphorylation effect on PtpA phosphatase activity, we constructed by SDM two variants of PtpA: a phospho-ablative $PtpA^{Y128AY129A}$ and a phospho-mimicking vari-

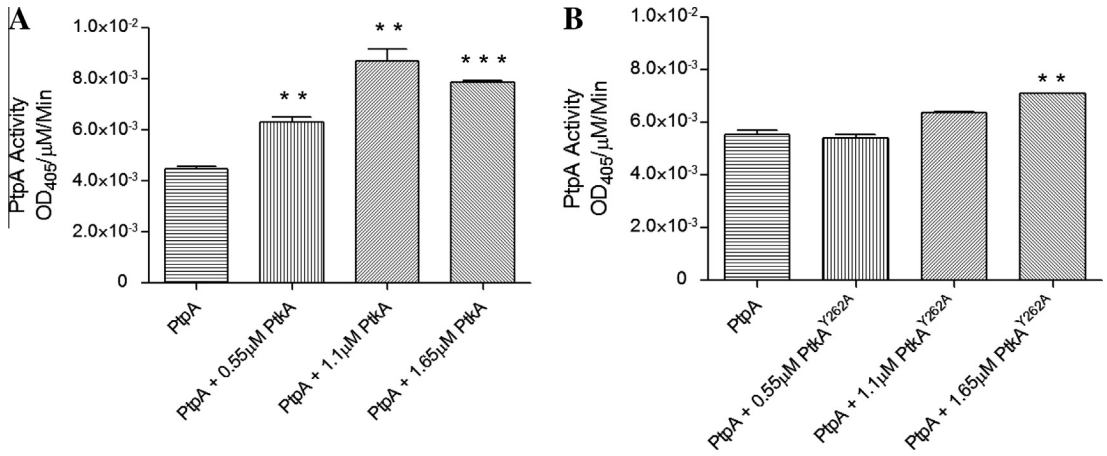

Fig. 1. PtkA enhances PtpA phosphatase activity *in vitro*. (A) Recombinant PtkA enhances PtpA phosphatase activity. (B) PtkA^{Y262A} contribution to PtpA phosphatase activity. 1.65 μM of PtkA or PtkA^{Y262A} were used as negative. 4.5 μM of PtpA was used in all other reactions. Values are the mean ± S.D. of PtpA phosphatase activity from three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ (significant difference compared to PtpA alone by Student *t* test).

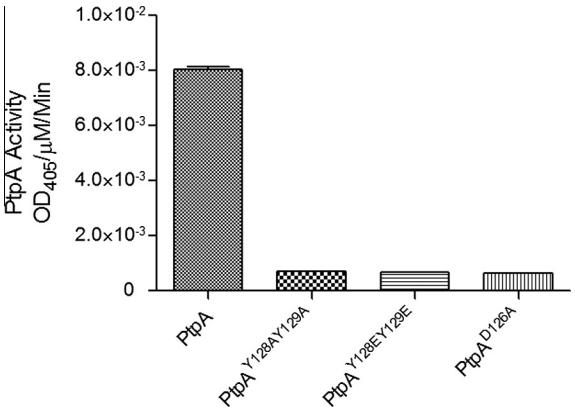

Fig. 2. Both Tyr^{128} and Tyr^{129} together are essential for PtpA phosphatase activity. Purified recombinant GST-tag PtpA or SDM proteins (4 μM) were used to test their phosphatase activity by using p-nitrophenyl phosphate (pNPP) as a substrate. Values represent mean ± S.D. of PtpA phosphatase activity from three independent experiments. $P < 0.001$ (significant difference compared to PtpA alone by Student *t* test).

ant of PtpA, PtpA^{Y128EY129E}. Interestingly, both these mutants were shown to be completely inactive in a phosphatase activity assay, similarly to the catalytically deficient mutant PtpA^{D126A} (Fig. 2).

3.3. Multi-STPKs phosphorylate and interact with PtpA

Protein kinases often phosphorylate multiple substrates, which in turn can serve as substrates to other kinases [15]. *Mtb* possess a set of 11 protein serine/thereonine kinases (STPKs) shown to control multiple processes in this pathogen [22]. To check whether PtpA can be phosphorylated by *Mtb* STPKs, we have cloned and expressed in *E. coli* recombinant proteins from all 11 kinase. We then performed auto phosphorylation assays and kinase assays with PtpA as a substrate. Two of the kinases, PknI and PknJ fail to show kinase activity and another two PknF and PknK were degraded to phosphorylated fragments in similar size to PtpA preventing us from properly analyzing their phosphorylation effect on PtpA. As seen in Fig. 3, we found that PknA, PknB, PknD, PknH, PknE and PknL can phosphorylate PtpA in an *in vitro* kinase assay. PknG did not phosphorylate PtpA.

Further analysis of protein–protein interactions using the mycobacterial protein fragment complementation assay (Fig. 4) clearly show that PtpA interact with seven out of the 10 examined kinases. PknI, PknJ and PknL failed to interact with PtpA. These results suggest that PtpA can potentially bind selected members of the STPKs family of proteins.

3.4. PknA enhances PtpA phosphatase activity through Thr^{45} phosphorylation

Protein phosphorylation is often linked to regulation of enzymatic activity. We tested the effect of STPKs phosphorylation on PtpA phosphatase activity. We found that PknA, PknB and PknH but not PknD and PknL (data not shown), enhanced PtpA phosphatase activity. However, due to cross activity whereby PknB, and PknH are also able to phosphorylate PtkA itself in addition to PtpA, we chose to focus our follow up studies on PknA contribution to PtpA activity. As seen in Fig. 5A, PknA enhance PtpA activity. PknA phosphorylate PtpA in dose and time dependent manner (Fig. 5B). Phosphoamino acid analysis confirmed that the phosphorylation sites of PknA on PtpA are composed of threonine residues (Fig. 5C).

To assess the contribution of PtpA threonine residues to the protein activity, we replaced all its seven-threonine residues with alanine residues using SDM. As seen in Fig. 6A, phosphatase activity of these SDM variants was compared with intact PtpA. We found that PtpA^{T12A} phosphatase activity was significantly reduced, while PtpA^{T41A} and PtpA^{T45A} were slightly attenuated compared to intact PtpA. The PtpA^{T8A}, PtpA^{T69A}, PtpA^{T78A} and PtpA^{T119A} mutant proteins were not significantly affected in their phosphatase activity (Fig. 6A).

In vitro kinase assay using PknA as an enzyme, and each of the recombinant SDM variants as substrates, suggests that Thr^{45} is the phosphorylation site for PknA on PtpA (Fig. 6B). Although PtpA^{T45A} is still an active phosphatase (Fig. 6A), T^{45} is important for PtpA enzyme catalytic activity on PtpA as it was shown that PknA enhance PtpA phosphatase activity by T^{45} phosphorylation.

Comparative enzyme kinetics analysis of PtkA and PknA on PtpA phosphorylation, determined a V_{max} of 9.2×10^{-8} μM Min^{-1} μM^{-1} and K_m of 4.379 μM for PtkA (Table 1). In comparison, PknA shows a V_{max} of 1.097×10^{-5}μM Min^{-1} μM^{-1} and K_m of 0.2590 μM for PtpA phosphorylation (Table 1).

4. Discussion

We have shown earlier that PtpA serves as a cognate substrate for PtkA [16] and auto-phosphorylation has been shown before to self regulates kinase activity [23]. Here, we extended this study and showed that PtpA can undergo phosphorylation by multiple

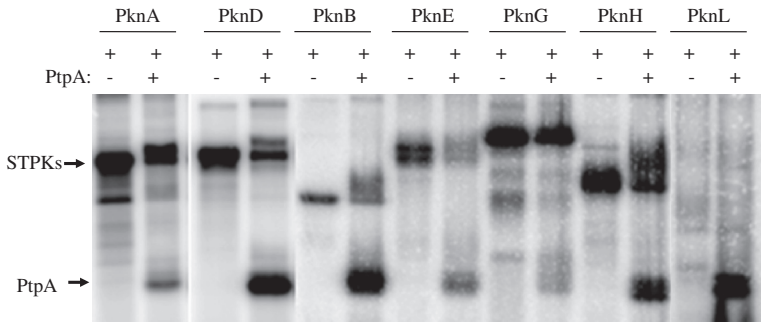

Fig. 3. Multi-STPKs phosphorylate PtpA *in vitro*. Seven of eleven recombinant kinase domains of STPKs encoded by the *Mtb* genome were expressed and purified and incubated with recombinant PtpA (2.82 μg) and γ-[^{32}P] ATP. STPKs levels varied from 0.15 μg to 8.1 μg to obtain visible autophosphorylation activity for each kinase. Samples were separated by 8% SDS–PAGE and visualized by autoradiography after overnight exposure to a film. The upper bands illustrate the auto-kinase activity of each STPK, and the lower bands represent phosphorylated PtpA.

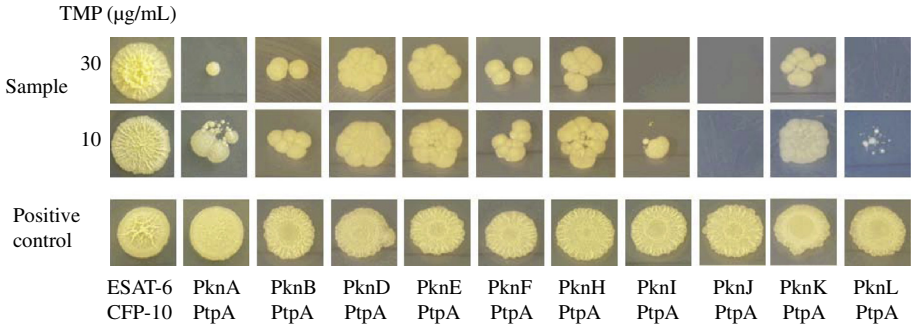

Fig. 4. Multi-STPKs interact with PtpA by M-PFC protein–protein assay *in vivo*. The interaction between the indicated kinases and PtpA were performed as described in materials and methods section. Growth in the presence of TMP suggests interaction between the two indicated proteins.

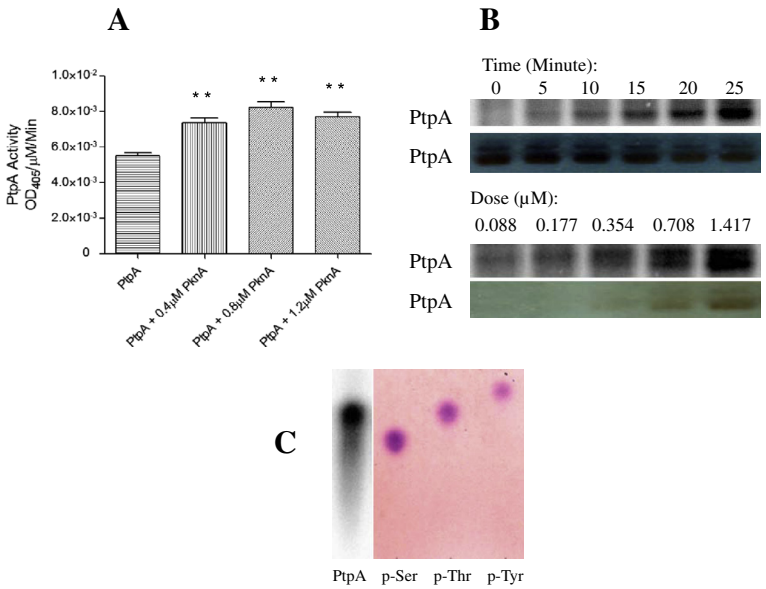

Fig. 5. PknA enhances PtpA phosphatase activity on threonine residues. (A) PknA enhances recombinant PtpA phosphatase activity. 4 μM of PtpA protein were used in each reaction. Values are the mean ± S.D. of PtpA phosphatase activity from three independent experiments. $^{**}P < 0.01$ (significant difference compared to PtpA alone by Student *t* test). (B) PknA phosphorylates PtpA in Time- and dose-dependent manner. The upper image show phosphorylated PtpA, and the lower image represents the silver stained PtpA. The number of dose belongs to PtpA. (C) PknA phosphorylates PtpA on Threonine residue. Control phospho-Tyr, -Thr, and -Ser were visualized by spraying with ninhydrin and radiolabeled PtpA residues were visualized by phosphor-imaging. Retention factors (RF) were calculated: p-Ser, 0.32; p-Thr, 0.38; p-Tyr 0.41; PtpA, 0.37.

Mtb kinases and those in turn can effect its enzymatic activity. PtkA phosphorylation on tyrosine residues enhances PtpA phosphatase activity which is completely lost when both Tyr^{128} and Tyr^{129} were replaced with either alanine or glutamate residues. This suggests that both the tyrosine residues together are intrinsically essential to PtpA activity. Nevertheless, it is possible that because these key amino acids are in the proximity of the active site, the introduction of the mutation could alter the structural characteristics of the protein such as charge distribution and hydrophobicity [24], which in turn affect its catalytic activity.

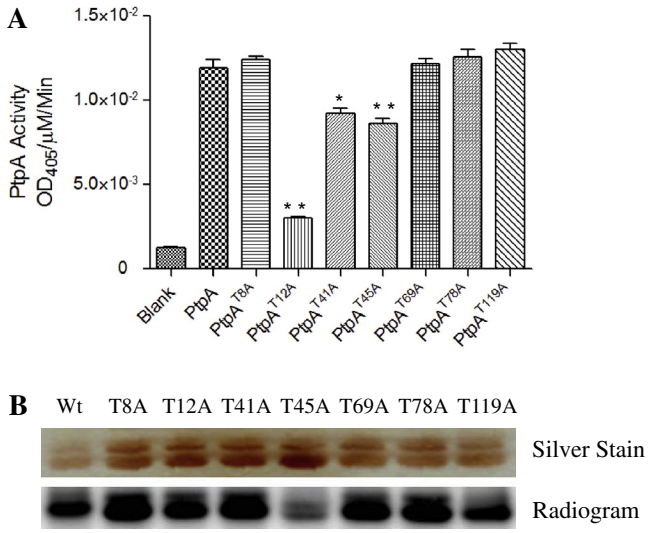

Fig. 6. Threonines involves in PtpA phosphatase activity. (A) Contribution of Threonine residues to PtpA phosphatase activity. Purified recombinant PtpA or SDM proteins (6 μM) were tested using p-nitrophenyl phosphate (pNPP) as a substrate. Values are the mean ± S.D. of PtpA phosphatase activity from three independent experiments. $^{*}P < 0.1$; $^{**}P < 0.01$ (significant difference compared to PtpA alone by Student *t* test). (B) PknA phosphorylate PtpA on Thr[45].

Table 1
Enzyme kinetics parameters of PknA and PtkA on PtpA.

	V_{max} ($\mu M\ Min^{-1}\ \mu M^{-1}$)	K_m (μM)	K_{cat} (S^{-1})	K_{cat}/K_m ($M^{-1}\ S^{-1}$)
PtkA (PtpA)	9.2×10^{-8}	4.379	1.53×10^{-9}	3.08×10^{-4}
PknA (PtpA)	1.097×10^{-5}	0.2590	1.828×10^{-7}	7.058×10^{-1}

Additionally, we have shown that PtpA can interact with and be phosphorylated by multi-STPKs. One of these STPKs, PknA can enhance PtpA phosphatase activity through phosphorylation of Thr[45]. Interestingly, PknA and PtkA, represent two different families of protein kinases in *Mtb*, and are both able to regulate PtpA phosphatase activity through phosphorylation of different amino acids, suggesting a possible role for multiple stages of regulation. Under *in vitro* conditions, PknA show higher effect on PtpA catalytic activity and has stronger affinity compared to PtkA. These two different kinases represent not only two different signal transduction families but also diverse physiological role in *Mtb*. PknA is predominantly expressed during exponential growth and is an essential for *Mtb* growth and control of cell division and morphology [25,26]. On the other hand, the *ptk*A gene is located within the same operon with *ptp*A and both genes may even be co transcribed. However, since PtpA was shown to be secreted and act on host macrophages proteins, a possible regulation of protein secretion or activity prior and during the secretion process should not be ruled out.

Signal transduction in *Mtb* is mediated primarily by the Ser/Thr kinases and the two components (TCs) families of proteins [27]. We have previously shown convergence of these two families in the process of controlling the DosR regulon in *Mtb* [17]. Here we show for the first time, that tyrosine phosphorylation, controlled by yet another family of functionally distinctive protein kinases can also converge with the STPKs. Overall, the key novel findings of this paper is that PtpA undergo phosphorylation by two different kinases suggesting delicate control of activity related to substrate specificity, or other enzyme characteristics such as stability and secretion into the macrophage. Future studies are needed to address the role of PtpA phosphorylation in each of the physiological processes it controls within the *Mtb* infected macrophage and expand our understanding as to the hierarchy of protein phosphorylation events in determining enzymatic and disease related activities.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.12.015.

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