ACCELERATED PUBLICATION

Mycobacterium tuberculosis PtkA is a novel protein tyrosine kinase whose substrate is PtpA

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In *Mycobacterium tuberculosis*, signal transduction is mediated by 11 serine/threonine kinases, but no tyrosine kinases have been identified thus far. The protein encoded by the ORF (open reading frame) *Rv2232* has been annotated as a member of the HAD (haloacid dehydrogenase-like hydrolase) superfamily, which includes phosphatases, phosphomanno- and phosphoglucomutases, and haloacid dehydrogenases. In the present paper, we report, on the basis of biochemical and mutational analyses, that the *Rv2232*-encoded protein, named protein tyrosine kinase A (PtkA) is a *bona fide* protein tyrosine kinase. The cognate substrate of PtkA is the secreted protein tyrosine phosphatase A (PtpA).

Key words: HAD superfamily, *Mycobacterium tuberculosis*, signal transduction, tyrosine phosphatase, tyrosine kinase.

INTRODUCTION

Protein phosphorylation is a versatile and sophisticated regulatory mechanism for cell adaptation to changing environments in both prokaryotic and eukaryotic organisms [1]. Protein kinases are the key mediators of phosphorylation signal transduction that transfer a phosphate group from a donor to an acceptor amino acid in a substrate protein. Protein kinases modulate the activity of substrate proteins by nucleoside triphosphate-dependent phosphorylation of specific serine, threonine and tyrosine residues. Dephosphorylation is mediated by protein phosphatases, which reset the system for the next signalling event.

Signal transduction in *Mycobacterium tuberculosis* involves 11 two-component systems driven by histidine kinases [2], 11 eukaryotic-like serine/threonine kinases [3] and one serine/threonine phosphatase [4]. In addition, *M. tuberculosis* produces and secretes two protein tyrosine phosphatases, PtpA and PtpB [5,6]. Recently, we showed that PtpA interferes with the signal transduction pathway of the host macrophage by dephosphorylating Vps (vacuolar protein sorting) 33B, a protein involved in the host vesicle trafficking [6]. Although other prokaryotes contain tyrosine kinases [7], none have been identified in mycobacteria. However, we have preliminary evidence suggesting the existence of a *M. tuberculosis* tyrosine-phosphorylated protein based on Western blot analysis using the anti-phosphotyrosine antibody 4G10 [8].

Our previous studies indicate that genes encoding serine/threonine protein kinases and phosphatases are frequently proximal to genes encoding substrates for those enzymes [3]. The *Rv2232* gene is clustered with the gene encoding PtpA and encodes a protein annotated as a member of the HAD (haloacid dehalogenase-like hydrolase) superfamily, which catalyse the hydrolysis of different molecules. With more than 3000 sequenced proteins, this superfamily comprises hydrolases such as phosphatases, dehalogenases, ATPases, phosphonatase (P-C bond hydrolysis) and sugar phosphomutases [9,10]. In the present study, we show that the *Rv2232* encodes a novel protein tyrosine kinase able to phosphorylate PtpA.

EXPERIMENTAL

Gene cloning and production of proteins

The *Rv2232* gene from *M. tuberculosis* H37Rv was amplified from genomic DNA using oligonucleotides detailed in Supplementary Table S1 (http://www.BiochemJ.org/bj/420/bj4200000add.htm). The fragment was purified and subsequently cloned into pET151D/TOPO (Invitrogen). The *Rv2232*/pET was then transformed into *Escherichia coli* DH5α cells, and, once the identity of the gene was confirmed by sequencing, it was transformed into *E. coli* BL-21 (DE3) cells for protein expression. The parental *Rv2232*/pET plasmid was used as a template to produce the mutated proteins by site-directed mutagenesis using the oligonucleotide-overlapping method [11]. Mutations were confirmed by DNA sequencing. PtpA was cloned into pALACE to obtain His_6-tagged protein as described earlier [6]. In order to check protein–protein interactions, PtpA was expressed fused to GST (glutathione transferase) after subcloning the gene into pGEX-4T3 (GE Healthcare) using BamHI and SalI restriction enzymes. Oligonucleotide sequences and vectors used in this work are listed in Supplementary Table S1. Luria–Bertani medium supplemented with 100 μg of ampicillin was used in all the bacterial cultures.

Purification of proteins and biochemical assays

Overnight starter cultures grown in a shaker at 37 °C were prepared by picking a single colony corresponding to *E. coli* BL-21 (DE3) strains harbouring the parental or mutated *Rv2232*/pET plasmids. The next day, the culture was diluted 1:100, and the cells were induced with 0.4 mM IPTG (isopropyl β-D-thiogalactoside) after reaching a D_{600} of 0.6–1.0. Induced cells were shaken overnight at room temperature (?? °C), harvested and stored at −20 °C until further processing. Proteins were purified by affinity chromatography using an Ni-NTA (Ni^{2+}-nitrilotriacetate) resin (Qiagen) following the instructions of the manufacturer. Kinase assays were carried out using [γ-^{32}P]ATP or [γ-^{32}P]GTP as the phosphate donor according to published protocols [12].

Abbreviations used: GST, glutathione transferase; HAD, haloacid dehydrogenase-like hydrolase; ORF, open reading frame; Ptk, protein tyrosine kinase; Ptp, protein tyrosine phosphatase.

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Proteins were separated by SDS/PAGE (12%) supplemented with 8 M urea and silver-stained. The incorporation of ^{32}P was measured either by a scintillation apparatus (Beckman) after drying the gel and excising the bands, or by autoradiography of the dried gel using a phosphoimager apparatus. Phosphatase assays were carried out by monitoring the hydrolysis of *p*-nitrophenyl phosphate according to published protocols [13]. The hydrolysis of β-glucose or mannose 1- and 6-phosphate was determined by monitoring the reduction rate of $NADP^+$ to NADPH at 340 nm according to published protocols [14]. Kinase inhibitors were supplemented to the kinase reaction at the following concentrations: staurosporine (100 nM), tartaric acid (10 mM), wortmannin (1 nM), methyl piperazine (100 nM) and phenyl piperazine (100 nM).

Phospho-amino acid analysis

Recombinant PtkA (protein tyrosine kinase A) was incubated with [γ-^{32}P]ATP in a kinase buffer. The sample was hydrolysed with 6 M HCl at 110 °C for 1 h and separated on a cellulose TLC plate following ascending chromatography [12]. For PtpA phosphorylation by PtkA, the reaction was stopped with sample buffer, loaded on to a SDS/12% PAGE gel containing 8 M urea, and electrophoresed. The gel was electroblotted on to an Immobilon PVDF membrane [15]. Phosphorylated proteins bound to the membrane were detected by autoradiography using a phosphoimager apparatus. The ^{32}P-labelled protein band corresponding to the migration of PtpA was excised from the membrane and hydrolysed following the same procedure as described above. After hydrolysis, samples were separated by TLC following ascending chromatography [12]. After migration, radioactive amino acids were detected by autoradiography. Phosphoserine, phosphothreonine and phosphotyrosine standards were also separated on a cellulose TLC plate in parallel and visualized by staining with ninhydrin.

Protein–protein interaction

The interaction between proteins was measured using a Fusion® αHT AlphaScreen apparatus (PerkinElmer). Reactions were prepared using the AlphaScreen histidine-detection kit according to the manufacturer's instructions. PtpA–GST proteins were biotinylated (Pierce) and coupled to the donor beads, and His_6-tagged PtkA was used for coupling to the acceptor beads.

RESULTS

PtpA interacts with the protein encoded by *Rv2232*

As described above, genes in the proximity of *M. tuberculosis* kinases and phosphatases are proposed to act as substrates or be under the control of these regulatory proteins. We used AlphaScreen technology to measure the interaction between PtpA and *Rv2232*. As shown in Figure 1(A), the protein encoded by *Rv2232* binds PtpA with a strong affinity ($K_d = 3\ \mu M$). Interestingly, in the presence of ATP, a stronger affinity was measured as reflected by a K_d of 1.268 μM. GTP was also a phosphate donor with a K_d of 1.740 μM. These results suggest that: (i) the presence of a phosphate donor increases the interaction affinity of the protein encoded by *Rv2232* to PtpA; (ii) GTP serves as an alternative phosphate donor; and (iii) ATP is the preferred substrate based on the lower K_d.

ORF (open reading frame) *Rv2232* encodes an autophosphorylated tyrosine protein kinase

The close proximity of *ptp*A and *Rv2232* suggests that the *Rv2232*-encoded protein might serve as a substrate for tyrosine phosphatase PtpA. Therefore the phosphorylation status of *Rv2232* was checked. Indeed, *in vitro* phosphorylation assays using purified recombinant protein expressed from ORF *Rv2232* revealed that this protein possesses time- and ATP-dependent autophosphorylation activity (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/420/bj4200000add.htm).

To determine the identity of the phosphorylated residues, a phospho-amino acid analysis of radiophosphorylated protein was performed under acidic conditions. As shown in Figure 1(B), this protein was found to be phosphorylated on tyrosine residues. These data identify the protein encoded by *Rv2232* as the first protein tyrosine auto-kinase found in *M. tuberculosis*, and thus it was renamed PtkA. The autophosphorylation activity of PtkA is not affected by the presence of a variety of kinase inhibitors such as staurosporine, tartaric acid, wortmannin and methyl and phenyl piperazine (results not shown).

Table 1 Autophosphorylation kinetic values of parental and mutated PtkA

PtkA	V_{max} (nmol · min^{-1} · mg^{-1})	K_m (nM)	K_{cat} (s^{-1})	K_{cat}/K_m (M^{-1} · s^{-1})
Wild-type	1.331	27.22	9.73×10^{-4}	3.60×10^{4}
Y146A	1.877	54.20	1.40×10^{-3}	2.59×10^{4}
Y150A	1.967	68.20	1.47×10^{-3}	2.16×10^{4}
Y262A	0.8545	59.08	6.38×10^{-4}	1.08×10^{4}
D85A	0.5326	118.2	3.98×10^{-4}	3.40×10^{3}
K184M	9.08×10^{-8}	40.24	6.79×10^{-11}	1.68×10^{-3}
K217M	0.1166	89.22	8.71×10^{-5}	9.76×10^{2}
K270M	0.302	378.6	2.26×10^{-4}	5.97×10^{2}

Sequence–function analysis of PtkA

Bacterial protein tyrosine kinases signatures, termed Walker A and B motifs [16], and the glycine-rich loop GXGXXGXV motif [17] are not present in PtkA. As we were not able to find any conserved kinase signature after *in silico* analysis of PtkA (see Supplementary Table S2 at http://www.BiochemJ.org/bj/420/bj4200000add.htm), we constructed a series of mutants to identify key functional residues in the protein. We replaced all three lysine residues in *Rv2232*, rationalizing that they are required for ATP binding. We also replaced all the three tyrosine residues to determine potential phosphorylation sites, as well as the first aspartate (Asp^{85}) in the DXD motif, which is needed for catalytic activity in the HAD superfamily members [14]. All of the mutated proteins were tested individually for their ability to undergo autophosphorylation by means of [γ-^{32}P]ATP incorporation in an *in vitro* kinase assay (Table 1).

Mutation of lysine residues

Binding of ATP in an enzymatic autophosphorylation reaction depends on lysine residues [18]. Thus we constructed in PtkA the mutants K184M, K217M and K270M and found that indeed all the mutants were unable to bind ATP in a dose-dependent manner (Figure 1C). Moreover, an associated 2–14-fold increase in the K_m values suggests a decrease in the affinity of these mutants for ATP as detailed in Table 1.

Mutation of tyrosine residues

We have shown in Figure 1(B) that PtkA is phosphorylated on tyrosine residues. To precisely map the tyrosine phosphorylation site, Y146A, Y150A and Y262A mutants were constructed. Only the Y262A mutant failed to undergo autophosphorylation, whereas Y146A and Y150A incorporated ^{32}P in a dose-dependent manner (Figure 1C). These results indicate that Tyr^{262} is the

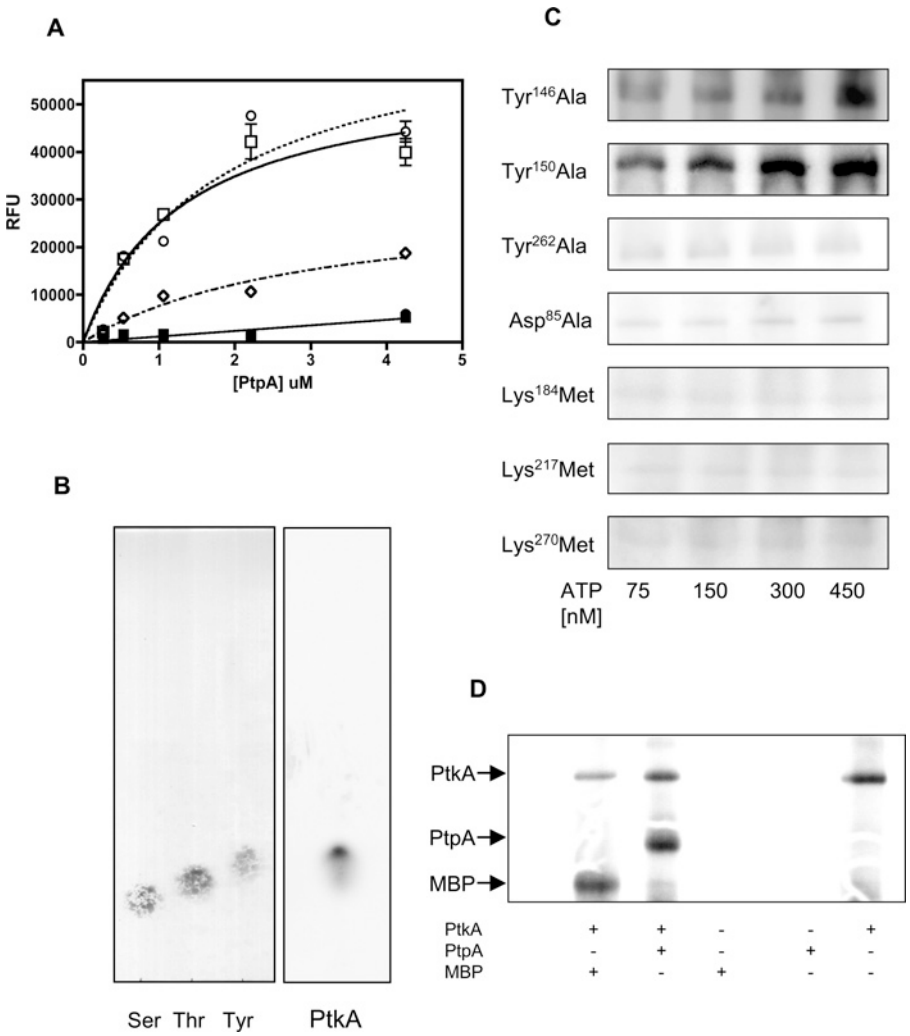

Figure 1 PtkA is a tyrosine kinase that interacts with PtpA

(**A**) Protein–protein interaction between PtkA and PtpA was measured using AlphaScreen technology. The K_d was calculated using biotinylated PtkA and His_6-tagged PtpA in presence or absence of 50 μM ATP or GTP. GST was used as negative control. K_d values (μM) of the interaction are given in parentheses. Open symbols represent interaction of PtpA and PtkA: □, PtkA + ATP (1.302); ○, PtkA + GTP (1.792); ◇, PtkA control without triphosphate added (3.004). Solid symbols represent the interaction of GST (negative control) and PtkA: ■, PtkA + ATP (2.9×10^7); ●, PtkA + GTP (no interaction); ◆, PtkA control without triphosphate added (no interaction). RFU, relative fluorescence units. Results are means ± S.D. for three independent experiments. (**B**) PtkA is phosphorylated on tyrosine residues as revealed by phospho-amino acid analysis; the migration of hydrolysed protein was detected by TLC against standards (R_F: phosphoserine = 0.22; phosphothreonine = 0.25; phosphotyrosine = 0.30; PtkA = 0.29). (**C**) Autophosphorylation activity of a series of point mutants of PtkA in increasing concentrations of [γ-^{32}P]ATP. (**D**) Gel autoradiography showing the phosphorylation of MBP (myelin basic protein) and PtpA by PtkA. [γ-^{32}P]ATP was used as phosphate donor.

autophosphorylated tyrosine in the PtkA backbone. Interestingly, the affinity of these proteins for ATP binding and the enzymatic efficiency of ^{32}P incorporation were not affected as reflected by similar values measured for K_m and K_{cat}/K_m respectively (Table 1).

Mutation of Asp^{85}

The mutation D85A, which represents the first aspartate residue in the conserved DXD motif within the HAD superfamily, was found to be essential for PtkA autophosphorylation activity (Figure 1C). This finding is supported by a considerable decrease in the enzymatic efficiency according to the measured K_{cat}/K_m for this mutant, as well as by the significant decrease in the ATP-binding affinity (Table 1).

PtpA is a substrate of PtkA

To test whether PtkA can serve as a substrate for PtpA, PtpA was supplemented in the autophosphorylation reaction. Interestingly, we found the opposite of the hypothesized relationship. PtkA autophosphorylation did not decrease upon addition of PtpA. Moreover, PtpA acted as a substrate of PtkA. As shown in Figure 1(D), PtkA phosphorylates PtpA (lane 2) and the generic tyrosine kinase substrate MBP (myelin basic protein) (lane 1). Moreover, PtkA phosphorylated PtpA in an ATP dose- and time-dependent manner (Figures 2A and 2B), demonstrating that the tyrosine phosphatase PtpA is a cognate substrate of PtkA.

To determine the amino acid residues affecting the interaction between PtkA and PtpA, we assayed the PtkA mutants in an AlphaScreen assay. Results listed in Table 2 show that mutants K184M, Y146A and Y150A interacted with PtpA as reflected with a K_d ranging between 0.85 and 1.5 μM. This range is similar to the K_d of 1.27 μM obtained for the wild-type protein. However, K217M, K270M, Y262A and D85A showed K_d values between 3- and 6-fold higher compared with PtkA in its native form ($K_d = 1.27$ μM) (Table 2). These results support the finding that Tyr^{262} is the phosphorylated residue ($K_d = 6.6$ μM for Y262A),

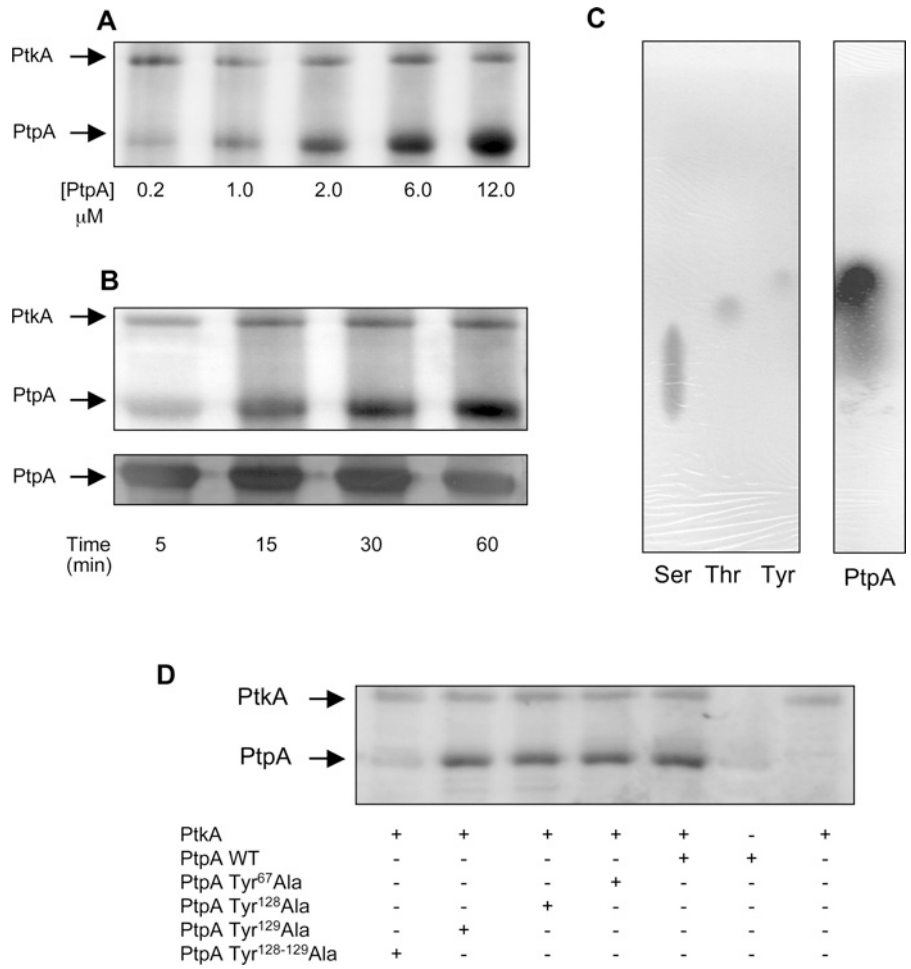

Figure 2 PtpA is a cognate substrate of PtkA

PtkA phosphorylates PtpA in ATP dose- (**A**) and time- (**B**) dependent manner. The lower image in (**B**) represents the silver-stained SDS/PAGE gel. (**C**) PtpA is phosphorylated at tyrosine residues as shown by phospho-amino acid TLC analysis (R_F: phosphoserine = 0.49; phosphothreonine = 0.55; phosphotyrosine = 0.59; PtpA = 0.61). (**D**) Site-directed mutagenesis shows that PtpA is phosphorylated on both Tyr^{128} and Tyr^{129}. WT, wild-type.

since a 5-fold decrease in the interaction was measured compared with the wild-type protein ($K_d = 1.27\ \mu M$). In addition, Lys^{217} and Lys^{270}, with K_d values of 4.98 and 7.08 μM for K217M and K270M respectively, appear to be two residues involved in the ATP binding. The mutant D85A ($K_d = 3.32\ \mu M$) also suggests that Asp^{85} residue is involved in the reaction, since a 3-fold weaker interaction compared to the wild-type protein ($K_d = 1.27\ \mu M$) was measured and the increase in K_d is similar to the experiment where ATP was not added. We cannot accurately determine whether Lys^{184} is an essential amino acid for the catalytic activity of the enzyme, as its autophosphorylation was not observed (Figure 1D). Nevertheless, an interaction similar to the wild-type protein was measured ($K_d = 1.47\ \mu M$).

Table 2 K_d values of PtpA interacting with parental and mutated PtkA proteins

PtkA	PtpA	K_d [μM]
Wild-type	Wild-type	1.268
Y146A	Wild-type	0.965
Y150A	Wild-type	0.854
Y262A	Wild-type	6.604
D85A	Wild-type	3.319
K184M	Wild-type	1.466
K217M	Wild-type	4.983
K270M	Wild-type	7.079
Wild-type	Y128A/Y129A	1.243

PtkA can phosphorylate both Tyr^{128} and Tyr^{129} of PtpA

Phospho-amino acid analysis of PtkA-phosphorylated PtpA followed by TLC showed that PtpA is phosphorylated on tyrosine residues (Figure 2C). To determine the specific tyrosine-phosphorylated residue, all three tyrosine residues in PtpA were replaced by alanine residues to generate Y67A, Y128A and Y129A forms of the protein. All three PtpA mutants were able to incorporate ^{32}P (Figure 2D, lanes 2–5). The proximity of both Tyr^{128} and Tyr^{129} residues suggest that, upon mutation of one residue, phosphorylation would take place on the adjacent residue. We therefore constructed a double mutant where both Tyr^{128} and Tyr^{129} were mutated to alanine. PtkA failed to phosphorylate this double mutant (Figure 2D, lane 1), indicating that both residues may exist in the phosphorylated form. The binding affinity between PtkA and the PtpA Y128A/Y129A double mutant was not affected by the residue replacement, since the measured

K_d of 1.24 μM was very similar to the K_d of 1.27 μM obtained between PtkA and the parental PtpA.

PtkA does not possess phosphoglucomutase nor phosphatase activity

On the basis of published reports [14], and to rule out multiple activities of PtkA, we aligned the conserved Motif I of different families of the HAD superfamily [14] to PtkA. Results showed that the highest homology of the Motif I of PtkA was with β-phosphoglucomutase (see Supplementary Figures S2 and S3 at http://www.BiochemJ.org/bj/420/bj4200000add.htm). Therefore the enzymatic activity of PtkA towards phosphoglucose was assayed. In addition, when PtkA was analysed *in silico*, high homology with phosphatases was observed as well (see Supplementary Table S2). When the catalytic activity of PtkA was assayed using the phosphosugar and *p*-nitrophenyl phosphate substrates, no activity was observed, suggesting that PtkA does not possess β-phosphoglucomutase or phosphatase activity (results not shown).

DISCUSSION

As protein phosphorylation plays a fundamental role in a wide range of cellular processes, it is not surprising that a large number of structurally distinct protein kinases have evolved [19,20]. The HAD superfamily comprises enzymes with hydrolytic activities, but kinase activities have not been reported to date. In the present study, we provide evidence that, although *M. tuberculosis* PtkA was annotated as a HAD superfamily member, it exhibits authentic tyrosine kinase activity.

Although earlier evidence suggests that *M. tuberculosis* possesses tyrosine phosphorylation activity [8], post-genomic bioinformatic analysis failed to identify a corresponding tyrosine kinase. Extensive *in silico* analysis revealed that PtkA does not possess any signature or pattern related to tyrosine kinases. For instance, the Walker A and B motifs [16], which are present in bacterial autophosphorylating tyrosine kinases, are lacking in PtkA. However, our findings are in line with studies describing novel tyrosine kinases. These are exemplified by the MasK protein from *Myxococcus xanthus* [21], and WaaP from *Pseudomonas aeruginosa* [22], both of which do not have the conserved pattern homologous with classical tyrosine kinases [16], but both have been reported as self-phosphorylated tyrosine kinases. We therefore conclude that protein tyrosine kinase activity does not necessarily require the canonical tyrosine kinase motifs.

In the present study, we carried out several independent experiments to demonstrate tyrosine kinase activity of PtkA. These included the incorporation of ^{32}P on tyrosine residues, detection of the radioactive phosphotyrosine residues in a TLC autoradiogram, and mutational analysis of PtkA. These experiments showed that Tyr^{262} is the phosphorylated tyrosine residue of PtkA, while the other two tyrosine residues located at positions 146 and 150 were phosphorylated to the same extent as the parental protein. This finding suggests that Tyr^{146} and Tyr^{150} are not involved in the catalytic activity of PtkA.

In an attempt to determine other residues involved in the catalytic activity of the protein, we carried out mutational analyses on all three lysine residues of PtkA. Mutations of these residues showed no incorporation of ^{32}P, suggesting that these residues could be a part of the mechanism of protein activity. Since individual mutations of the three lysine residues reduced the enzymatic activity to nearly undetectable levels at this stage, we are not able to determine which one of these residues provides the hydrogen bonds to both the phosphate and the nucleophile [23].

Interestingly, we found that, although the K184A mutant was not phosphorylated, it binds PtpA similarly as the wild-type protein. The inability of all three lysine mutants to incorporate ^{32}P is in agreement with that reported for the tyrosine kinase Ptk of *Acinetobacter johnsonii*, where two lysine residues were shown to be involved in ATP binding [16].

The core mechanism of HAD proteins is the transfer of a phosphoryl group from a specific phosphate ester to an active-site aspartate residue, and then to a water molecule. The nucleophilic mechanism in HAD superfamily members is driven by the first aspartate residue in the DXD motif. To demonstrate that this residue is involved in the catalytic activity of PtkA, Asp^{85} was replaced by alanine. This mutated protein did not show any kinase activity, suggesting that this residue indeed is involved in the catalytic mechanism of the enzyme, and it supports further the essentiality of the DXD motif in the catalytic activity of the HAD superfamily members [9] and the inclusion of PtkA in this superfamily.

In retrospect, our finding that PtpA is the cognate substrate of PtkA is in agreement with the fact that PtpA was shown to be secreted and act on host proteins; it therefore seems unlikely that PtkA would serve as its substrate within the bacilli. Although preliminary reports have showed that *Rv2232* is up-regulated in SCID (severe combined immunodeficient) mouse model of infection [24], and in murine macrophages [25], the role of PtpA phosphorylation by PtkA still needs to be defined. Mutational analyses of PtpA showed that PtkA could phosphorylate two adjacent tyrosine residues of PtpA. This result is in concordance with human low-molecular-mass protein tyrosine phosphatase B expressed in T-lymphocytes, where two adjacent tyrosine residues can be phosphorylated alternatively by a tyrosine kinase [26]. Thus we hypothesize that the phosphorylation of PtpA might be needed to retain its activity within host macrophages. Alternatively, phosphorylation might be needed for PtpA secretion from the pathogen through the phagosome membrane to the host cytosol. Nevertheless, owing to the discrepancy between the molecular mass of PtpA, (approx. 18 kDa) and the reported tyrosine-phosphorylated 55 kDa protein [8], we do not rule out broader activity for PtkA, depending on differential environmental signals. PtpA and its secretion are essential to tuberculosis disease progression [6]. This, together with the large industrial knowhow, and small-molecule libraries of kinase inhibitors, suggest that PtkA might be an attractive target for drug development against this notorious disease.

To conclude, our data provide evidence that: (i) PtkA is a new tyrosine kinase belonging to the HAD superfamily; (ii) PtkA is able to alternatively use ATP or GTP as phosphate donors; (iii) PtkA neither possesses kinase signatures nor resembles a tyrosine kinase, but is autophosphorylated on tyrosine residues; and (iv) PtkA is able to autophosphorylate and transfer phosphate groups to the tyrosine phosphatase PtpA, although the role of PtpA phosphorylation in *M. tuberculosis* physiology remains to be determined.

ACKNOWLEDGEMENTS

We thank Rodgoun Attarian, Mary Ko and Jim Sun for technical assistance.

FUNDING

This research was supported by the Canadian Institute of Health Research [grant number MOP-68857 (to Y. A.-G.)] and TB Veterans Charitable Foundation (to Y. A.-G.).

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Received 24 March 2009/9 April 2009; accepted 15 April 2009
Published as BJ Immediate Publication 15 April 2009, doi:10.1042/BJ20090478

Biochem. J. (2009) **420**, 00–00 (Printed in Great Britain) doi:10.1042/BJ20090478

online data

SUPPLEMENTARY ONLINE DATA

Mycobacterium tuberculosis PtkA is a novel protein tyrosine kinase whose substrate is PtpA

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Table S1 Oligonucleotide sequences and vectors used in the present study

Vector or oligonucleotide	Characteristics
Vectors	
pET151D/TOPO (Invitrogen)	
pALACE [1]	*ace* promoter, hygromycin^r
Oligonucleotides	
Rv2232-forward	5′-atagatatcgtgtcttcgcctcgtgaac-3′
Rv2232-reverse	5′-taaaagcttctcgagtcagacacctagcgcctc-3′
Rv2232-D85A-forward	5′-cagctggtgatcttcgctctggacggcacgctg-3′
Rv2232-D85A-reverse	5′-cagcgtgccgtccagagcgaagatcaccagctg-3′
Rv2232-K184M-forward	5′-gccgtcgccacctccatggcagagccgaccgca-3′
Rv2232-K184M-reverse	5′-tgcggtcggctctgccatggaggtggcgacggc-3′
Rv2232-K217M-forward	5′-ggctcgcgaggcagcatggtcgacgtgctggcc-3′
Rv2232-K217M-reverse	5′-ggccagcacgtcgaccatgctgcctcgcgagcc-3′
Rv2232-K270M-forward	5′-ggcatctttatcgacatgacctccaccaccgtc-3′
Rv2232-K270M-reverse	5′-gacggtggtggaggtcatgtcgataaagatgcc-3′
Rv2232-Y146A-forward	5′-gaggcgatcgtagccgcccgggccgactacagc-3′
Rv2232-Y146A-reverse	5′-gctgtagtcggcccgggcggctacgatcgcctc-3′
Rv2232-Y150A-forward	5′-gcctaccgggccgacgccagcgcccgcggttgg-3′
Rv2232-Y150A-reverse	5′-ccaaccgcgggcgctggcgtcggcccggtaggc-3′
Rv2232-Y262A-forward	5′-gtggtcggctggggcgccgggcgcgccgacttt-3′
Rv2232-Y262A-reverse	5′-aaagtcggcgcgcccggcgccccagccgaccac-3′
PtpA-Y67A-forward	5′-ttgcgagcccacggcgcccctaccgaccaccgg-3′
PtpA-Y67A-reverse	5′-ccggtggtcggtagggggcgccgtgggctcgcaa-3′
PtpA-Y128A-forward	5′-gatgtcgaggatcccgcctatggcgatcactcc-3′
PtpA-Y128A-reverse	5′-ggagtgatcgccataggcgggatcctcgacatc-3′
PtpA-Y129A-forward	5′-gtcgaggatccctacgccggcgatcactccgac-3′
PtpA-Y129A-reverse	5′-gtcggagtgatcgccggcgatgggatcctcgac-3′
PtpA-Y128-129A-forward	5′-gatgtcgaggatcccgccgccggcgatcactccgac-3′
PtpA-Y128-129A-reverse	5′-gtcggagtgatcgccggcggcgggatcctcgacatc-3′

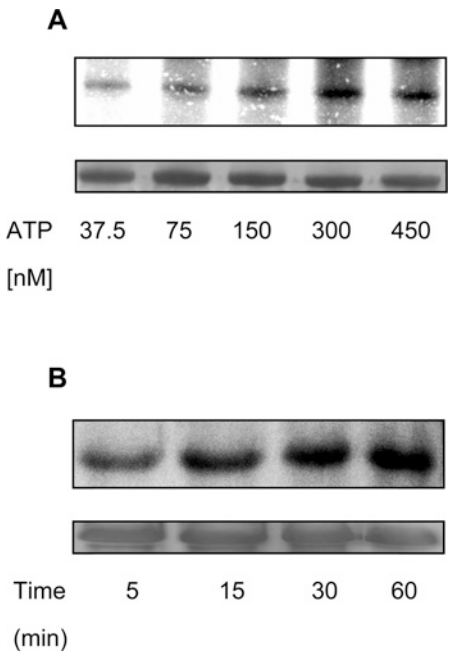

Figure S1 PtpkA autophosphorylation

Recombinant PtkA is autophosphorylated in an ATP dose- (**A**) and time- (**B**) dependent manner. The upper images show phosphorylation visualized by autoradiography, and the lower images represents the silver-stained gel.

Table S2 Sequence alignments of PtkA

Protein ID	Strain	Function	Accession number	Identity (%)	Expect
Mb2257	*Mycobacterium bovis*	Hypothetical	NP_855906.1	99	$6e^{-164}$
MMar3308	*Mycobacterium marimun* M	Hypothetical	ACC_41734.1	74	$3e^{-86}$
MSMEG4308	*Mycobacterium smegmatis* mc^{2}155	5′-nucleosidase	YP_888585.1	70	$2e^{-82}$
Mav2207	*Mycobacterium avium* 104	Hypothetical	YP_881415.1	76	$2e^{-79}$
Map1984	*Mycobacterium paratuberculosis* K10	Hypothetical	NP_960918.1	75	$1e^{-77}$
MAB1901c	*Mycobacterium abcessus*	Hypothetical	YP_0017026	62	$2e^{-66}$
RO01185	*Rhodococcus jostii*	Phosphoglycolate phosphatase	YP_701170.1	52	$2e^{-44}$

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COLOUR

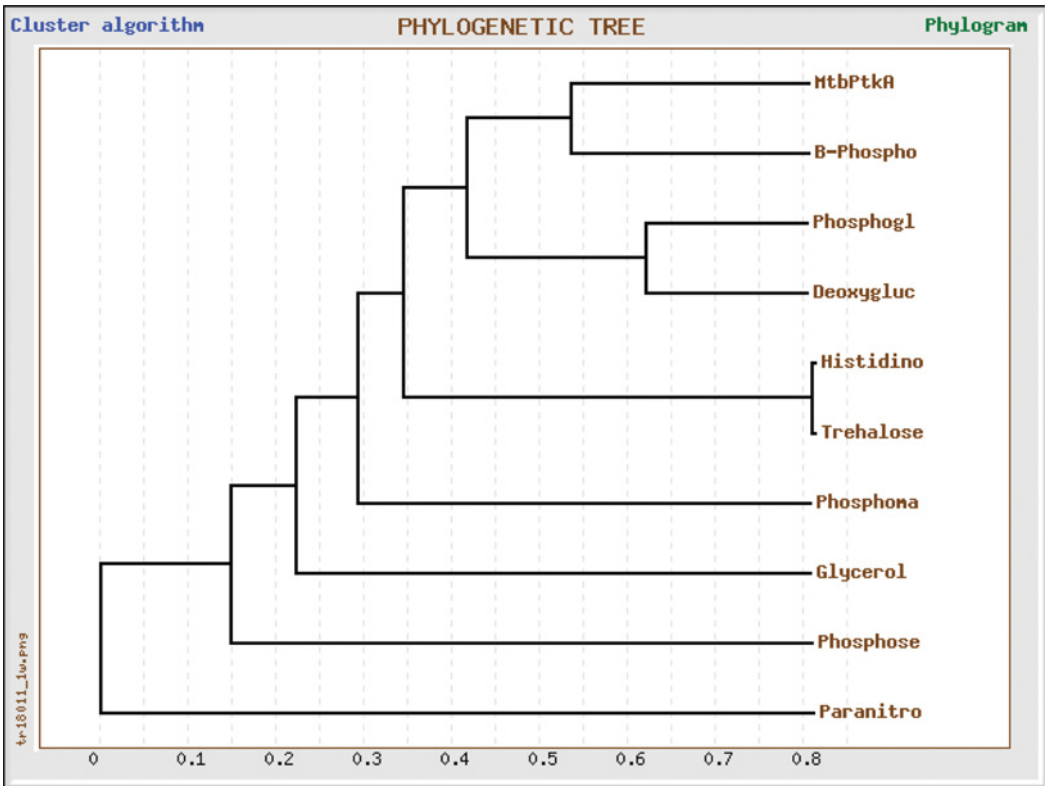

Figure S2 Phylogenetic tree of PtkA

A phylogenetic tree was created (http://www.genebee.msu.su) using the Motif I conserved in the HAD superfamily. The conserved motifs of each family are listed in Supplementary Figure S3.

COLOUR

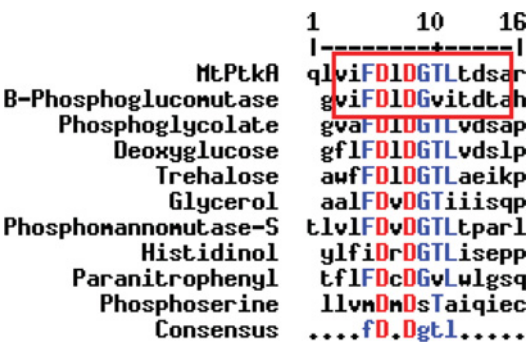

Figure S3 Sequence alignment of Motif I in the HAD superfamily

Motifs I from representative families [2] of the HAD superfamily were aligned. The highest homology with PtkA is marked in red and corresponds to the β-phosphoglucomutase family.

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Received 24 March 2009/9 April 2009; accepted 15 April 2009
Published as BJ Immediate Publication 15 April 2009, doi:10.1042/BJ20090478