Forkhead-associated (FHA) Domain Containing ABC Transporter Rv1747 Is Positively Regulated by Ser/Thr Phosphorylation in Mycobacterium tuberculosis

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One major signaling method employed by Mycobacterium tuberculosis, the causative agent of tuberculosis, is through reversible phosphorylation of proteins mediated by protein kinases and phosphatases. This study concerns one of these enzymes, the serine/threonine protein kinase PknF, that is encoded in an operon with Rv1747, an ABC transporter that is necessary for growth of M. tuberculosis in vivo and contains two forkhead-associated (FHA) domains. FHA domains are phosphopeptide recognition motifs that specifically recognize phosphothreonine-containing epitopes. Experiments to determine how PknF regulates the function of Rv1747 demonstrated that phosphorylation occurs on two specific threonine residues, Thr-150 and Thr-208. To determine the in vivo consequences of phosphorylation, infection experiments were performed in bone marrow-derived macrophages and in mice using threonine-to-alanine mutants of Rv1747 that prevent specific phosphorylation and revealed that phosphorylation positively modulates Rv1747 function in vivo. The role of the FHA domains in this regulation was further demonstrated by isothermal titration calorimetry, using peptides containing both phosphothreonine residues. FHA-1 domain mutation resulted in attenuation onine protein kinases (STPKs), a system originally thought to indicate that phosphorylation activates Rv1747, which may have important consequences in regulating growth of M. tuberculosis.

Tuberculosis (TB), caused by Mycobacterium tuberculosis, remains one of the world’s most rampant infective agents, and despite preventative and therapeutic measures, this pathogen killed 1.8 million people in 2008 alone. Furthermore, an estimated two billion people are latently infected with TB bacilli (1). Globally, this disease burden has escalated because of its deadly synergy with human immunodeficiency virus (HIV). This HIV burden coupled with the emergence and increase of multidrug and extensively drug-resistant M. tuberculosis strains have made the search for new TB drugs ever more important.

Signal transduction in M. tuberculosis has become a target for the development of novel therapeutics in the treatment of TB. Protein kinases and phosphatases allow reversible protein phosphorylation to transduce extracellular signals into cellular responses, and this has been implicated in nearly all basic cellular processes (2). Consequently, small molecule kinase inhibitors represent attractive candidates as drug targets (3, 4). M. tuberculosis has a repertoire of both the classical bacterial two-component systems involving histidine kinases and response regulators and also a second family comprising the serine/threonine protein kinases (STPKs), a system originally thought to be only present in eukaryotes. Studies performed to date have demonstrated the presence of a complex network of phosphorylation-dependent interactions mediated by STPKs in M. tuberculosis (5–7). A total of 11 STPKs have been identified (8, 9), significantly 4 of which lie in putative operons with forkhead-associated (FHA) domain-containing proteins.

FHA domains are modular phosphopeptide recognition motifs, conserved from bacteria to humans, which are between 95 and 150 amino acid residues in size and demonstrate a strik-
ing specificity for phosphothreonine (Thr(P))-containing epitopes (10–12). A total of six FHA-containing proteins have been found encoded within the *M. tuberculosis* genome (9). Rv1747, a predicted ATP-binding cassette (ABC) transporter, encodes two FHA domains, a feature unique to the FHA modules of *M. tuberculosis*. The presence of an FHA domain is now indicative that the protein is likely to interact with a phosphorylated protein partner (13). ABC transporters bind and hydrolyze ATP providing energy for uptake or export of a diverse array of substrates across cell membranes. Rv1747 is a presumed ABC exporter required for the growth of *M. tuberculosis* in *vivo* (14, 15) and forms a putative operon with its upstream adjacent gene, *pknF*, encoding a Ser/Thr protein kinase. A number of recent investigations have examined phosphorylation by Ser/Thr protein kinases *in vitro* and have identified substrates based on these assays. Thus, previous studies have demonstrated that PknF can phosphorylate the FHA domains of two other proteins, Rv0020c and Rv1747 (16), and also the heat-shock protein GroEL1 (17). Furthermore, PknF has previously been implicated in regulating glucose uptake in *M. tuberculosis* (18), as well as in sliding motility and biofilm formation in *Mycobacterium smegmatis* (19). Thus, mycobacterial Ser/Thr protein kinases have been identified as promising therapeutic targets. However, for a kinase to be a suitable drug target, it is necessary not only to identify a target for the kinase that is required for the growth of the bacterium but also to determine the functional consequences of phosphorylating the target protein. We have therefore sought to combine the approach of analyzing the molecular details of Ser/Thr-mediated phosphorylation with *in vitro* studies designed to elucidate what functional consequences flow from PknF-mediated phosphorylation.

In previous studies, we and others showed that Rv1747 exhibited ATPase activity and was a substrate for PknF *in vitro*; furthermore, the FHA domains of Rv1747 were shown to be required for specific interaction with PknF in a yeast two-hybrid assay (15, 20). Moreover, we demonstrated that deletion of *Rv1747* results in a growth defect in macrophage and mouse infections (15). However, whether phosphorylation is directly involved in regulating *Rv1747* function *in vivo* has not been clearly established. This study was undertaken to determine whether *Rv1747* function might be influenced by STPK-dependent regulatory mechanisms and how PknF could modulate *Rv1747*. Therefore, we have characterized *Rv1747* phosphorylation sites to decipher how the PknF- *Rv1747* signal transduction system functions in *M. tuberculosis*. Then, through the use of mutants in macrophage and mouse infections, we provide for the first time evidence that phosphorylation of *Rv1747* is required for its function, *i.e.* phosphorylation positively regulates *Rv1747* function.

**EXPERIMENTAL PROCEDURES**

**Strains, Growth Conditions, and Reagents—** *M. tuberculosis* H37Rv cultures were grown at 37 °C in Dubos broth supplemented with 0.05% (v/v) Tween 80, 0.2% (v/v) glycerol, and 4% (v/v) Dubos medium albumin (BD Biosciences). *M. tuberculosis* liquid cultures were grown in 50-ml Falcon tubes in a wheel at 20 rpm (Corning Glass) or in 1,000-ml polycarbonate roller bottles (Nalgene) in a Bellco roll-in incubator (2 rpm). Kanamycin and hygromycin were used at a final concentration of 25 and 50 μg/ml, respectively. *M. tuberculosis* was grown on 7H11 agar plates supplemented with 10% Middlebrook oleic acid-albumin-dextrose-catalase enrichment and 0.5% (v/v) glycerol. All *Escherichia coli* strains (Table 1) were grown on L-agar and in L-broth overnight at 37 °C, with shaking for liquid cultures (250 rpm). Kanamycin and ampicillin were used at a final concentration of 50 and 100 μg/ml, respectively. Adult (6–8 weeks old) female BALB/c mice were obtained from the Biological Services specific pathogen-free animal facility at the National Institute for Medical Research.

**RNA Isolation from *M. tuberculosis* Liquid Cultures—** Total RNA was isolated from 100 ml of exponential phase (A 600 0.6) rolling cultures using the Fast RNA Pro Blue kit (Qiobogene). Contaminating DNA was removed by DNase digestion with 2 units of RNase-free DNase (Promega) in 5 mM magnesium sulfate and 100 mM sodium acetate with 80 units of RNase inhibitor and incubated at 37 °C for 1 h. A further 2 units of RNase-free DNase was then added and incubated as described previously. Proteins and other contaminants were then removed from RNA samples using the RNeasy kit (Qiagen) as per the manufacturer’s guidelines. 200 ng of RNA was then run on a 2100 Bioanalyzer (Agilent Technologies) to assess integrity.

**Reverse-transcription PCR (RT-PCR)—** A reverse transcription reaction contained 1 μg of DNA-free RNA in 1× Quantiscript RT buffer containing magnesium and dNTPs, RT primer mix, and 1 μl of Quantscript reverse transcriptase (Qiagen). Reactions were incubated at 42 °C for 30 min. Samples were then incubated at 95 °C for 3 min to inactivate the reverse transcriptase enzyme. PCR was then performed using the cDNA template with HotStarTaq as per the manufacturer’s guidelines (Qiagen). Primers used in the RT-PCR study can be found in Table 2.

**Cloning, Expression, and Purification of Recombinant PknF and Rv1747 Proteins—The** kinase domain of PknF and the nucleotide binding domain along with FHA-1 and FHA-2 domains of *Rv1747* were amplified by PCR using *M. tuberculosis* H37Rv chromosomal DNA as a template and ligated into pGEX-6P-1. Full-length proteins were not purified because of the presence of single or multiple transmembrane domains. All plasmids were verified using DNA sequencing. All constructs were expressed as 3C protease-cleavable GST fusions in *E. coli* BL21 (DE3) Star competent cells (Invitrogen) or *E. coli* BL21 (DE3) pRep4 cells as follows. Recombinant strains harboring the different constructs were used to inoculate 4 liters of LB medium supplemented with ampicillin, and the resulting cultures were incubated at 37 °C with shaking until the A 600 reached 0.6. Isopropyl 1-thio-β-D-galactopyranoside was then added at a final concentration of 0.1 mM, and growth was continued for 16 h at 18 °C. Cells were harvested by centrifugation, washed in PBS plus 10% glycerol, and then resuspended in lysis buffer (50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 10% glycerol) containing DNase, RNase, lysozyme, and a mixture of protease inhibitors (Roche Applied Science). Bacteria were disrupted by sonication (VibraCell, Sonics) on ice with 10 bursts of ~20 s at amplitude 10. The soluble lysate was applied to an appropriate amount of prepared glutathione-Sepharose 4B resin (3–5 ml).
Positive Regulation by M. tuberculosis Kinase

**TABLE 1**

| E. coli strains | Genotype or description | Source or Ref. |
|-----------------|-------------------------|---------------|
| E. coli TOP10    | F' mcrA Δ(mcrA-mcrB-ΔmcrBC) q880lac2ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galL galK rpsL endA1 nupG | Invitrogen |
| E. coli BL21(DE3)/Star | Δomt1 lasB(BeR, m, see below) dcm m131(DE3) | Stratagene |
| E. coli BL21(DE3)/Star pRepl-groEL | Δomt1 lasB(BeR, m, see below) dcm m131(DE3) | Stratagene |
| XL1-Blue        | Δomt1 lasB(BeR, m, see below) dcm m131(DE3) | Stratagene |

**M. tuberculosis strains**

| H37Rv | M. tuberculosis WT strain | 45 |
| ΔRv1747 | H37Rv with deletion of Rv1747 constructed by homologous recombination with targeting construct pRW69 | 15 |
| Rv1747 complement | ΔRv1747 containing complementing plasmid pRW76 | 15 |
| Rv1747 complement T150A/T208A | ΔRv1747 containing complementing plasmid pRW76 with mutations T150A and T208A | This study |
| Rv1747 complement S47A | ΔRv1747 containing complementing plasmid pRW76 with mutation S47A | This study |
| Rv1747 complement S248A | ΔRv1747 containing complementing plasmid pRW76 with mutation S248A | This study |
| ΔpknF | H37Rv with deletion of pknF constructed by homologous recombination with targeting construct pRW51 | This study |
| ΔpknF complement | ΔpknF containing complementing plasmid pRW95 | This study |

**M. tuberculosis shuttle plasmids**

| p2Nl | Suicide gene delivery vector, oriF, KanR | 22 |
| pKP186 | Suicide vector containing integrase, AmpR | 46 |
| pBS-lnt | Suicide vector containing integrase, AmpR | 25 |
| pRW69 | p2Nl containing a 2-kb region of H37Rv DNA flanking each side of the Rv1747 gene, HygR | 15 |
| pRW76 | Rv1747 complementing plasmid. pKP186 derivative containing 609 bp Rv1745c, pknF, and Rv1747, KanR HygR | 15 |
| pRW51 | p2Nl containing a 2-kb region of H37Rv DNA flanking each side of the pknF gene, KanR | This study |
| pRW95 | pknF complementing plasmid. pKP186 derivative containing 609 bp Rv1745c, pknF, and 20 bp Rv1747, KanR | This study |

**E. coli plasmids**

| pGEX-6P-1 | Replicating protein expression vector. N-terminal GST tag, tac promoter, lacI repressor, AmpR | GE Healthcare |
| pVS_02 | pGEX-6P-1 containing pknF-292 | This study |
| pVS_03 | pGEX-6P-1 containing Rv17471-559 | This study |
| pVS_04 | pGEX-6P-1 containing FHA-11-240 | This study |
| pVS_05 | pGEX-6P-1 containing FHA-11-240 S47A | This study |
| pVS_06 | pGEX-6P-1 containing FHA-11-240 S248A | This study |
| pVS_07 | pGEX-6P-1 containing Rv17471-559 T150A | This study |
| pVS_09 | pGEX-6P-1 containing Rv17471-559 T208A | This study |
| pVS_11 | pGEX-6P-1 containing Rv17471-559 T150A/T208A | This study |

(GE Healthcare) equilibrated in lysis buffer. Resin was incubated overnight at 4 °C with gentle mixing. The resin was collected and then washed with 1 liter of wash buffer (50 mM Tris/HCl, pH 8.0, 500 mM NaCl, 10% glycerol). Washed resin was equilibrated with PreScission protease cleavage buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 10% glycerol). 50 μl of PreScission protease was then added to the resin and incubated overnight with mixing as described previously. Elutions were concentrated in a 20-ml Vivaspin ultrafiltration concentrator (VivaScience) with an appropriate molecular weight cutoff filter (3–10 kDa). Each sample was purified by size exclusion chromatography with a pre-packed HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare) using an AKTA Prime system. The column was equilibrated in gel filtration buffer (40 mM Tris/HCl, pH 8.0, 200 mM NaCl, 10% glycerol); the sample was loaded, and the column was developed at flow rates between 0.5 and 1 ml/min collecting fractions. Appropriate fractions containing the protein of interest were checked by SDS-PAGE, pooled, concentrated, and snap-frozen at −80 °C in 20–100-μl aliquots.

**In Vitro Phosphorylation Assays**—In vitro phosphorylation was carried out in 20-μl reactions containing the recombinant PknF (1 μg), Rv1747 derivatives (5 μg), and 200 μCi/ml (65 nm) [γ-32P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences) in phosphorylation buffer (25 mM Tris/HCl, pH 6.8, 1 mM DTT, 5 mM MgCl2, 1 mM EDTA). The reaction was carried out for 30 min at 37 °C and stopped by addition of Laemmli Sample Loading Buffer and incubated at 100 °C for 5 min before analysis by SDS-PAGE. After electrophoresis, gels were washed in 10% trichloroacetic acid for 10 min at 90 °C, stained with InstantBlue (Invitrogen) and separately cloned into pCRBlunt-TOPO (Invitrogen) and sequenced. The primer pairs for the flanking regions were 5′-ATGAAGATGC-GGCGCGACTGTGATCGGGGCAAACGAC-3′ (5′-NotI restriction site underlined), 5′-GATATCCACACTGCGGACC-GATGGTGACG-3′ (5′-EcoRV site underlined), 5′-ATAA-
GAATTCGCTGGTGGCTCATC-3′ (5′-EcoRI site) (Fig. 1). The PCR product was then cloned into the vector pKP186 (23), a pHV306 (24) derivative lacking the integrase gene, and used to electroporate the M. tuberculosis ΔpknF mutant along with the mycobacterial suicide vector, pPS-Int, which contains the integrase gene (Table 1) (15, 25).

The Rv1747 deletion mutant (hygromycin marked) was described previously (15). Complementation of the Rv1747 deletion mutant was achieved by PCR, amplifying the genes Rv1747, pknF (Rv1746), and 609 bp of Rv1745c using primers 5′-AAAGCTTGACCGCTTGTAGCGGAAAT (5′-EcoRV site) (Fig. 1). The PCR product was then cloned into the vector pKP186 and transformed into the M. tuberculosis ΔRv1747 mutant as above.

**Site-directed Mutagenesis**—Site-directed mutagenesis was carried out according to the Stratagene QuikChange XL site-directed mutagenesis manual, using SoloPack Gold Supercompetent E. coli for transformation. Mutation of the phosphorylated threonine residues of Rv1747 was created by substitution of Thr-150 and Thr-208 with alanine. Sequences of primers used are shown in Table 2. After site-directed mutagenesis, constructs were re-cloned into pKP186 or pGEX-6P-1 to ensure no mutations had occurred within the plasmid backbone (Table 1). The presence of the desired mutations was confirmed by sequencing.

**Growth of M. tuberculosis in Murine Bone Marrow-derived Macrophages**—Monocytes were isolated from the hind legs of 6–8-week-old female BALB/c mice. The cells were suspended in 10 ml of RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 2 mm l-glutamine, 1 mm sodium pyruvate, 10 mm HEPES, and 50 μM β-mercaptoethanol (RPMI complete) in a Petri dish. A
Positive Regulation by M. tuberculosis Kinase

10-ml aliquot of 0.83% ammonium chloride was added for 5 min at 37 °C to lyse the red blood cells. Cells were harvested again, and ammonium chloride was removed. Cells were washed in 10 ml of 1× PBS (Invitrogen) and then resuspended in 10 ml of RPMI complete containing 20% by volume of supernatant from L929 cells that produce macrophage colony-stimulating factor. Monocytes were plated out in Petri dishes at a concentration of 4×10^5 cells per plate in RPMI complete plus 20% L929 supplement in a total volume of 10 ml. Cells were incubated at 37 °C with 5% CO₂. After 72 h, a further 10 ml of RPMI complete plus 20% L929 supplement was added to each Petri dish. After a further 48 h, macrophages were ready to use. All media and nonadherent cells were removed from the Petri dishes. 4 mM EDTA in 1× PBS was added to flush the cells from the plate. The supernatant was discarded, and the cell pellet was resuspended in RPMI complete containing 5% L929 supplement. 2×10⁶ macrophages were seeded per well in a 1-ml volume in a 24-well cell culture plate (tissue culture-treated plates, Costar). When required, mouse interferon-γ (IFNγ) was added at 20 ng/ml (Roche Applied Science) to activate the macrophages, and activation was confirmed using the Greiss assay. Cells were plated out in triplicate for each M. tuberculosis strain and post-infection time point. Prior to infection, cells were incubated for 18 h at 37 °C with 5% CO₂ to allow adherence and activation. The macrophages were infected with M. tuberculosis with a multiplicity of infection of 0.5 bacteria: 1 macrophage for 6 h. Survival and multiplication of M. tuberculosis within the macrophages were assessed at 6, 24, 72, 120, and 168 h post-infection. At each time point, medium was removed from each well, and macrophages were lysed in 500 µl of distilled H₂O plus 0.05% Tween 80 for 30 min. Appropriate dilutions of bacteria were then plated onto 7H11 agar plates and incubated at 37 °C for 2–4 weeks. Colonies were then counted and cfu/ml calculated.

Growth of M. tuberculosis in Mice—M. tuberculosis cultures were grown in Dubos medium to an A₆₀₀ of 0.6. Cultures were pelleted and resuspended in DMEM (Sigma) plus 2 mM L-glutamine and 50% fetal calf serum to a concentration of 10⁷ bacteria per ml. Subsequently, 10-ml bacterial stocks were prepared containing ~10⁶ cfu/ml, and mice were infected with ~100 cfu each, using a Glas-Col aerosol infection system. Aerosol infections were performed at the National Institute for Biological Standards and Control. The infection was monitored by removing the lungs and spleens of infected mice at various intervals, homogenizing the tissues, and plating 10-fold serial dilutions to determine numbers of cfu of M. tuberculosis. The growth curves were compared by graph and statistical analysis. The results for each time point are the means of cfu determinations performed on organs from five mice, and the error bars show the standard error of the means.

Isothermal Titration Calorimetry—Experiments to determine binding affinities and stoichiometries were performed with the MicroCal iTC200 system (GE Healthcare). 200 µl of purified FHA-1 or FHA-2 protein (at 50 µM) in 20 mM Tris/HCl, pH 8, and 150 mM NaCl was loaded into the sample cell. 100 µl of phosphopeptide (at 500 µM) in the same buffer was loaded into the syringe. During the experiment, 2 µl of ligand was titrated into the sample cell every 180 s with a total of 20 injections. The sequence of the synthesized Thr(P)-150 peptide was KKYAGQQLPPApTTRIPAA and the sequence of the Thr(P)-208 peptide was KKYAGTEAGNLApTSMMK, where pT is the phosphothreonine residue.

RESULTS

pknF and Rv1747 Form a Two Gene Operon—The genomic localization of pknF and Rv1747 (Fig. 1A) suggested that the two genes could potentially form an operon as the intergenic region between pknF and Rv1747 is only 62 bp. Therefore, to confirm this hypothesis, we performed RT-PCR showing that there was a transcript extending from pknF through the intergenic region into Rv1747, thus demonstrating that these two genes are indeed co-transcribed (Fig. 1B). A transcript was also present for each investigated gene when using gene-specific internal primers, although no transcript was detected in the intergenic region between Rv1747 and Rv1748 when using a forward primer within Rv1747 and with a reverse primer within Rv1748, indicating that these two genes are not co-transcribed. In addition, no transcript was present between Rv1748 and the convergent gene Rv1749c. Therefore, these results confirmed that pknF and Rv1747 are co-transcribed. Such co-localization of genes within the same operon is usually taken to indicate that there is a functional relationship between the gene products; this prompted us to study the role of a putative interaction between PknF and Rv1747.

Rv1747 Is Phosphorylated by PknF on Two Threonine Residues—To decipher the role of phosphorylation on Rv1747, it was necessary to determine the PknF-mediated phosphorylation sites. First of all, we confirmed the phosphorylation of Rv1747 by PknF with the recombinant proteins newly generated. Thus, only the kinase domain of PknF (residues 1–292) and the nucleotide binding domain of Rv1747 (residues 1–559) were cloned, expressed, and purified as recombinant proteins from E. coli. Phosphorylation of Rv1747 did not occur with PknG and was extremely weak with PknB (supplemental Fig. S1A). Using the recombinant proteins, we showed that PknF was an active kinase in an in vitro assay and was indeed able to specifically phosphorylate Rv1747, whereas Rv1747 did not have any autophosphorylation activity (Fig. 2A), thus confirming our previous results (20). In a second step, a mass spectrometry strategy was used to identify the number and nature of the phosphorylation sites on Rv1747. Such a method has been successfully used to elucidate the phosphorylation sites in a sequence-specific fashion for several M. tuberculosis STPK substrates (17, 26–29). Rv1747 was incubated with unlabeled ATP in the presence of PknF and subjected to mass spectrometric analysis after tryptic digestion. Spectral identification and phosphorylation determination were achieved with the paragon algorithm from the ProteinPilot® 2.0 data base-searching software (Applied Biosystems) using the phosphorylation emphasis criterion against our locally constructed data base that included the sequences of Rv1747 and derivatives. The phosphopeptides identified by the software were then validated by manual examination of the corresponding MS/MS spectra. Manual validations were performed based on neutral loss of H₃PO₄ from the precursor ion and the assignment of major fragment ions to b- and y-ion series or to the corresponding
neutral loss of H$_3$PO$_4$ from these ions. The sequence coverage of the protein was 96% and phosphorylation occurred only on peptides 119–164 and 199–212 as the MS/MS spectra unambiguously confirmed the presence of a phosphate group on Thr-150 and Thr-208 (Fig. 3), which are situated between the FHA-1 and FHA-2 domain for Thr-150, although Thr-208 is just upstream of the FHA-2 domain.

Moreover, to confirm the phosphorylation sites identified, we co-expressed the PknF kinase and its substrate Rv1747 in E. coli using a strategy described recently (30). The PknF kinase domain and its cognate substrate Rv1747 were cloned into the pCDF-Duet vector and overexpressed, and therefore the His-tagged phosphorylated Rv1747 was purified as described previously, whereas the PknF kinase was not tagged and therefore...
not co-purified. The phosphorylated Rv1747 isoform was directly analyzed by mass spectrometry as described above, and the MS/MS spectra confirmed the presence of phosphate groups on each of the phosphorylation sites identified previously (data not shown).

Definitive identification of the Thr-150 and Thr-208 residues determined by mass spectrometry was achieved by site-directed mutagenesis by introducing mutations that prevented their specific phosphorylation. Thus, single and double mutations were performed replacing threonine residues by alanine, yielding the mutants Rv1747_T150A, Rv1747_T208A, and Rv1747_T150A/T208A. These mutant proteins were expressed in E. coli, purified, and incubated with \[^{32}P\]ATP and PknF (Fig. 2B). Importantly, phosphorylation of the double mutant (phospho-ablative mutant), Rv1747_T150A/T208A, was almost totally abrogated compared with phosphorylation of Rv1747_WT (Fig. 2B), indicating that Rv1747 is phosphorylated only on these two residues, at least in vitro, in the presence of PknF. This was further supported by analysis of an additional round of mass spectrometry of Rv1747_T150A/T208A pretreated with ATP and PknF, which failed to identify any additional phosphate groups (data not shown). However, as shown in Fig. 2B, the T150A or T208A substitutions reduce the phosphorylation signal of the mutants compared with Rv1747_WT but not as much as the double mutant. Taken together, these results indicate that Thr-150 and Thr-208 are the primary targets for PknF phosphorylation in vitro, suggesting that they are likely to play critical roles in the regulation of Rv1747 in vivo.

Growth of the ΔRv1747 Mutant and the Phospho-ablative Mutant Strains Was Attenuated in Macrophages and in Mice—We have previously demonstrated that deletion of Rv1747 resulted in a growth defect in macrophage and mouse infections (15). Therefore, with the phosphorylation sites identified above, it became possible to investigate the in vivo consequences of Thr-150 and Thr-208 phosphorylation on Rv1747 activity. Thus, site-directed mutagenesis was used to introduce a double T150A/T208A mutation into the Rv1747-complementing plasmid previously used (15), which includes Rv1747, pknF (Rv1746), and 609 bp of Rv1745 (Fig. 1A). This construct was then integrated into the attB site of the ΔRv1747 mutant to produce a phospho-ablative Rv1747-complementing strain. These different strains were used in macrophage and mouse infections. As shown in Figs. 4 and 5, growth of the ΔRv1747 strain in BMDMs and in mice was significantly attenuated for growth. In fact, growth of the ΔRv1747 mutant was approximately 1 log lower than the WT strain at 168 h post-infection in naive and IFN-γ-activated BMDMs (Fig. 4) and was more than 1 log lower in the lungs and almost 4 logs lower in the spleens.
than the WT strain at 90 days post-infection (Fig. 5). However, growth in vitro of the \Delta Rv1747 mutant and the complemented strain, as measured by OD_{600} readings, did not differ from that of the WT strain thus indicating that growth attenuation is specific to the host-pathogen environment and that Rv1747 is critical for growth in macrophages and mice rather than an in vitro growth defect (15). Importantly, growth in vivo of the T150A/T208A phospho-ablative strain was attenuated, displaying an intermediate growth phenotype between that of the \Delta Rv1747 mutant and WT complement in both BMDMs and in mice (Figs. 4 and 5). These data confirm that phosphorylation of Thr-150 and Thr-208 plays a critical role in positively regulating Rv1747 activity.

Growth of the \Delta pknF Mutant Strain Was Not Attenuated in Macrophages—Because of the growth attenuation phenotype observed with the \Delta Rv1747 mutant in both infection models (Figs. 4 and 5) and because PknF phosphorylates Rv1747 (Figs. 2 and 3), it was therefore interesting to investigate the consequences of \textit{pknF} deletion on the growth of \textit{M. tuberculosis} in BMDMs. Growth in vitro of the \Delta pknF mutant and the complemented strain, as measured by OD_{600} readings, did not differ from that of the WT strain (data not shown). As shown in Fig. 6, growth of the \Delta pknF strain in BMDMs was not significantly different compared with growth of the WT or \textit{pknF} complementing strain (Fig. 6) suggesting that Rv1747 is probably able to be phosphorylated \textit{in vivo} by other STPK(s) when its preferred kinase, PknF, is missing.

\textbf{FHA-1 Is Required for Rv1747 Function, and Both FHA Domains Bind the Rv1747 Phosphothreonine Epitopes with Similar Affinities}—Our \textit{in vivo} assays in macrophages and mice demonstrated that Rv1747 was tightly regulated by phosphorylation. However, the specific role of the Rv1747 FHA domains in Rv1747 regulation by PknF remained to be investigated. The structure of the Rad53 FHA-1 domain from \textit{Saccharomyces cerevisiae} in complex with a phosphothreonine peptide has indicated that there are six highly conserved residues in the FHA domain (31). Five are located around the peptide-binding site, three of which make interactions with the peptide. Of these three, only two, Arg-70 and Ser-85, bind directly to the Thr(P) residue itself and are essential for this interaction. We have therefore mutated the equivalent serine residues in the two FHA domains of Rv1747 to alanines, and generated two additional Rv1747-complementing strains. We showed that mutation of FHA-1 results in a growth attenuation phenotype in BMDMs (Fig. 7) suggesting that Rv1747 is probably able to be phosphorylated \textit{in vivo} by other STPK(s) when its preferred kinase, PknF, is missing.

\textit{FHA-1 Is Required for Rv1747 Function, and Both FHA Domains Bind the Rv1747 Phosphothreonine Epitopes with Similar Affinities}—Our \textit{in vivo} assays in macrophages and mice demonstrated that Rv1747 was tightly regulated by phosphorylation. However, the specific role of the Rv1747 FHA domains in Rv1747 regulation by PknF remained to be investigated. The structure of the Rad53 FHA-1 domain from \textit{Saccharomyces cerevisiae} in complex with a phosphothreonine peptide has indicated that there are six highly conserved residues in the FHA domain (31). Five are located around the peptide-binding site, three of which make interactions with the peptide. Of these three, only two, Arg-70 and Ser-85, bind directly to the Thr(P) residue itself and are essential for this interaction. We have therefore mutated the equivalent serine residues in the two FHA domains of Rv1747, Ser-47 in FHA-1 and Ser-248 in FHA-2 to alanines, and generated two additional Rv1747-complementing strains. We showed that mutation of FHA-1 results in a growth attenuation phenotype in BMDMs (Fig. 7) suggest-
ing that FHA-1 is essential for Rv1747 function. In fact, growth of the FHA-1 mutant was more than 1 log lower than the WT strain at 168 h post-infection in naive and IFNγ-activated BMDMs (Fig. 7). Interestingly, growth of the FHA-2 domain mutant was not significantly different from the growth of the WT strain (Fig. 7) suggesting that this domain is not essential for Rv1747 function.

From these BMDM infections, it therefore appeared interesting to investigate the role of this FHA-1 domain at the molecular level. Moreover, we wanted to determine the binding affinities and stoichiometries between both the Rv1747 FHA domains and the Thr(P)-150 and Thr(P)-208 phosphopeptides to understand more about the interplay between the FHAs and the PknF-phosphorylated motifs within Rv1747 in controlling the Rv1747 signaling system. Using isothermal titration calorimetry, both FHA domains were shown to bind both Rv1747 phosphothreonine epitopes with similar affinities in the micro-molar range (Fig. 8). However, the FHA-1 interaction with both the phosphopeptides was abolished upon mutation of Ser-47 to Ala in the peptide binding pocket of FHA-1, thus strongly suggesting that the interactions between the FHA domains and the epitopes are specific. These isothermal titration calorimetry data coupled with the fact that we observed growth attenuation in an FHA-1 S47A mutant (Fig. 7) and in a Rv1747 T150A/ T208A strain in macrophages and mice (Fig. 4 and Fig. 5) allows us to speculate that the FHA domains, particularly FHA-1, play dual roles in Rv1747 regulation through both recruitment of PknF and, potentially, through phospho-dependent interactions with PknF target sites within the dimeric Rv1747 assembly.

**DISCUSSION**

Reversible phosphorylation is a ubiquitous mechanism of signaling transduction often used to transduce extracellular signals into cellular responses (2). Intriguingly, mycobacterial genomes contain relatively few histidine kinases and response regulators compared with other bacterial genomes of a similar size (32). Although 30 genes encoding putative two-component system proteins have been identified in the M. tuberculosis genome (9), only 11 systems paired in operons have been characterized. This is far fewer than in E. coli where over 30 pairs have been characterized (33). However, this paucity of histidine kinase-based signal transduction systems in M. tuberculosis may be compensated by the relatively large number of STPKs (11) in the genome (8, 9). Therefore, it is thought that in M. tuberculosis, STPKs fulfill the role of the classical bacterial two-component systems (8, 32).

Interestingly, STPKs have been discovered in many species of pathogenic bacteria, including Listeria monocytogenes, Pseudomonas aeruginosa, and Streptococcus pneumoniae implicating STPKs in the regulation of virulence and pathogenesis of these organisms (34). Furthermore, STPKs are present particularly in organisms such as Streptomyces...
where proteins are phosphorylated in response to developmental phases, including secondary metabolism (35). The *M. tuberculosis* STPKs have been implicated to have diverse regulatory effects, and potential functions of some of the proteins have been deduced experimentally (6). For example, PknA and PknB (the two essential STPKs) have been implicated to have roles in cell morphology and shape (36, 37); phosphorylation of Rv1827 by PknB (or PknG) abrogates binding of Rv1827 to three proteins that are all involved in α-ketoglutarate metabolism (38); PknE is involved in the nitric oxide stress response and apoptosis of *M. tuberculosis* in a human macrophage model of infection (39), and PknG promotes mycobacterial survival within macrophages by preventing phagosome-lysosome fusion (40).

Clearly, the *M. tuberculosis* STPK signaling network is highly complex and still rather poorly understood. Here, we have identified two specific threonine residues on Rv1747 that are phosphorylated *in vitro* by PknF and that have *in vivo* modulatory effects on the function of the Rv1747 ABC transporter. We showed that mutation of Thr(P)-150 and Thr(P)-208 results in almost complete loss of phosphorylation of Rv1747 *in vitro*. This result demonstrated that the two residues identified are both important for PknF phosphorylation of Rv1747, although other minor sites may play additional roles (Fig. 2). We have further investigated the consequences of Rv1747 phosphorylation by assessing the *in vivo* growth of *M. tuberculosis* mutated at these critical Thr residues and showed that growth is attenuated in both BMDMs, as well as in the lungs and spleens of mice. This latter result indicates that phosphorylation of Rv1747 is required for its function, *i.e.* that phosphorylation positively regulates its function. The alternative possibility of negative regulation whereby phosphorylation inhibited Rv1747 function would not be expected to result in growth attenuation of
Positive Regulation by M. tuberculosis Kinase

the Thr-150/Thr-208 mutant and neither would a situation where phosphorylation had no effect on Rv1747 function.

The ΔpknF mutant showed no clear growth phenotype in BMDMs thus confirming that Rv1747 is probably able to be phosphorylated in vivo by other STPK(s) when its preferred kinase is absent. This point is supported by the fact that PknB can slightly phosphorylate Rv1747, although PknF is clearly more intense in vitro (supplemental Fig. S1A). Incidentally, a similar result was obtained using 50 μM cold ATP, thus removing the possibility that PknF appears to be the most effective because it has the lowest K_m value for ATP (supplemental Fig. S1C). Therefore, one could imagine that in vivo, when the preferred PknF kinase is missing, another STPK could phosphorylate Rv1747. The fact that various M. tuberculosis STPKs appear able to phosphorylate domains of Rv1747 (16) suggests that its activity might be regulated in vivo by multiple environmental signals. This hypothesis is based on observations by different groups that show mycobacterial Ser/Thr kinases are able to cross-talk and recognize the same substrate (and probably also in vitro) (5–7, 16) in order for the mycobacteria to integrate different kinds of signals emitted in distinct environmental conditions.

Furthermore, we have shown that the function of Rv1747 was disrupted by mutation of Ser-47 of FHA-1, which is a highly conserved phospho-interacting residue in FHA domains (31, 41). In contrast, growth of M. tuberculosis with mutation of S248A of FHA-2 in BMDMs was the same as the WT. These data greatly strengthen our previous observations that the interaction of Rv1747 with PknF was reduced by 99% by substitution of Ser-47 by Ala in FHA-1 of Rv1747 using yeast two-hybrid analysis but was only approximately one-third reduced by substitution of Ser-248 by Ala in FHA-2 (15). Furthermore, in our previous experiments, phosphorylation of Rv1747 by PknF was reduced to ~5% of the WT levels by substitution of Ser-47 by Ala in FHA-1 and to ~10% of the WT levels by substitution of Ser-248 by Ala in FHA-2 (20), again highlighting the relative importance of both domains.

Taken together, our results suggest that in order for the Rv1747 ABC transporter to function fully, it is a requirement for phosphorylation on residues Thr-150 and Thr-208 that may, in turn, mediate protein-protein interactions with the FHA domains and/or other protein partners to sponsor full Rv1747 activation. Furthermore, our data suggest that Rv1747 must contain a functional FHA-1 domain to mediate interactions with PknF (or other kinases) and/or with Rv1747 itself. The degree of the growth attenuation phenotype of the Rv1747 complement strain mutated in residues Thr-150 and Thr-208 is not as severe as the growth phenotype seen in the Rv1747 deletion strain. This indicates that in the absence of phosphorylation on residues Thr-150 and Thr-208 of Rv1747, this ABC transporter can still function to some extent and that phosphorylation serves to positively modulate the transporter. Additionally, our results show that FHA-1 and FHA-2 bind both Thr(P)-150 and Thr(P)-208 phosphopeptides with similar affinities suggesting that in vivo both of the FHA domains may be able to bind either of the Thr(P) epitopes to regulate protein function. These interactions could occur within an Rv1747 monomer or across the dimer. Indeed, both intra- and intermolecular regulatory mechanisms have been observed in other FHA domain interactions (38, 42).

A recent study used a mass spectrometry-based approach to identify phosphorylated proteins in M. tuberculosis (7). A combined bioinformatic analysis of the in vivo phosphorylation sites with data from in vitro kinase assays led to identification of phosphorylation site motifs for PknA, PknB, PknD, PknE, PknF, and PknH (7). The motif of the six investigated kinases all included a threonine residue as the phosphoacceptor and hydrophobic residues at the Thr(P)+3 and Thr(P)+5 positions. Residues Thr-150 and Thr-208 of Rv1747 both share some of the features of the PknF preferred phosphorylation motif supporting the identified phosphoacceptor residues as significant in terms of Rv1747 protein function. In fact, the Thr-208 motif has a methionine residue at Thr(P)+3 and an isoleucine residue at Thr(P)+5, both of which appear in the PknF preferred phosphorylation site motif, and the Thr-150 site contains an isoleucine residue at the Thr(P)+3 position, which also appears in the predicted motif (7). Indeed, the FHA domains present in Rv1827 and Rv0020c both select for hydrophobic residues at the Thr(P)+3 position suggesting a common FHA domain recognition mechanism in M. tuberculosis that overlaps with that of the STPKs themselves (31, 43).

In conclusion, this work has uncovered significant roles for pknF and Rv1747 genes in M. tuberculosis growth in vivo and has demonstrated the critical importance of the FHA-1 domain for Rv1747 protein function. However, because the pknF mutant has no growth phenotype in an infection, this suggests that this particular kinase would not be a useful target for drug inhibition. Our results suggest that phosphorylation positively modulates the function of the ABC transporter possibly through conformational changes associated with the two FHA domains interacting with Thr(P)-150 and Thr(P)-208 on Rv1747. These data provide evidence for the first time that an STPK can modulate the function of an ABC transporter required for the growth of M. tuberculosis in vivo. Future studies are required to determine the PknF stimulus and the precise nature of the phospho-dependent regulatory mechanism of Rv1747 to complete the dissection of this important mycobacterial signaling system.

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