Review

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2005;9:167–181
DOI: 10.1159/000089645

Structure/Function Studies of Ser/Thr and Tyr Protein Phosphorylation in *Mycobacterium tuberculosis*

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Key Words
Ser/Thr protein kinase · Protein tyrosine phosphatase · Protein kinase substrates

Abstract
Many bacterial species express 'eukaryotic-like' Ser/Thr or Tyr protein kinases and phosphatases that are candidate mediators of developmental changes and host/pathogen interactions. The biological functions of these systems are largely unknown. Recent genetic, biochemical and structural studies have begun to establish a framework for understanding the systems for Ser/Thr and Tyr protein phosphorylation in *Mycobacterium tuberculosis (Mtb)*. Ser/Thr protein kinases (STPKs) appear to regulate diverse processes including cell division and molecular transport. Proposed protein substrates of the STPKs include putative regulatory proteins, as well as six proteins containing Forkhead-associated domains. Structures of domains of receptor STPKs and all three *Mtb* Ser/Thr or Tyr phosphatases afford an initial description of the principal modules that mediate bacterial STPK signaling. These studies revealed that universal mechanisms of regulation and substrate recognition govern the functions of prokaryotic and eukaryotic STPKs. Several structures also support novel mechanisms of regulation, including dimerization of STPKs, metal-ion binding to PstP and substrate mimicry in PtpB.

Introduction

Normal cellular physiology requires metabolic responses to external and internal cues. Through reversible protein phosphorylation, protein kinases and phosphatases provide fundamental systems for environmental sensing and physiological signaling. Humans express over 500 putative Ser/Thr or Tyr protein kinases [Manning et al., 2002] and over 130 putative protein phosphatases [Alonso et al., 2004]. In contrast, through histidine phosphorylation and relays that involve protein-aspartyl phosphates, bacterial two-component kinases regulate processes including transcription, cell division, virulence and chemotaxis [Hoch, 2000; Galperin et al., 2001]. In

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addition, in concert with cognate phosphatases, sigma-factor inhibitors with Ser kinase activity regulate bacterial transcription [Hughes and Mathee, 1998]. Biochemical and genomic studies also revealed bacterial homologs of eukaryotic Ser/Thr and Tyr kinases [Zhang et al., 1992; Leonard et al., 1998; Kennelly, 2002]. Somewhat unexpectedly, genes for these 'eukaryotic-like' phospho-signaling systems have been found in dozens of bacterial genera, particularly in pathogens [Cozzone, 2005] or other species that grow in diverse environments.

The discovery of eukaryotic-like signaling systems – including Ser/Thr protein kinases (STPKs), Ser/Thr phosphatases, protein tyrosine phosphatases (PTPs) and proteins containing pThr-binding Forkhead-associated (FHA) domains – raised central questions about their physiological roles and biochemical mechanisms. These functions likely rest on key differences between His-Asp phospho-relays and Ser/Thr/Tyr phosphates. Phospho-ser, -thr and -tyr are much more stable chemically than phospho-asp and -his. At neutral pH, aspartyl-phosphate hydrolysis occurs with a half-time of a few hours, while the ser, thr and tyr phospho-esters are stable for weeks [Sickmann and Meyer, 2001]. Thus, while two-component signals are attenuated rapidly through hydrolysis of phosphoryl-asp, ser, thr and tyr phosphorylation can produce long-term signals that require phosphatases to be reversed on a biological time scale. In addition, residues such as ser and thr are relatively abundant and offer the scope to regulate many proteins and pathways. Some eukaryotic STPKs, for example, are thought to regulate hundreds of substrate proteins, and estimates suggest that as many as one third of eukaryotic proteins are phosphorylated [Meggio and Pinna, 2003]. Moreover, because ser, thr and tyr phosphorylation are ubiquitous in eukaryotes, many pathogens produce specific enzymes that modulate these host signaling pathways.

Koonin and co-workers presented two models for the evolution of the bacterial STPKs: divergence from a common ancestor or horizontal gene transfer from an early eukaryote [Leonard et al., 1998]. The relative paucity of STPK genes in archaea provided initial support for horizontal gene transfer, but this idea has been undermined by the discovery of additional STPK genes in recently sequenced archaeal genomes [Kennelly, 2003]. Many of the bacterial STPK genes encode predicted receptors with the N-terminal kinase domain inside the cell joined through a single predicted transmembrane helix to a C-terminal domain that presumably binds signaling ligands outside the cell. This unusual orientation is the opposite of that seen in most receptor kinases in metazoans. In contrast, the characteristic arrangement featuring an intracellular, N-terminal kinase domain occurs in the TGFβ receptor and in hundreds of receptor-like kinases in plants [McCarty and Chory, 2000]. This domain organization emphasizes that the origins of the bacterial receptor STPKs and the mechanism of membrane insertion remain to be elucidated.

Recent work has aimed at discovering the signals that regulate the STPKs, the mechanisms of kinase activation, the identity of the protein kinase and phosphatase substrates, the structures of the signaling modules and the functions of the protein kinases and phosphatases in vivo. Here we will review recent progress on these questions, focusing on the STPKs and phosphatases in *Mycobacterium tuberculosis (Mtb)*. Cozzone [2005] puts these studies into the context of the general roles of Ser/Thr and Tyr phosphorylation in bacterial virulence. *Mtb* expresses 11 predicted STPKs (named PknA-PknL) [Av-Gay and Everett, 2000], at least one Ser/Thr phosphatase (PstP) and two PTPs (PtpA and PtpB) [Koul et al., 2000]. These proteins are candidates for the switches that regulate diverse stages of growth, development and pathogenesis. The medical impact of tuberculosis, which is currently the leading infectious disease in terms of worldwide annual mortality [Russell, 2001], coupled with the potential for broad functions afforded by the 14 distinct regulatory proteins [Cole et al., 1998], provide strong rationales for investigating the functions and mechanisms of the *Mtb* pSer/pThr and pTyr signaling systems.

Av-Gay and Everett [2000] reviewed beautifully the discovery and initial functional analysis of the *Mtb* STPKs. Progress has been substantial in the last five years. Genetic studies have provided new clues about the functions of these systems. The first *Mtb* STPK-substrate proteins have been defined in vitro, enabling initial comparisons to STPK targets discovered in other bacteria. These studies point to the involvement of the STPKs in diverse pathways in *Mtb*. The crystal structures of various signaling modules – including STPK catalytic and sensor domains and three *Mtb* Ser/Thr and Tyr phosphatases – were determined. These structural studies suggest that dimerization regulates the STPKs through an allosteric interface that is universal in the STPK family. The PTPs appear to be secreted into host cells where they function as virulence factors, and PtpB defines a new structural family. Overall, these studies lay the groundwork to discover the metabolic functions and molecular mechanisms of Ser/Thr and Tyr phosphorylation systems in *Mtb*.

Knockouts of *Mtb* STPK and Protein Phosphatase Genes

Sassetti, Rubin and coworkers carried out genome-wide transposon mutagenesis to identify genes essential for *Mtb* growth in culture and in vivo [Sassetti et al., 2003; Sassetti and Rubin, 2003]. Genes essential in vitro were characterized by transposon insertions depleted from populations of bacteria grown in culture. To gauge function in vivo, pools of bacteria carrying individual transposon insertions were sampled from the spleens of infected mice [Sassetti and Rubin, 2003]. The essential genes were identified as those carrying transposons depleted from the input pool. Interpretation of the results is influenced by the possibility that genes essential for growth vary in different tissues and different animal models. Moreover, transposon insertions can exert pleiotropic effects. In addition, screens have yet to be carried out to identify genes required for long-term infection (persistence) or reactivation. These caveats notwithstanding, the transposon-insertion experiments suggested that PknA, PknB and PknG were individually required for growth in culture, indicating that these three STPKs perform essential functions within *Mtb*. In contrast, none of the other STPKs or the PtpA, PtpB and PstP phosphatases appeared to be essential for growth in vitro or in vivo [Sassetti and Rubin, 2003].

These results remain to be confirmed and amplified by individual gene knockouts. For example, a targeted *PknG* knockout, the first for any *Pkn* gene in *Mtb*, displayed a slow growth phenotype in culture and reduced virulence in immunocompetent mice [Cowley et al., 2004]. Cell fractionation experiments performed on *Mtb* grown in rich media indicated PknG was present in the bacterial cytoplasm, membrane, and culture filtrate. Because of the proximity of the *PknG* gene to the gene for the putative glutamine transporter *GlnH*, PknG was hypothesized to affect glutamine transport. Consistent with this idea, glutamate, glutamine, and amine sugar-containing molecules accumulated in the *PknG* knockout strain. Moreover, glutamine synthetase expression and de novo glutamine synthesis were significantly reduced in the PknG knockout relative to wild-type strains grown in culture. This work implicated PknG in sensing and regulating glutamate/glutamine levels [Cowley et al., 2004]. The mechanism(s) of these effects and the physiological targets, including potential phosphorylation of the GlnH transporter, remain to be defined.

In apparent contradiction to its key functions for growth in culture, PknG from *M. bovis* BCG was reported to be secreted into host macrophages and to inhibit phagosome-lysosome fusion, presumably by phosphorylating host protein substrates [Walburger et al., 2004]. Evidence for PknG secretion included visualization within the phagosome by immunocytochemistry and detection of PknG in the soluble fraction of phagosome preparations from Mtb-infected cells. Moreover, ectopic expression of PknG in *Mycobacterium smegmatis*, a fast-growing, nonpathogenic species that lacks a PknG ortholog, increased resistance to lysosomal targeting. A specific chemical inhibitor of PknG reversed this effect [Walburger et al., 2004]. The idea that PknG functions in the host represents a new paradigm for *Mtb* STPK function. Further work is needed to establish secretion of PknG into host cells, to establish the export mechanism and to identify PknG substrates. In any case, the attenuated growth phentoype of the *PknG* knockout in culture implies that PknG has important functions within *Mtb* growing in the absence of eukaryotic host cells [Cowley et al., 2004].

In contrast to the attenuated growth of the *PknG* knockout strain, Av-Gay and coworkers showed that deletion of *PknH* resulted in a hypervirulent phenotype in BALB/c mice [Papavinasasundaram et al., 2005]. In samples from lung and spleen, the *PknH* deletion strain grew to densities 10-fold (spleen) or nearly 100-fold (lung) higher than congenic wt or Δ*PknE* strains. In culture, the *PknH* deletion strain was more resistant to acidified-nitrite stress, suggesting that PknH mediates NO toxicity and affording a hypothesis for the basis for hypervirulence. In vitro, PknH phosphorylated EmbR, a DNA-binding protein involved in regulating expression of genes that mediate resistance to the anti-mycobacterial drug, ethambutol [Molle et al., 2003]. Moreover, RT-PCR analysis indicated that ethambutol treatment of wt cells induced the embCAB and iniBAC operons, while antibiotic treatment repressed these operons in the Δ*PknH* strain [Papavinasasundaram et al., 2005]. Unexpectedly, however, wild-type and Δ*PknH* strains displayed a similar minimum inhibitory concentration (MIC) for ethambutol. These results suggested that PknH regulates the *embBAC* and *iniCAB* operons, but left open the question of why drug sensitivity was not affected by the *PknH* deletion. The similar (but not identical) phenotypes of mutants of *PknH* and certain two-component regulators (e.g. DevR) [Parish et al., 2003] raise the possibility that these distinct protein kinase systems converge on common growth regulators.

Deletion of the PknD [Piers et al., 2005] or PknE genes [Papavinasasundaram et al., 2005], in contrast, produced

no growth phenotypes in culture or in mice. These results suggested that these STPKs might mediate redundant functions. The PknD and PknE sensor domains, however, comprise unique sequences not found in any other *Mtb* Pkn protein. This singularity implies that PknD and PknE respond to distinct signals that are not sensed by other STPKs. Rather than mediating redundant functions, the lack of in vitro or in vivo phenotypes of the *PknD* and *PknE* knockouts may suggest that these STPKs mediate signals that are required neither in culture nor in mice. Candidate processes that are modeled poorly in mice include persistence, survival in granulomas and reactivation [Russell, 2001].

In contrast to these gene-knockout experiments, Tyagi, Singh and coworkers explored the function of PknF by ectopic expression in *M. smegmatis* and by knocking down expression with antisense RNA in *Mtb* [Deol et al., 2005]. *M. smegmatis* lacks the gene for PknF, and expression of the *Mtb* PknF protein (but not a kinase-dead mutant) reduced the growth rate in culture and produced defects in septation and cell morphology. When the level of the PknF protein was reduced in *Mtb*, the resulting bacilli showed an increased growth rate, defects in septation and shorter, bulbous cell morphology. Because PknF appears to phosphorylate the ABC transporter encoded by the adjacent Rv1747 gene in the *Mtb* genome [Molle et al., 2004; Grundner et al., 2005; Curry et al., 2005], Deol et al. [2005] also measured the effect of the PknF knock-down on nutrient uptake. While no changes were seen in the uptake of oleic acid, leucine or glycerol, PknF depletion increased glucose uptake in a 15- to 30-min window 16-fold. These results implied that the Rv1747 protein phosphorylated by PknF may function as a sugar transporter. This work sets the stage to determine how PknF influences cell division, growth, morphology, sugar transport and metabolism.

The *Mtb* STPKs PknA and PknB are expressed most strongly in log phase, and overexpression of wild-type but not kinase-dead mutants, especially of PknB, reduced growth rate and viability [Kang et al., 2005]. Most striking was the change in cell morphology upon PknA or PknB overexpression. When PknA was overexpressed in *M. smegmatis* or *M. bovis* BCG, the cells took on an elongated or branched morphology [Kang et al., 2005]. PknB overexpression in these mycobacterial species resulted in bulging cells. When antisense RNA was used to deplete PknA or PknB, *M. smegmatis* growth in rich medium decreased. Depletion of either STPK resulted in elongated, narrow cells. These data implicated PknA and PknB in the control of cell shape, septation and/or cell division [Kang et al., 2005].

Considering both transposon mutagenesis and targeted genetic studies, the STPKs PknA, PknB, PknF, PknG and PknH appear to mediate functions that affect the growth of *Mtb* in culture. These results support the idea that these STPKs regulate multiple aspects of bacterial metabolism. The effects of deleting the Ser/Thr phosphatase, PstP, have not been reported.

Unlike the *Mtb* STPKs, the protein tyrosine phosphatases (PTPs) are thought to function exclusively in infected host cells [Koul et al., 2000, 2004]. While the *Yersinia* YopH and *Salmonella* SptP PTPs function early in infection to inhibit phagocytosis [Fu and Galan, 1999; Cornelis, 2002], the *Mtb* PTPs likely function in distinct processes that occur after uptake by host cells. PtpA expression in *M. bovis* BCG increased upon entry into stationary phase in vitro or upon infection of human monocytes [Cowley et al., 2002]. Moreover, expression of PtpA by transient transfection in macrophages and NIH3T3 cells suggested that PtpA regulates phagocytosis and actin dynamics [Castandet et al., 2005]. Transient PtpA overexpression reduced the percentage of macrophages that showed phagocytosis of *Mtb* and *M. smegmatis* cells. In contrast, phagocytosis of IgG-coated *M. smegmatis* was unaffected by PtpA overexpression, suggesting that PtpA does not regulate uptake mediated by Fcγ receptor. Compared to control cells, macrophages transiently overexpressing PtpA also showed increased actin polymerization at phagosomes. These changes were proposed to inhibit phagosome-lysosome fusion. Additional studies are required to establish the relationship between normal PtpA functions and the effects of transient overexpression.

Tyagi and coworkers showed that a deletion of the PtpB protein tyrosine phosphatase showed no growth phenotype in culture [Singh et al., 2003]. Defects were apparent, however, in activated macrophages (but not resting macrophages) and in the guinea pig model. Although Δ*PtpB Mtb* proliferated normally in spleen at three weeks post infection, the splenic load of Δ*PtpB* bacteria was reduced 70-fold compared to wild-type (and the complemented strain) at six weeks post infection. Coupled with the findings that PtpA and PtpB are secreted and with the absence of a recognizable tyrosine kinase in the *Mtb* genome, these studies supported the idea that the *Mtb* PTPs function to disrupt signaling pathways in host cells [Koul et al., 2000, 2004; Cowley et al., 2002]. The late phenotype of the Δ*PtpB* mutation is consistent with effects on the adaptive immune response [Singh et al., 2003]. This phenotype makes PtpB an interesting drug target. Elucidating the PtpB sub-

strates in the host cell could provide valuable insights into the mechanisms of long-term bacterial survival in macrophages.

Mtb STPK Substrates

The functions of the STPKs and protein phosphatases depend critically on their substrates in vivo. Consequently, discovering the substrates of the bacterial Ser/Thr and Tyr phosphosignaling systems has been the focus of many recent studies. While the purified *Mtb* STPK catalytic domains have provided reagents to define substrates in vitro, demonstrating substrate targeting in vivo has been more challenging. Nonetheless, the discovery of the first STPK and phosphatase substrates has afforded new ideas about the potential mechanisms of STPK functions and provided estimates of the total number of potential substrate proteins.

The STPKs from a variety of different bacteria catalyze autophosphorylation [Zhang et al., 1992; Matsumoto et al., 1994; Peirs et al., 1997; Av-Gay et al., 1999; Gaidenko et al., 2002]. The isolated catalytic domains of *Mtb* PknA, B, D, E, F, G, H and I [Piers et al., 1997; Av-Gay et al., 1999; Koul et al., 2001, Chaba et al., 2002, Molle et al., 2003a, b; Gopalaswamy et al., 2004] were found to be catalytically active, suggesting that the sensor domains may not be required to turn on the associated kinase domain. Compared to most eukaryotic STPKs, the *Mtb* kinase domains are phosphorylated on more sites. Three to six autophosphorylation sites were discovered on PknB, for example, including phosphorylation of two threonines and up to two serines in the segment called the activation loop [Young et al., 2003; Boitel et al., 2003]. Phosphorylation of the activation loop segment, located between the conserved DFG motif and the following APE sequence, activates many eukaryotic STPKs [Huse and Kuriyan, 2002], suggesting that bacterial and eukaryotic STPKs are regulated by a similar chemical mechanism [Young et al., 2003]. Alzari and coworkers showed that PknB dephosphorylation by the PstP phosphatase inactivated PknB, and phosphorylation of Thr171 and Thr173 in the activation loop each enhanced kinase activity by approximately 20-fold [Boitel et al., 2003].

Consistent with this idea, the autophosphorylation sites on *Mtb* PknD (up to 14 sites), PknE (up to 13 sites) and PknF (up to 6 sites) were shown to include sites in the activation loop regions of these STPKs [Duran et al., 2005]. Although the effects of mutating individual sites in these kinases have not been reported, dephosphorylating the kinase domains using PstP reduces their activity in vitro (unpublished results). These findings suggested that phosphorylation of the activation loop represents a common mechanism of STPK activation.

Bacterial STPK phosphorylation also may serve a second function, to create binding sites for proteins containing FHA (Forkhead-associated) domains [Umeyama et al., 2002; Molle et al., 2003]. FHA domains comprise pThr-peptide binding motifs that target numerous eukaryotic proteins in a phosphorylation-dependent manner [Durocher and Jackson, 2002]. *Mtb* contains seven FHA domains in six different proteins [Cole et al., 1998]. One of these occurs in EmbR, a transcription factor that regulates the embBAC operon. Following the demonstration that the *Streptomyces* STPK, AfsK, phosphorylates the FHA-domain containing transcription factor, AfsR [Matsumoto et al., 1994; Umeyama et al., 2002], Prost and coworkers showed that the orthologous *Mtb* EmbR protein is phosphorylated by PknH (fig. 1a), which is encoded by the adjacent gene [Molle et al., 2003]. EmbR FHA-domain mutations that abolished the pThr binding site also blocked phosphorylation, indicating that phosphorylation was dependent on the FHA domain.

To explore the generality of this model, Prost and coworkers demonstrated that PknF phosphorylates in vitro constructs containing the tandem FHA domains of the Rv1747 ABC transporter [Molle et al., 2004]. This interaction required autophosphorylation of PknF. Like *EmbR* and *PknH*, the *Rv1747* and *PknF* genes are adjacent in the *Mtb* genome. A targeted deletion of *Rv1747* reduced *Mtb* viability in macrophages and in mice [Curry et al., 2005], suggesting a physiologically important role for Rv1747 phosphorylation. These results supported the idea that the *Mtb* FHA domains target substrates to the activated STPKs in vitro and in vivo.

This model was expanded by testing the in vitro phosphorylation specificity of PknB, PknD, PknE and PknF for the FHA-domain-containing proteins Rv0020c, Rv1747 [Grundner et al., 2005] and GarA [Villarino et al., 2005]. These studies showed that these kinase domains phosphorylate a specific subset of these FHA proteins in vitro (fig. 1b). GarA and Rv1747-FHA-A mediated phosphorylation by all four kinase domains, while the other FHA domains were more restrictive. As seen also for FHA-domain-mediated phosphorylation in *Streptomyces* [Sawai et al., 2004], these studies revealed a web of phosphorylation reactions in vitro in which STPKs target specific FHA-domain proteins encoded by nearby or distant genes.

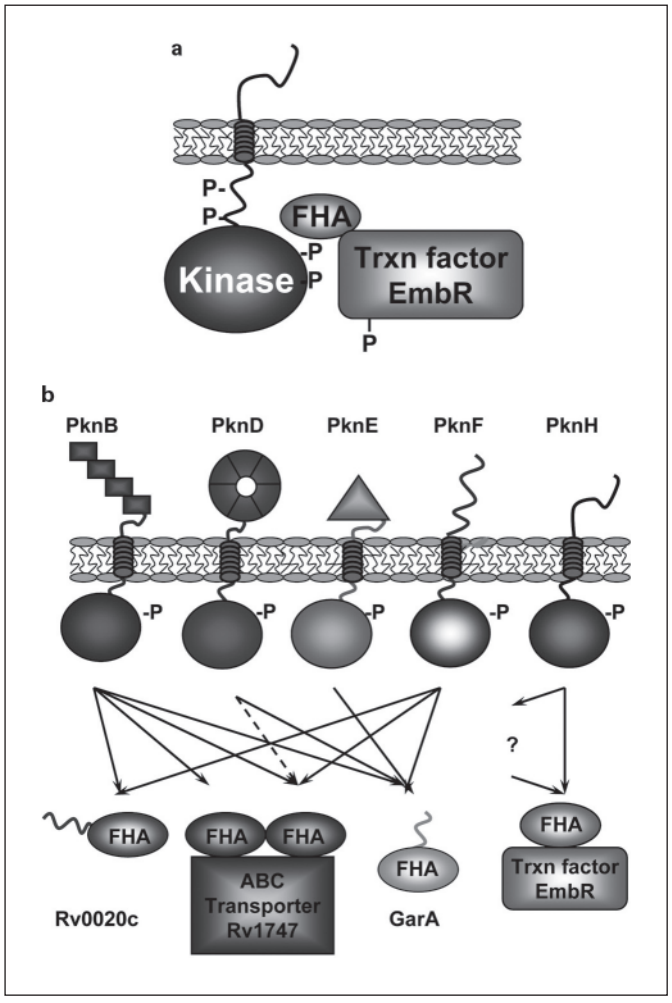


Fig. 1. Specific phosphorylation of FHA-domain proteins by *Mtb* STPK domains in vitro. **a** Schematic drawing of EmbR FHA-domain binding to the phosphorylated activation loop of PknH [Molle et al., 2003a; Duran et al., 2005]. **b** A web of specific interactions between FHA-domain proteins and STPKs predicted on the basis of in vitro phosphorylation activities of the isolated STPK domains [Molle et al., 2003a, b; Grundner et al., 2005; Duran et al., 2005]. The sensor domains of PknB and PknD are comprised respectively of PASTA domains and a β-propeller motif. While PknB, PknD, PknE and PknF phosphorylate GarA and Rv1747 in vitro, Rv0020c phosphorylation is more restricted. EmbR was assayed only with PknH. The FHA domains may serve as kinase inhibitors or effectors, and the phosphorylation may promote autoinhibition of FHA-domain-containing proteins or stimulate the effector functions.

Using a proteomic approach, Alzari and coworkers showed that GarA (Rv1827) is the most abundant soluble phosphoprotein produced by treating *Mtb* soluble protein extracts with the purified catalytic domain of PknB [Duran et al., 2005]. The phosphorylation site was mapped to Thr22 in a conserved region of the protein N-terminal to the FHA domain. The only pThr residues in the PknB construct were in the activation loop, and Ala substitutions at these sites reduced simultaneously GarA binding, kinase activity and GarA phosphorylation. The authors proposed that the FHA domains bind directly to the phosphorylated activation loop of the cognate STPKs. Because activation-loop phosphates are generally buried in a conserved receptor site in activated eukaryotic STPKs [Huse and Kuriyan, 2002], this model suggests a distinct mechanism of substrate recognition in which the activation-loop phosphates are accessible to engage the FHA domain. To test this model, several groups are working to cocrystallize *Mtb* STPK segments with FHA-domain constructs.

The roles of FHA-domains have yet to be defined. The FHA domains may mediate binding of a subset of cellular substrates (such as Rv1747). Alternatively, the FHA domains may function as kinase inhibitors by blocking the binding of other substrates to the activation-loop platform in activated STPKs. The functions of FHA-domain protein phosphorylation also have not been determined. Phosphorylation may mediate the effector functions of FHA-domain proteins through allosteric changes or by creating additional FHA-domain binding sites. On the other hand, phosphorylation may promote autoinhibition of the FHA-domain by favoring intramolecular binding to the pThr binding site. These alternative functions are not mutually exclusive, and different mechanisms may operate in different systems in diverse bacteria.

Husson and coworkers recently reported the first *Mtb* STPK substrates lacking a FHA domain [Kang et al., 2005]. A peptide-specificity screen indicated that PknA and PknB showed a preference for phosphorylation of Ser/Thr followed by Gln [Kang et al., 2005]. Using p(Ser/Thr)Gln antibodies and mass spectrometry, phosphoproteins enriched in *Mtb* extracts overexpressing PknA and PknB in early stationary phase were detected. Two in vivo substrates were identified: Rv1422 (a hypothetical protein) and Rv2145c (Wag31, a homolog of *B. subtilis* DivIVA) [Kang et al., 2005]. In vitro, the kinase domains of either PknA or PknB phosphorylated purified Rv1422c [Kang et al., 2005]. While PknB did not phosphorylate Wag31 and PknA phosphorylated it weakly, Wag31 phosphorylation was enhanced when PknA and PknB acted in concert [Kang et al., 2005]. The phosphorylation site of Wag31 was mapped to Thr73, the morphologies of *Mtb* strains overexpressing wild-type, Thr73Ala, or Thr73Glu Wag31 variants were analyzed [Kang et al., 2005]. While overexpression of wild-type and the Thr73Glu

phosphorylation mimic produced the same bulbous-cell phenotype as PknB overexpression, expression of the Thr73Ala Wag31 mutant resulted in normal cell shape [Kang et al., 2005]. This combination of in vivo and in vitro phosphorylation data with corroborating genetic data indicated that PknA and PknB regulate cell division or morphology by phosphorylating Rv1422 and Wag31.

Identification of *Mtb* STPK substrates, including the FHA-domain proteins, Rv1422 and Wag31, enables a comparison with putative STPK substrates discovered in other bacteria. The most comprehensive identification of presumptive bacterial STPK substrates has been reported for the actinomycete *Corynebacterium glutamicum* [Bendt et al., 2003]. Cells were grown to exponential phase in minimal medium, and soluble proteins with isoelectric points in the 4–7 range were analyzed using 2D gel electrophoresis. Using in vivo labeling with ^{33}P phosphoric acid or detection with pSer/pThr-specific antibodies, ~60 radiolabeled proteins or ~110 antibody-reactive proteins were detected on 2D gels of soluble extracts. These two methods detected ~31 proteins in common, and 41 different phosphoproteins were identified by peptide mass fingerprinting. These pSer/pThr proteins included enzymes of major pathways of energy metabolism and nitrogen fixation, as well as biosynthetic pathways for pyrimidines and methionine. Phosphorylated translation factors and folding chaperones also were detected. The identification of phosphorylated Ef-G, inorganic pyrophosphatase and Ef-Tu matched reports that orthologs of these proteins are presumptive STPK substrates in *Bacillus subtilis* [Gaidenko et al., 2002], *Streptomyces agalactiae* [Rajagopal et al., 2003] and *Listeria monocytogenes* [Archambaud et al., 2005], respectively. The PurA protein identified as an in vivo STPK substrate in *Streptomyces* [Rajagopal et al., 2005] was not among the substrates detected in *Corynebacterium*. The identified *C. glutamicum* pSer/pThr proteins also did not include the homologs of any of the proteins identified as substrates in *Mtb*.

These global studies of the *C. glutamicum* phosphoproteome enable an initial estimate of the number of Ser/Thr phosphoproteins in *Mtb*. The ~100 phosphoprotein spots detected provide a lower limit for the size of the *C. glutamicum* pSer/pThr phosphoproteome. Only about ~1/3 of the proteins encoded in the genome were expected to be present in the analysis, with the other proteins predicted to be membrane associated or to have been excluded from the 2D gel system. Moreover, the phosphoproteins detected were generally abundant and the phosphorylation pattern was insensitive to nitrogen starvation, supporting the idea that rare or transiently phosphorylated proteins fell below the limits of detection. Assuming conservatively that approximately half the pSer/pThr proteins were detected and that the ~1,300 proteins analyzed represent a typical fraction of the ~3,700 predicted ORFs leads to an estimate of ~600 pSer/pThr phosphoproteins (2 × 3 × 100 proteins) in *C. glutamicum*.

C. glutamicum and *Mtb* are both Actinomycetes with ~4,000 genes, but *C. glutamicum* contains only four STPK genes, in contrast to 11 in *Mtb*. The four orthologous kinases are PknA, PknB, PknG and PknL. These shared STPKs may phosphorylate the majority of the substrate proteins in both species, or at the other extreme, each STPK may phosphorylate approximately the same number of unique proteins. (This latter assumption clearly breaks down for *Myxococcus* or *Streptomyces* species, which contain ~100 or ~36 STPKs, respectively.) These considerations support the idea that *Mtb* may contain on the order of 500–1,000 pSer/pThr proteins. This number is much larger than the handful of *Mtb* phosphoproteins detected by in vitro phosphorylation of lysates [Villarino et al., 2005] or Western blotting of *Mtb* lysates with pSer/pThr-reactive antibodies [Kang et al., 2005]. The determination of the size of the *Mtb* phosphoproteome requires further experimental testing. In contrast to the current picture provided by the small number of identified *Mtb* STPK substrates, a large phosphoproteome would provide scope for STPK regulation of the majority of metabolic processes in the cell.

Structural Studies of S/T/Y Protein Kinases and Phosphatases

Recent studies of *Mtb* proteins have yielded the first five structures of pSer/pThr/pTyr phospho-signaling domains from bacteria. These structures provide an initial mechanistic understanding of the principal STPK and phosphatase modules. Because nine of the *Mtb* STPKs and the Ser/Thr phosphatase PstP are predicted membrane proteins, structural analyses of these systems have relied on protein dissection strategies to produce soluble domains. These studies leave open the question of how the sensor domains regulate the respective kinase domains. In contrast, the intact, soluble PtpA and PtpB proteins were amenable to crystallographic analysis.

PknB Kinase Domain

Studies of *Mtb* PknB yielded the first structure of a kinase domain from a bacterial STPK [Young et al., 2003;

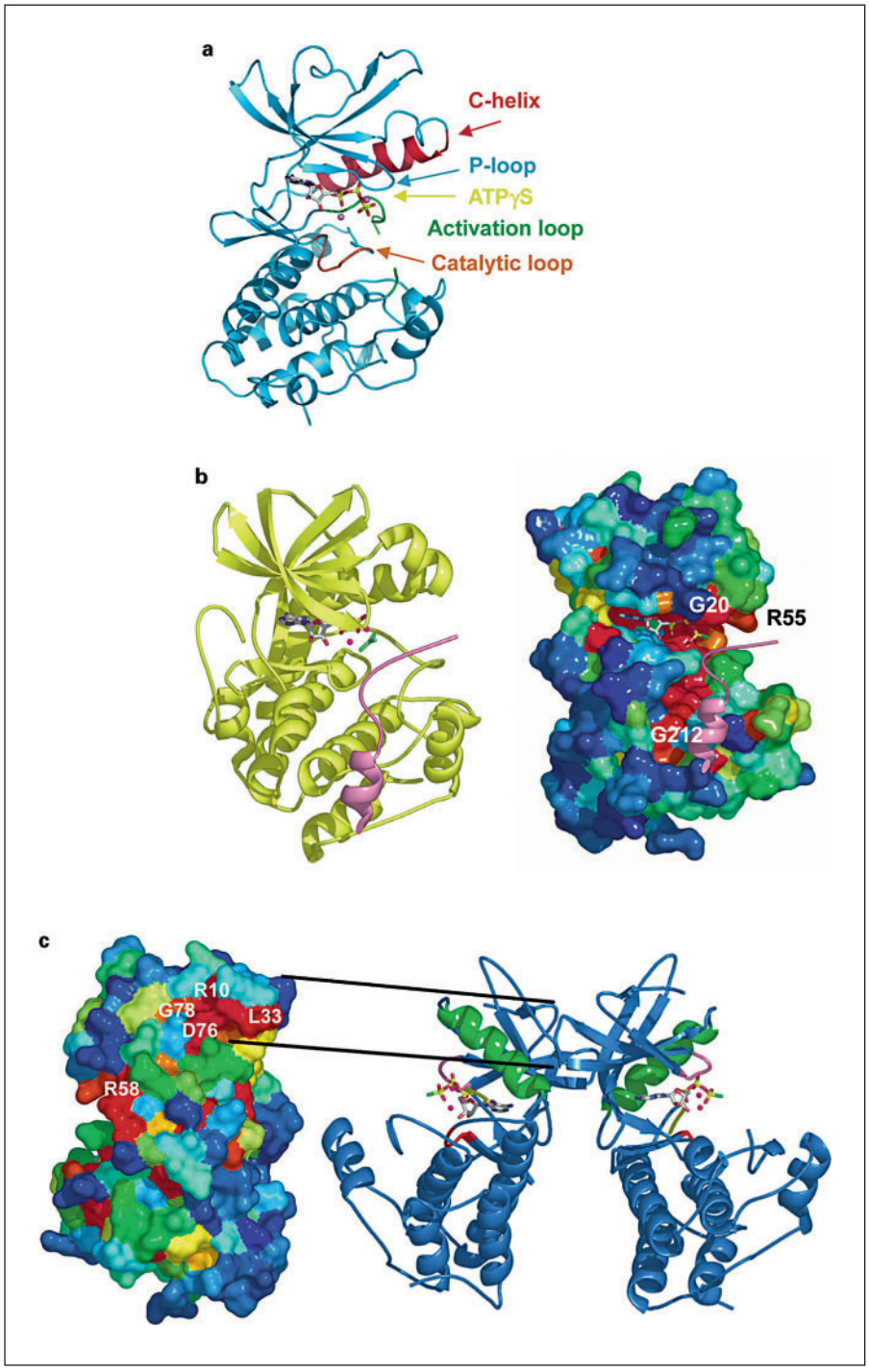


Fig. 2. PknB adopts a classic STPK structure that dimerizes through a conserved interface. **a** Ribbon drawing showing the conserved fold and functional motifs of the PknB:ATPγS complex. Characteristic features of the STPK fold include the P-loop (which engages the phosphate groups of the nucleotide), the C helix (which orients key residues in the active site and forms an allosteric docking site for protein substrates), and the catalytic loop (which contains residues essential for catalysis). Most of the activation loop is disordered in the PknB crystal structures. The high structural similarity to the eukaryotic STPK fold suggests catalysis and regulation occur by similar mechanisms in eukaryotic and prokaryotic STPKs. **b** Conserved nucleotide binding site and substrate-binding groove in PknB orthologs. Sequence conservation in PknB orthologs is displayed from red (high) to blue (low) in the surface of the PknB structure (right). Conserved surfaces include the ATP-binding site and a groove corresponding to the peptide binding site in the transition-state analog complex of mouse PKA (yellow, left). The peptide from the PKA complex (mauve) was superimposed on the PknB structure using residues in the kinase C-terminal domains. The conservation in the peptide binding groove suggests that this surface of PknB may function in substrate recognition. **c** PknB dimerizes (right) through a conserved surface (red, left surface drawing) at the C-terminal end of the C helix on the opposite side of the N-terminal domain relative to the active site. Allosteric interactions at a position analogous to this dimerization interface control the position of the C helix in several eukaryotic protein kinases, such as c-Src. These structural similarities in light of the great functional divergence of PknB and c-Src suggest that the PknB dimer interface defines a universal allosteric site in STPKs.

Ortiz-Lombardia et al., 2003]. Despite <30% sequence identity to the most related human STPK, the PknB kinase domain conformed closely to the classical architecture associated with this large protein family (fig. 2a). Compared to the transition-state-analog complex of mouse cAMP-dependent protein kinase (PKA) [Madhusudan et al., 2002], the backbone root-mean-square-deviation (rmsd) of the ATPγS complex of the PknB kinase domain was only 1.4 Å. As in other members of the STPK family, the nucleotide was sandwiched be-

tween the N- and C-terminal lobes, engaging characteristic sequence motifs including the P-loop, catalytic loop, DFG motif and a regulatory helix called the C helix. Despite phosphorylation of the activation loop at 2–4 sites, this segment was disordered in the crystals of both PknB:nucleotide complexes [Young et al., 2003; Ortiz-Lombardia et al., 2003]. As a result, the C-helix, which forms part of the binding site for the phosphorylated activation loop in activated STPKs [Huse and Kuriyan, 2002], was tipped away from the active site in a characteristic inactive conformation [Goldberg et al., 1996]. In this position, the conserved Glu59 in the C-helix did not form an ion pair with Lys40 in the active site, also distinguishing the conformation of the PknB kinase domain from activated STPK structures. Thus, the structure of the PknB:ATPγS complex showed features of both inactive and active STPKs, and revealed that eukaryotic and prokaryotic STPKs share common catalytic and regulatory mechanisms.

PknB orthologs are widely distributed in prokaryotes. Consequently, unlike more unique bacterial STPKs or orthologous eukaryotic STPKs (which show little sequence divergence), putative functional sites in the PknB kinase domain could be detected by mapping sequence conservation in orthologs onto the structure (fig. 2b, c) [Young et al., 2003]. Conserved surfaces of the PknB kinase domain include the ATP binding cleft, residues adjacent to the ATP γ-phosphate and a groove on the C-terminal lobe adjacent to the catalytic site. These sites are analogous to the substrate-binding sites of PKA [Madhusudan et al., 2002], suggesting that nucleotide and polypeptide substrates may be recognized by mechanisms common to PknB and PKA [Young et al., 2003].

Unexpectedly, PknB orthologs also showed conservation of a site on the opposite side of the N-terminal lobe relative to the active site (fig. 2c) [Young et al., 2003]. This conserved surface forms a dimerization interface in both crystal forms of the nucleotide complexes of the PknB STPK domain [Young et al., 2003; Ortiz-Lombardia et al., 2003]. Covering ~1,045 Å^2 of each monomer, the dimer interface includes the conserved Leu33, as well as the docking site for Leu33 at the C-terminus of the C helix in the adjacent monomer.

The existence of this conserved dimer interface strongly suggested that it is functionally important. The Leu33 binding pocket is analogous to an autoinhibitory site in c-Src [Xu et al., 1999; Young et al., 2001], suggesting that dimerization of PknB regulates the kinase domain and that the dimer interface contains a universal allosteric site that controls the position of the C helix. This idea is consistent with a report that the membrane-spanning and extracellular domains of the *B. subtilis* PknB ortholog, PrkC, may mediate dimerization in vivo [Madec et al., 2002]. Immediate challenges include determining if other bacterial STPKs form similar dimers, testing the formation of heterodimers of the different kinase domains and determining the function of the dimer interface in vitro and in vivo.

PknD Sensor Domain

Mtb PknD, which contains an intracellular kinase domain and a predicted extracellular β-propeller domain, is unique to pathogenic mycobacteria. The structure of the PknD extracellular sensor domain was determined to explore the mechanism of kinase regulation by extracellular stimuli [Good et al., 2004]. The PknD sensor domain formed a six-bladed, β-propeller motif (fig. 3). This domain was the most symmetric β-propeller characterized to date. Because β-propeller proteins mediate a wide variety of catalytic and binding functions, however, the sensor-domain structure did not provide strong clues about the identity of the regulatory ligand(s).

Most β-propeller proteins bind ligands at a structurally conserved site, which is called the 'cup' because of its concave shape. In contrast to other parts of the structure, most residues in the cup of the PknD sensor differ in each blade [Good et al., 2004]. The cup surface showed the hallmarks of a protein-protein interaction interface, including a number of exposed hydrophobic residues (fig. 3b). The lack of evidence for homodimerization of this or any other isolated β-propeller motif suggested that the PknD sensor forms heterotypic interactions with the signaling ligand(s) [Good et al., 2004]. Disorder of the N-terminal 10 residues and the high similarity of independent structures obtained from two crystal forms are consistent with the idea that the PknD sensor domain forms a rigid structure that is flexibly tethered to the predicted transmembrane helix. In such a structure, a ligand-promoted conformational change of the extracellular domain is unlikely to initiate signaling. Instead, the structure of the PknD sensor domain supports a transmembrane-signaling model in which extracellular ligand binding alters the localization or oligomerization of the kinase.

PstP Phosphatase Domain

Mtb encodes the Ser/Thr phosphatase, PstP, which is a member of the PPM/PP2C-subfamily. PstP contains a 237-amino-acid phosphatase domain followed by a 67-amino-acid, low-complexity sequence, a predicted transmembrane helix and a 191-amino-acid extracellular do-

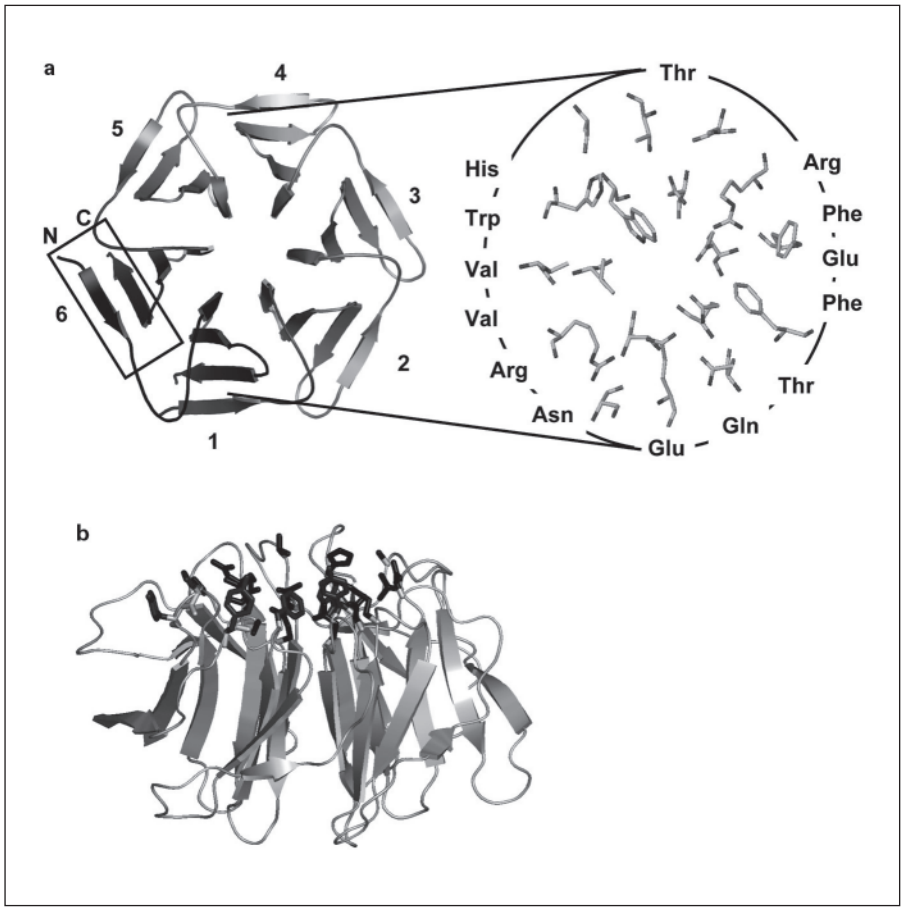


Fig. 3. The PknD sensor domain structure and predicted interaction surface. **a** The PknD sensor domain forms a β-propeller with six blades arranged sequentially around a central pore. The N-terminus forms the fourth strand of blade 6, making a topological latch (box). The large 'cup' surface of the PknD sensor contains exposed hydrophobic groups and aromatic residues consistent with a protein recognition site. **b** All residues that differ in at least 3 of the six blades (black sticks) form the surface of the cup. This localization of sequence divergence in the structure suggests that the cup can mediate recognition of an asymmetric signaling ligand or that a symmetric ligand binds to surfaces that are conserved among the blades [Good et al., 2004].

main with no predicted homology to a known fold. Like other PP2C phosphatases, PstP requires the divalent cations Mn^{2+} or Mg^{2+} [Boitel et al., 2003] and is insensitive to classic phosphatase inhibitors such as okadaic acid. PstP acts as a cellular antagonist to the 11 STPKs in *Mtb*, and it has been found to dephosphorylate PknA, PknB, PknD, PknE, PknF and their substrates [Boitel et al., 2003; Chopra et al., 2003; Duran et al., 2005]. The catalytic mechanism of PP2C phosphatases was elucidated based on a single structure, that of the human PP2Cα [Das et al., 1996]. In this mechanism, a nucleophilic water molecule or hydroxide ion coordinated by two divalent cations in the active site attacks the phospho-ester bond, and a histidine serves as a general base to protonate the leaving group [Das, 1996; Jackson and Denu, 2001; Jackson et al., 2003].

By comparison, the high-resolution crystal structure of the PstP phosphatase domain (fig. 4) showed a conserved fold that unexpectedly contained a third Mn^{2+} ion in the active site and a large difference in the position of an adjacent flap subdomain [Pullen et al., 2004]. The conservation of two of the active-site metals and their ligands (fig. 4b) supported the concept of an activated water (or hydroxide) nucleophile, but no analog of the general acid proposed to protonate the leaving group was apparent in PstP. Conserved residues, Asp118, Asp191 and Ser160 (Asp146, Asp239 and Ser190 in PP2Cr) coordinated the third Mn^{2+} ion. Ser160, which provided both an equatorial and an axial ligand for the third Mn^{2+} ion, was located on the flap adjacent to the active site. The difference in the flap regions in the PstP and PP2Cα structures suggested that the flap may assume different positions in different PP2C-family members or that it forms a flexible domain that moves in response to the binding of substrates. Such alternate conformations of the flap may afford a versatile substrate-binding surface that enables PstP to regulate the pathways of the 11 different STPKs in *Mtb*.

Protein Tyrosine Phosphatase PtpA

Sequence comparisons suggested that the two PTPs in the *Mtb* genome, PtpA and PtpB, belong to different PTP subfamilies, the low-molecular-weight (LMW) and the conventional/dual-specificity subfamilies, respectively. The crystal structure of PtpA (fig. 5) revealed a classic LMW PTP fold [Madhurantakam et al., 2005]. PtpA (like PknB and PstP) has close structural homologs in eukaryotes, despite modest sequence homology. The Cα rmsd of PtpA and the human HCPTA is 0.99 Å, with the two PTPs sharing 38% sequence identity. The structures of PtpA and HCPTA differ most in loop regions and an insertion of approximately two turns in helix 5 of PtpA. Hydrophobic residues thought to contact the incoming phosphotyrosine line the substrate-binding cleft. Two residues that probably contribute to substrate specificity, His49 and Ser52, are notably different in homologs. Whereas His49 is conserved or deleted from bacterial homologs, Ser52 is highly variable. The conformation of the catalytic Cys side chain is similar to that recently proposed to confer resistance to oxidation by H_2O_2 [Groen et al., 2005], a property that may help preserve the activity of the enzyme within host macrophages.

PtpB

The crystal structure of PtpB in complex with the product phosphate [Grundner et al., 2005] revealed a number of surprising features and provided the first clues to the mechanisms of regulation and function (fig. 6). Compared to conventional PTPs, PtpB is deeply diverged and simplified structurally. Apart from the signature core motif of all PTP structures (a four-stranded parallel beta sheet that connects to an α-helix through the catalytic P-loop), PtpB does not show significant similarity to other PTPs. Unlike conventional PTPs that feature antiparallel β-strands flanking the central β-sheet, PtpB contains several additional α-helices (fig. 6a) and a disordered, 31-residue loop adjacent to the active site. The differences between PtpB and other PTPs justified the classification of PtpB as a member of a new protein tyrosine phosphatase subfamily, named the YSPs [Grundner et al., 2005].

A striking structural feature of PtpB is a two-helical insertion that is unique to mycobacterial homologs (fig. 6) [Grundner et al., 2005]. This 'lid' segment is positioned on top of the catalytic site, effectively blocking access to the active site cleft. The arrangement of Phe222 from the lid and the bound product phosphate mimics the expected position of a substrate phosphotyrosine (fig. 6b). The lid structure is likely to open rapidly, however, because PtpB efficiently catalyzed the hydrolysis of the noncognate substrate, *p*-nitrophenyl phosphate in solution [Koul et al., 2000, Grundner et al., 2005].

The occurrence of the autoinhibited conformation in the PtpB crystals raised the possibility that lid opening and closing may be regulated in vivo. Due to the reactivity of the cysteine nucleophile in tyrosine phosphatases, PTPs are generally sensitive to regulation or inactivation by reactive oxygen species (ROS) [van Montfort et al., 2003; Salmeen et al., 2003]. PtpB is likely to encounter ROS in host macrophages. When treated with increasing amounts of H_2O_2, PtpB showed extraordinary resistance to inactivation [Grundner et al., 2005]. This resistance to oxidative inactivation supported the idea that the PtpB lid may provide a dynamic filter that excludes ROS from the active site. If corroborated by additional studies, this model would represent a remarkable molecular adaptation to the conditions encountered in infected macrophages.

Conclusions

Recent genetic and biochemical studies have begun to establish the functions and substrates of the *Mtb* pSer/pThr and pTyr signaling proteins. The STPKs appear to control diverse targets – including the STPKs themselves, an ABC transporter, mediators of glutamate/glutamine metabolism, a transcription factor and proteins involved in regulating cell division and morphology. Many additional protein substrates likely remain to be identified.

Compared to eukaryotic homologs, the bacterial STPKs share conserved sequence motifs and structures, as well as universal mechanisms of activation by phosphorylation, ATP binding and protein substrate binding. STPK phosphorylation also creates binding sites for FHA-domain proteins of unknown function. The *Mtb* PknB kinase domain forms a dimer through an allosteric interface analogous to an inhibitory site in the Src protein kinase. Together with the rigid structure of the PknD sensor domain, this dimeric arrangement supports the idea that the transmembrane STPKs are regulated by localization or oligomerization (fig. 7).

The PstP phosphatase antagonizes the STPKs. The PstP catalytic domain structure revealed a conserved catalytic fold with an unexpected third Mn^{2+} ion in the active site and a large change in the position of a flap that coordinates the third metal ion. The *Mtb* PTPs, PtpA and PtpB, are thought to function within host cells to inactivate host signaling pathways. The LMW PTP, PtpA,

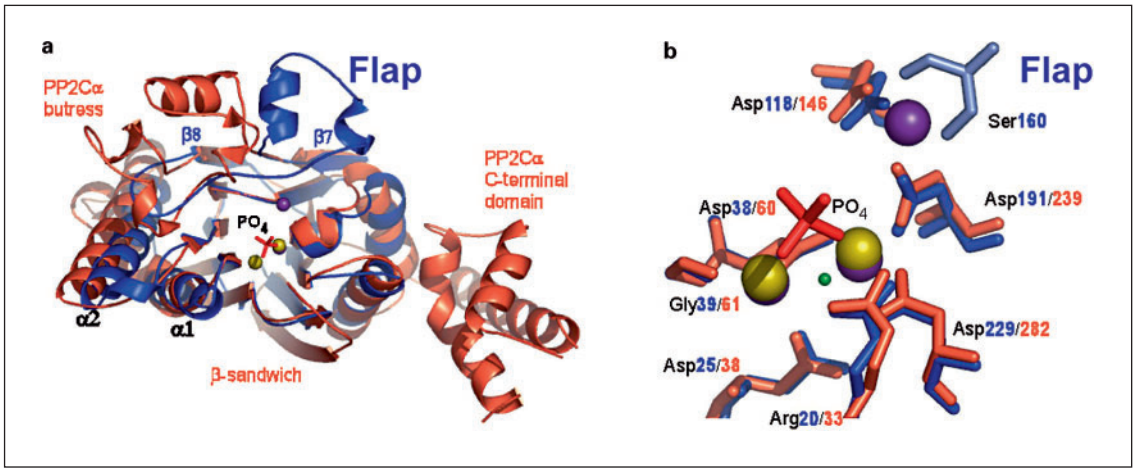


Fig. 4. Structural comparison of the *Mtb* PstP protein Ser/Thr phosphatase (blue with Mn^{2+} ions in yellow and purple) with human PP2Cα (red with Mn^{2+} ions in yellow). **a** Superimposed ribbon drawing showing the conserved central c-sandwich flanked by antiparallel-helices. Notable differences included the presence of a third metal in PstP (purple), the substantial movement of the flap region, extra turns in the PstP helices α1 and α2, and the absence of the C-terminal domain in PstP. **b** The PstP active site contains a third Mn^{2+} ion. The positions of key catalytic residues and two metals are conserved between the prokaryotic (blue) and eukaryotic Ser/Thr phosphatases (red), supporting a common catalytic mechanism. One of the most striking differences between PstP (blue) and PP2Cα (red) was the presence of a third Mn^{2+} ion in the PstP active site (purple). Mutations of the residues that bind the third Mn^{2+} ion in PstP had little effect on the activity of the noncognate substrate, *p*-nitrophenylphosphate. These results suggested alternative roles in for the third Mn^{2+} ion in substrate recognition or in promoting the reactions of pSer and pThr substrates [Pullen et al., 2004].

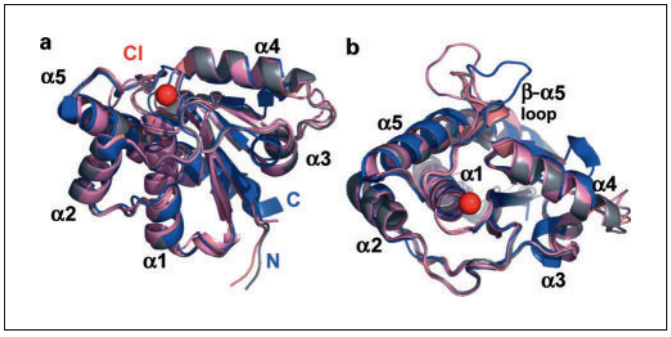
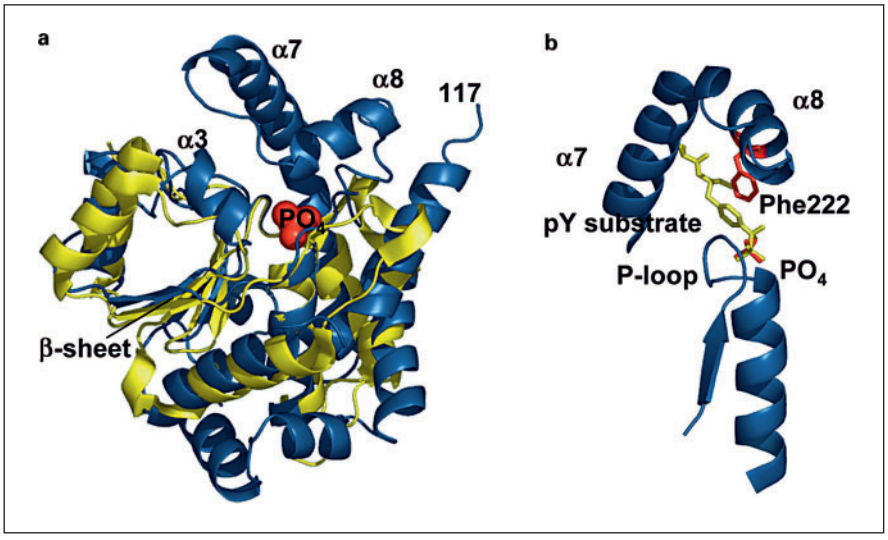


Fig. 5. Close structural similarity of *Mtb* PtpA with mammalian homologs. Superposition of the ribbon drawings of *Mtb* PtpA (blue, 1U2P; Madhurantakam et al., 2005), human LMW PTP (gray; 5PNT) and bovine LMW (mauve; 1DG9) reveals the largest backbone differences in the segment from α4 through α5. The active site (marked by the red Cl^- ion in 1 U2P) is open and accessible to substrates.

Fig. 6. The PtpB protein tyrosine phosphatase adopts a unique fold with a buried active site. **a** Superposition of PtpA:PO_4 (blue, with the phosphate shown in red) with the closest structural homolog in the Protein Data Bank, a dual-specificity phosphatase from *Arabidopsis thaliana* (1XRI, yellow). The structures were aligned using the core PTP motif consisting of the beta-sheet, the P-loop, and the following helix. Little similarity apart from this core was apparent. The PtpB active site is buried under a lid (α7 and α8) that is formed by a sequence insertion unique to homologs in pathogenic mycobacteria. The lid opens rapidly in solution, as judged by the catalytic activity of the enzyme [Grundner et al., 2005]. By comparison, the shallow active-site of the dual-specificity phosphatase is accessible to solution. PtpB helix α3 frames a deeper active-site cleft and may contribute to the specificity of PtpB for pTyr substrates [Grundner et al., 2005]. **b** The lid and Phe222 block the PtpB active site. Helices α7 and α8 form the lid (blue), with Phe222 (red) reaching down into the active site. Overlay with a structure of substrate-bound *Yersinia* YopH (1QZ0, yellow) reveals that Phe222:PO4 mimic the substrate pTyr.



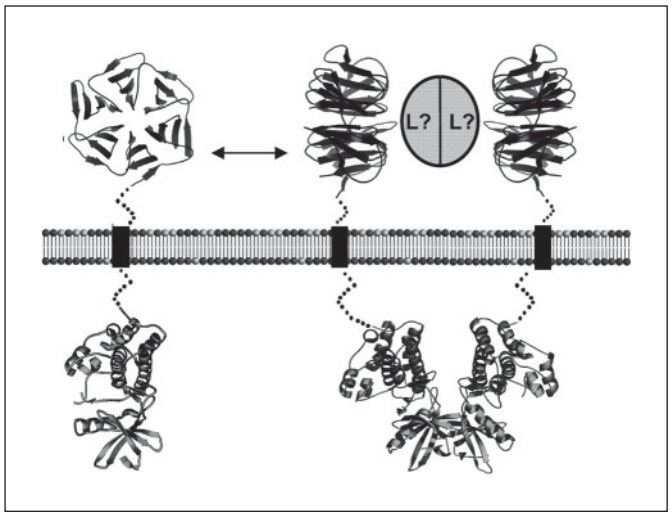


Fig. 7. A model for bacterial Ser/Thr kinase transmembrane signaling. Crystallographic studies support an architecture for a composite, intact, receptor kinase in which the sensor domain (e.g. the PknD β-propeller, top) and the kinase domain (PknB catalytic domain, bottom) are flexibly tethered (dots) to the transmembrane helix. The conservation of the PknB dimerization interface (fig. 2c) [Young et al., 2003] suggests that oligomerization regulates STPK activity. Binding of an oligomeric ligand (L) to the extracellular sensor domain may approximate two kinase monomers, promoting dimerization of the intracellular kinase domains. Alternatively, a sufficiently large ligand or a large excess of ligand may trap the monomeric kinase domain. This oligomerization switch could activate or inhibit the kinase domain, depending on the specific structural effects transmitted from the dimer interface through the C helix to the active site.

causes changes in actin dynamics in macrophages. PtpB likely inhibits the immune response. The PtpB structure showed a simplified PTP fold and an unprecedented lid associated with protection from inactivation by reactive oxygen species in vitro.

These studies set the stage to determine the signaling inputs, activation mechanisms and in vivo substrates of these phospho-signaling proteins. Defining the in vivo functions and signaling mechanisms of the *Mtb* STPKs will provide a deeper understanding of general mechanisms of kinase signaling and test the validity of targeting bacterial STPKs with new classes of antibiotics. Establishing the functions of the *Mtb* PTPs will sharpen the molecular picture of pathogen interactions with host defense pathways.

Acknowledgements

We are indebted to the TB Structural Genomics Consortium for encouragement and collaboration. This work was supported by grants to T.A. from the National Institutes of Health. C.G. gratefully acknowledges a Postdoctoral Fellowship from the New York Community Trust-Heiser Fund. K.E.P. and A.G. were supported respectively by pre-doctoral fellowships from the Ford Foundation and the National Science Foundation.

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