Phosphorylation of proteins by Ser/Thr protein kinases (STPKs) has recently become of major physiological importance because of its possible involvement in virulence of bacterial pathogens. Although Mycobacterium tuberculosis has eleven STPKs, the nature and function of the substrates of these enzymes remain largely unknown. In this work, we have identified for the first time STPK substrates in enzymes involved in mycolic acid biosynthesis: the malonyl-CoA::AcpM condensing part of the type II fatty acid synthase (FAS-II) system. The elongation process of long chain fatty acids is controlled by two elongation systems, the eukaryotic-type fatty acid synthase (FAS-I) and the prokaryotic-like FAS-II. FAS-I consists of a single multifunctional polypeptide, catalyzing de novo synthesis of medium length acyl-CoA chains (C₁₆₋₂₆), whereas FAS-II comprises several distinct enzymes. It catalyzes similar types of reactions to FAS-I, but functions on acyl carrier protein (AcpM)-bound chains and is incapable of de novo synthesis. The initial substrates of FAS-II are KasA and KasB. All three enzymes were phosphorylated in vitro by different kinases, suggesting a complex network of interactions between STPKs and these substrates. In addition, both KasA and KasB were efficiently phosphorylated in M. bovis BCG each at different sites and could be dephosphorylated by the M. tuberculosis Ser/Thr phosphatase PstP. Enzymatic studies revealed that, whereas phosphorylation decreases the activity of KasA in the elongation process of long chain fatty acids synthesis, this modification enhances that of KasB. Such a differential effect of phosphorylation may represent an unusual mechanism of FAS-II system regulation, allowing pathogenic mycobacteria to produce full-length mycolates, which are required for adaptation and intracellular survival in macrophages.

Mycobacterium tuberculosis has a unique cell wall structure that accounts for the ability of the bacterium to grow in several contrasting environments and which is responsible for its low membrane permeability, contributing to its resistance to common chemotherapeutic agents (1). The cell wall has been implicated as a direct modulator of interactions between mycobacteria and the environment (2). This envelope, characterized by its high lipid content, comprises an inner membrane barrier composed of mycolic acids anchored to arabinogalactan, linked to peptidoglycan. Mycolic acids are a hallmark of the mycobacterial waxy coat: they represent key virulence factors required for intracellular survival (3, 4) and contribute to the physiopathology of tuberculosis. They consist of very long chains of α-branched β-hydroxy fatty acids (C₉₀₋₁₅₀), whose biosynthesis is controlled by two elongation systems, the eukaryotic-type fatty acid synthase (FAS-I) and the prokaryotic-like FAS-II (5, 6). FAS-I consists of a single multifunctional polypeptide, catalyzing de novo synthesis of medium length acyl-CoA chains (C₁₆₋₂₆), whereas FAS-II comprises several distinct enzymes. It catalyzes similar types of reactions to FAS-I, but functions on acyl carrier protein (AcpM)-bound chains and is incapable of de novo synthesis. The initial substrates of FAS-II are KasA and KasB resulting from the condensation by mtFabH of the acyl-CoA products of FAS-I with malonyl-AcpM (7, 8). Following reduction by MabA, elimination of water by a yet unidentified dehydratase, and reduction by the enoyl-AcpM reductase InhA, the β-ketoacyl-AcpM synthases KasA and KasB catalyze further condensations with malonyl-AcpM in the FAS-II cycle (9, 10). Although changes in the mycolic acid profile seem to be regulated by various environmental stimuli, such as those encountered within the infected macrophage, very little is known at a molecular basis about how pathogenic mycobacteria modulate mycolate composition in response to these changes. Whether regulation of FAS-II enzymes occurs at the transcriptional and/or the translational level is not known. Elucidation of mechanisms modulating mycolic acid biosynthesis would shed some light on the capacity of M. tuberculosis to adapt and survive within the infected host.

Reversible protein phosphorylation is a key mechanism by which environmental signals are transmitted to cause changes in protein expression or activity in both eukaryotes and prokaryotes. Genes encoding functional serine/threonine protein kinases (STPKs) are ubiquitous in prokaryotic genomes, but little is known regarding their physiological substrates and their participation in bacterial signal transduction pathways (11). Understanding prokaryotic kinase biology has been seriously hampered by the failure to identify relevant kinase substrates. Signaling through Ser/Thr...
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TABLE 1
Primers used in this study

| Kinase       | Primer*  | 5’ to 3’ sequencea,b,c | Primers pair               |
|-------------|----------|------------------------|----------------------------|
| PknA-(1–338) | 132 (+)  | TATGAGCCCCCGAGTTGGCGTGACGC | PCR1 = 200/201             |
|             | 184 (−)  | TATAAGCTTTACAGACTGGAGGAAAGGACGAGCC | PCR2 = PCR1/273            |
| PknB-(1–331) | 133 (+)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
|             | 86 (−)   | TATAAGCTTTACAGACTGGAGGAAAGGACGAGCC |                            |
| PknD-(1–378) | 206 (+)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
|             | 207 (−)  | TATAAGCTTTACAGACTGGAGGAAAGGACGAGCC |                            |
| PknE-(1–337) | 220 (+)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
|             | 222 (−)  | TATAAGCTTTACAGACTGGAGGAAAGGACGAGCC |                            |
| PknF-(1–300) | 212 (+)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
|             | 211 (+)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
| PknG-(1–360) | 141 (−)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
|             | 200 (−)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
| PknH-(1–399) | 187 (+)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
|             | 88 (−)   | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
| PknI-(1–351) | 198 (+)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
|             | 199 (−)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
| PknJ-(1–340) | 211 (+)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
|             | 208 (−)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
| PknK-(1–300) | 209 (+)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
|             | 211 (+)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
| PknL-(1–369) | 274 (+)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |
|             | 275 (+)  | TATGAGCCCCCGAGTTGGCGTGACGC |                            |

* Forward and reverse primers are represented by plus (+) or minus (−), respectively.
*b Restriction sites are italicized.
*c The bases mutated from those present in the wild type are underlined.

Phosphorylation has emerged as a critical regulatory mechanism in various bacteria, including pathogenic mycobacteria. The genome of M. tuberculosis contains eleven coding regions with significant similarity to eukaryotic STPKs (11, 12). Nine of these gene products are predicted to be membrane proteins, presenting a sensor domain to the extracellular face and a kinase catalytic domain to the cytoplasm (11). All mycobacterial Ser/Thr kinases described to date display autophosphorylation activity, and several exogenous substrates have been reported to be phosphorylated by these enzymes (13–21). Our understanding of STPKs/substrate interactions in mycobacteria remains limited, because only a few endogenous substrates have been reported, most of them being recognized by virtue of being encoded by genes close to their cognate STPK genes (22–26).

A recent proteomic study with Corynebacterium glutamicum revealed that the vast majority of the phosphorylated proteins are metabolic enzymes rather than regulatory proteins, suggesting that protein phosphorylation plays a much broader function in the physiology of the bacteria than was previously expected (27). This observation, along with several pieces of data brought us to suspect that activity of the tightly interconnected FAS-II components (28) might depend on post-translational modifications, such as phosphorylation. Therefore, as a first step toward the identification of regulatory mechanisms governing mycolic acid biosynthesis, we investigated whether metabolic FAS-II components from M. tuberculosis may represent substrates of STPKs. In this study, we show for the first time that several FAS-II components, including KasA and KasB, are phosphorylated in vitro and in vivo by STPKs and provide evidence that phosphorylation differentially affects their condensing activity.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Strains used for cloning and expression of recombinant proteins were Escherichia coli DH5α (Clontech Laboratories), E. coli TOP-10 (Invitrogen), E. coli BL21(DE3)pLysS (Novagen) and E. coli strain C41(DE3) (29). All strains were grown and maintained in LB medium at 37 °C. When required, media were supplemented with 100 μg/ml ampicillin, and/or 50 μg/ml chloramphenicol, and/or kanamycin 25 μg/ml. M. bovis BCG strain Pasteur 1173P2 was grown on Middlebrook 7H10 agar plates supplemented with OADC enrichment (Difco) or in Sauton medium.

Cloning, Expression, and Purification of the Eleven Recombinant GST-tagged STPKs of M. tuberculosis—PCR fragments encoding the intracellular region corresponding to the kinase core and the juxtamembrane linker of PknA (residues 1–338), PknB (residues 1–331), PknD (residues 1–378), PknE (residues 1–337), PknF (1–300), PknG (residues 1–360), PknH (residues 1–399), PknI (residues 1–351), PknJ (residues 1–340), PknK (residues 1–300), and PknL (residues 1–369) were amplified by using M. tuberculosis H37Rv genomic DNA as template. Site-directed mutagenesis based on PCR amplification was carried out for the cloning of PknG, PknI, and PknJ. This strategy, as already described by Molle et al. (22), consisted of creating substitutions in the BamHI restriction site naturally present in those genes to create a mismatch in the specific restriction sequence without changing the coding sequence. Therefore, PknG, PknI, and PknJ could be digested and cloned as BamHI/HindIII DNA fragments. DNA fragments corresponding to the 11 intracellular regions of the different kinases were amplified with their specific primers (Table 1), digested by BamHI and HindIII, and ligated into vector pGEX(M).
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Recombinant strains harboring the kinase-expressing constructs were used to inoculate 100 ml of LB medium supplemented with ampicillin and were incubated at 37 °C with shaking until $A_{600}$ reached 0.5. IPTG was then added at a final concentration of 1 mM, and growth was continued for an additional 3 h at 37 °C, with shaking. Purification of the GST-tagged recombinant proteins was performed with glutathione-Sepharose 4B matrix (Amersham Biosciences), as already described (22).

Construction and Purification of His$_6$-tagged mtFabD, KasA, KasB, Holo-AcpM, and OmpATb—Plasmids designed to express mtFabD and KasA (pET28a-mtFabD and pET28a-kasA) were described earlier (10, 30). The kasB gene (Rv2246) was amplified by PCR using M. tuberculosis H37Rv genomic DNA as a template and the following primers: kasB-up 5’-GGG TAC CAC CAC TTG CGG GGG CGA GT-3’ and kasB-lo 5’-GGG GCC CAA GCT TGT CAT CGC AGG TCT-3’. This 1361-bp DNA fragment was directly ligated into the pET28a (Novagen), which had been cut with NdeI and filled-in with Klenow enzyme, thus giving rise to pET28a-kasB. E. coli C41(DE3) cells transformed with pET28a-kasB were used to inoculate 100 ml of Terrific Broth medium supplemented with 25 μg/ml kanamycin. Cultures were incubated at 37 °C with shaking until $A_{600}$ reached 1. IPTG was then added at a final concentration of 1 mM, and growth was continued overnight at 16 °C with shaking. Purification of recombinant mtFabD, KasA, KasB was performed as described earlier (30). Expression and purification of holo-AcpM and OmpATb was done as reported previously (30, 31).

Analysis of the Phosphoamino Acid Content of Proteins—Phosphoamino acid analysis of the labeled protein reaction products of the in vitro protein kinase reactions were performed as previously described (32).

Overexpression and Purification of the M. tuberculosis KasA and KasB Proteins in M. bovis BCG—Standard PCR strategies were used to amplify the M. tuberculosis H37Rv kasA or kasB genes, using the following primers: pVV16-kasA-up 5’-TGA GTC AGC CTT CCA CCG CTA-3’ and pVV16-kasA-lo 5’-TCT TGG TCC CCC CGC TTG CGG-3’ (containing a HindIII site underlined), pVV16-kasB-up 5’-TAT AAG CTT TCA GCC CGA CGA-3’ (containing a HindIII site underlined), and pVV16-kasB-lo 5’-TGG GCC AAG CTG GTA GCC GGC GAT TGC-3’ (containing a HindIII site underlined). The PCR products were cut with HindIII, enabling direct cloning into the pVV16 expression vector cut with MscI/HindIII (33). This plasmid is a derivative of pMV261 (34), containing both a kanamycin and a hygromycin resistance cassette, harboring the hsp60 promoter as well as a His tag for expression of C-terminal His-tagged fusion proteins. The resulting expression vectors, named pVV16-kasA and pVV16-kasB, were used to transform M. bovis BCG. Transformants were selected on Middlebrook 7H10 supplemented with OADC enrichment and 25 μg/ml kanamycin and grown in Sauton containing kanamycin. Purification of soluble KasA-(His)$_6$ and KasB-(His)$_6$ was performed on Ni-NTA agarose beads as described previously (23).

Cloning, Overexpression, and Purification of PstP—The PCR fragment encoding the cytoplasmic region of the PstP phosphatase (residues 1–298), containing the phosphatase catalytic core, was amplified by using M. tuberculosis genomic DNA as a template. The 894-bp pstP gene fragment with appropriate sites at both ends was synthesized by PCR amplification with the following primers: 5’-TAT GGA TCC GTG CGG CGC GTG ACC CTG GTC-3’ and 5’-TAT AAG CTT TCA GCC CGA CCA CCG TGG CGG ACT-3’. This DNA fragment was digested with BamHI and HindIII, and ligated into vector pET43, digested with the same enzymes, to yield pET43-pstP-1(1–298). E. coli BL21(DE3) pLysS cells were transformed with pET43-pstP-1(1–298) and the recombinant E. coli strain was used to inoculate 100 ml of LB medium supplemented with ampicillin and chloramphenicol, and was incubated at 37 °C with shaking until $A_{600}$ reached 0.5. IPTG was then added at a final concentration of 1 mM, and growth was continued for an additional 3 h period at 37 °C, with shaking. Recombinant HspA3tagged PstP-(1–298) protein was purified on Ni-NTA beads (Qiagen) as described previously (23).

Two-dimensional Gel Electrophoresis—To detect the different phosphorylated isoforms of proteins that were phosphorylated in vitro, 5 μg of KasA, KasB, or mtFabD (purified from E. coli and phosphorylated in the presence of [γ-$^32$P]ATP and PknA) were electrophoresed on immobilized 7-cm pH 5–8 gradient strips on a Protean IEF Cell (Bio-Rad) in the first dimension and on a 10% SDS-PAGE in the second dimension. The Coomassie Blue-stained gels were dried onto filter paper (Whatman), and radioactivity was revealed by autoradiography. For in vivo detection, wild-type M. bovis BCG was grown to early stationary phase. Cells were harvested, washed twice with 20 mM Tris·HCl, pH 7.5, and resuspended in lysis buffer (20 mM Tris·HCl pH 7.5, 10% glycerol, antiprotease mixture, Roche Applied Science), followed by sonication. The lysate was cleared by centrifugation at 14,000 rpm for 30 min at 4 °C. Approximately 150 μg of total soluble proteins were loaded onto a 7-cm immobiline strip (Bio-Rad, pH 3–6) and electrophoresed in a Protean IEF Cell in the first dimension and on a 10% SDS-PAGE in the second dimension.

Immunoblotting—Two-dimensional gels of M. bovis BCG total soluble proteins were blotted on PVDF membrane, and probed with a rat anti-KasA antibody raised against the M. tuberculosis KasA protein, which also strongly cross-reacts with KasB (1:1000 dilution) (35). Horseradish peroxidase-conjugated anti-rabbit serum was used as a secondary antibody (1:5000 dilution), and detection was carried out using the Western Lightening Reagent (PerkinElmer Life Sciences) according to the manufacturer’s instructions. For immunoblotting of purified KasA and KasB proteins from E. coli or M. bovis BCG resolved on 1D PAGE, 2 μg were loaded on a 10% polyacrylamide gel, electrophoresed, blotted on PVDF, and detected using either polyclonal rabbit anti-phosphothreonine or polyclonal rabbit anti-phosphoserine (Invitrogen ImmunoPeroxidase) antibodies used at 1:200 dilution. Horseradish peroxidase-conjugated anti-rabbit serum was used as a secondary antibody (1:5000 dilution), and detection was carried out using the Western Lightening Reagent according to the manufacturer’s instructions.

KasA and KasB Activity Assay—KasA and KasB enzymatic activities were assayed as described (10). Briefly, Holo-AcpM (40 μM) was incubated at 37 °C for 30 min with 1 mM β-mer-
RESULTS AND DISCUSSION

STPK-mediated Phosphorylation of Mycobacterial FAS-II Enzymes—The main locus of the mycobacterial FAS-II system is an operon comprising five genes, all transcribed in the same orientation (6, 36). The third and fourth ORFs, kasA and kasB, encode the β-ketoacyl-ACP synthases that elongate the growing meromycolate precursor, whereas the first gene, mtfabD, encodes the malonyl-CoA:ACP transacylase that provides them with the malonyl-ACP substrate, the carbon donor during the elongation steps (6, 10, 30). AcpM, the mycobacterial acyl carrier protein, is encoded by a gene located between mtfabD and kasA (10). A systematic approach was used to investigate whether the eleven STPKs of M. tuberculosis (PknA to PknJ) phosphorylate these FAS-II components. All eleven STPKs were expressed as GST fusions and purified from E. coli and incubated with the purified His-tagged mycobacterial FAS-II enzymes in the presence of [γ-32P]ATP. The samples were separated by SDS-PAGE and visualized by autoradiography. Upper panel, KasA; second panel, KasB; third panel, mtfabD; fourth panel, AcpM; and lower panel, OmpATb. The right lane in each panel corresponds to the Coomassie Blue-stained gel showing purity and migration of each subunit. The asterisks represent the phosphoforms of KasA, KasB, and AcpM. A kinetic analysis (0–30 min) was performed with [γ-32P]ATP. Proteins were analyzed by SDS-PAGE, and radioactive bands were revealed by autoradiography. Upper panel, KasA; middle panel, KasB; lower panel, mtfabD.

We next examined whether mtfabD may be phosphorylated in vitro, an enzyme that has previously been shown to be phosphorylated in M. bovis BCG (37). The autoradiograph clearly shows [33P]ATP in the presence of the different STPKs (Fig. 1A, third panel). More surprising was the finding that the phosphorylation profile was comparable to those obtained for KasA and KasB. Altogether, these results suggest that the three FAS-II components can be phosphorylated by a specific set of M. tuberculosis kinases, and are preferred substrates for PknA in vitro, suggesting that PknA (and presumably the other STPKs) could phosphorylate all three enzymes using a similar mechanism.

Removal of the N-terminal His tag of KasA, KasB, or mtfabD by cleavage with thrombin did not alter the phosphorylation profile of the “mature” proteins, thus indicating that phosphorylation did not occur on the additional His tag residues (data not shown).

AcpM is a crucial FAS-II component that carries on the growing fatty acyl chain during the elongation step. We therefore investigated whether holo-AcpM may also be phosphorylated. Fig. 1A (fourth panel) shows that all STPKs failed to phosphorylate AcpM. We next addressed the question whether OmpATb, the major porin found in the cell wall of M. tuberculosis (31, 38) and which is not related to FAS-II, could be a substrate of the kinases. Our results indicate that none of the
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Kinases were able to phosphorylate OmpATb in vitro (Fig. 1A, fifth panel). Together, these results indicate that the kinases exhibit substrate specificity, although they present overlapping activities with regard to KasA, KasB, or mtFabD.

The results of kinetic analyses of PknA on the three FAS-II substrates, KasA, KasB, and mtFabD is summarized in Fig. 1B. In all three cases, the phosphorylation activity reached a maximum after 20 min. The “overlapping” substrate specificity of different kinases is consistent with previous studies showing that PknB, PknD, PknE, and PknF can all phosphorylate the FHA-containing protein GarA to some extent (24), as well as the FHA-containing proteins Rv0020c and Rv1747 (39). Most studies have reported interactions between FHA-containing proteins and STPKs belonging to either the same or different operons. This work shows that the same kinases can also phosphorylate substrates that lack the FHA domain. Thus, in addition to FHA-containing proteins, FHA-independent mechanisms involve direct binding to STPKs, emphasizing the complexity of STPKs signaling in M. tuberculosis. Another finding arising from these experiments is that KasA, KasB, and mtFabD can interact with multiple STPKs, suggesting that these enzymes may be regulated by multiple signals. However, it remains to be established whether this STPK cross-talk occurs in vivo, which would argue for a very complex signaling mechanism.

PknA Phosphorylates All Three FAS-II Enzymes on Multiple Threonine Residues—First, to investigate which amino acid residues were phosphorylated by PknA, we analyzed the phosphoamino acid content of phosphorylated KasA, KasB, and mtFabD. The different proteins (1 μg) were labeled with [γ-32P]ATP in vitro, separated by SDS-PAGE, excised, and subjected to acid hydrolysis as described in Molle et al. (22). Fig. 2 shows that all three substrates are preferentially phosphorylated at threonine residues with minor amounts of phosphoserine being detected.

We have recently shown that PknH-mediated phosphorylation of EmbR in vitro leads to five phosphorylation states in EmbR (40), whereas PknB phosphorylated GarA at a single phosphate acceptor residue (24). Therefore, to determine the in vitro phosphorylation profile of all three FAS-II enzymes, we performed two-dimensional gel electrophoresis. First, following in vitro phosphorylation by PknA, gel analysis indicated up to three phosphorylation states in KasA (Fig. 3A). The conclusion that the different phosphorylation states of KasA correspond to mono-, di-, or tri-phosphorylated forms of the protein relies on the fact that each spot on the two-dimensional gel is equidistant to the next one and can only correspond to a post-translational modification such as phosphorylation. In fact, each phosphate group changes the charge of the protein and makes it to migrate toward the acidic end of the two-dimensional strip (41). Spot intensities from autoradiographs indicated a dominant phosphorylated state (labeled 1), accounting for 88% of the total integrated spot intensity, with states 2 and 3 accounting for 10 and 2% each. Similarly, three phosphorylation sites were observed following PknA-mediated phosphorylation of KasB (Fig. 3B) and mtFabD (Fig. 3C).

In Vivo Phosphorylation of KasA and KasB in M. bovis BCG—To investigate whether KasA and KasB phosphorylation occurs in vivo, we have adopted a proteomic approach. Total soluble M. bovis BCG proteins were resolved on a two-dimensional gel, which were subsequently transferred to a membrane and probed with rat anti-KasA antibodies, which also cross-reacts with KasB. Western blot analysis shows the presence of 3 or 4 isoforms of KasA and KasB, presumably corresponding to various phosphorylation states (Fig. 4). These results clearly demonstrate that both KasA and KasB are very efficiently phosphorylated in vivo and that it is more significant in vivo than in vitro (following treatment with purified STPKs added individually, Fig. 3). The basis for the difference in the phosphorylation profiles observed in vitro and in vivo is presently unknown but suggests that the intracellular environment plays a key role in the phosphorylation efficiency of KasA and KasB. Some mycobacterial factors, which are absent from in vitro assays, may be required for better presentation of the substrate to the STPK(s). Alternatively, some STPKs may act first in order to mono-phosphorylate the substrate, thus allowing other kinases to phosphorylate the enzymes in a sequential manner. Moreover, this approach constitutes a powerful tool to look for phosphorylation of any mycobacterial protein. In addition, it may be useful to analyze how growth conditions can affect the phosphorylation profile. More importantly, these results...
Overproduction and Purification of KasA and KasB Phosphorylated in Vivo in M. bovis BCG—To overproduce and purify in vivo phosphorylated KasA, a His tag was attached at the C terminus of the KasA polypeptide and the recombinant protein was expressed in M. bovis BCG. Cultures of M. bovis BCG/pVV16-kasA were collected, lysed, and fractionated to separate the soluble cytosolic compartment from the cell envelope. Soluble KasA was then purified to homogeneity by affinity chromatography on Ni-containing beads under native conditions (Fig. 5D, left panel). We reasoned that analysis of this purified KasA sample by two-dimensional gel electrophoresis would allow us to determine whether phosphorylation of KasA takes place in mycobacteria, and whether it corresponds to a constitutive or growth phase-dependent phenomenon. Samples were collected at various time points during M. bovis BCG growth, corresponding to early-log, mid-log, late-log, or early stationary phases and KasA was purified at each time point and subjected to two-dimensional gel electrophoresis. Three spots, the first one presumably corresponding to the unphosphorylated form of KasA followed in the acidic direction by mono- and di-phosphorylated KasA, were clearly observed (Fig. 5A), demonstrating that KasA is phosphorylated in vivo. Here, the vast majority of KasA was phosphorylated at either one or two sites (mono-phosphorylation being the more prominent spot), and only a small proportion of KasA remained unphosphorylated. This contrasts with our in vitro analyses in which the majority of KasA remained unphosphorylated (Fig. 3A). Furthermore, the phosphorylation state of KasA remained constant, regardless of the growth phase. However, it is noteworthy that the phosphorylation profile was slightly different during stationary phase (Fig. 5A), where the mono-phosphorylated species was less pronounced than in actively replicating mycobacteria and characterized by the appearance of another spot, corresponding to the tri-phosphorylated form of KasA. This contrasts with recent findings using an anti-phosphothreonine antibody suggesting that mtFabD phosphorylation can only be detected in growing M. bovis BCG cultures (37). Like KasA, the vast majority of KasB was mono-phosphorylated (Fig. 5C) in M. bovis BCG. These results clearly confirm that both condensing enzymes are phosphorylated in mycobacteria to a significant level.

To determine whether in vivo phosphorylation of KasA and KasB occurs on threonine and/or serine residues, Western blot analysis were performed using specific anti-phosphothreonine or anti-phosphoserine antibodies. It was found that KasA and KasB purified from their respective M. bovis BCG strains were only phosphorylated on threonine residues (Fig. 5E). This is consistent with the results obtained by phosphoaminoacid analysis when the proteins were phosphorylated in vitro using PknA (Fig. 2). It clearly establishes that both condensing enzymes are highly phosphorylated in vivo on threonines. In contrast, neither the anti-phosphothreonine nor the anti-phosphoserine antibodies did react with recombinant KasA or KasB proteins purified from E. coli thus confirming that the phosphorylation of the condensing enzymes is a specific phenomenon of M. bovis BCG (Fig. 5E).

M. tuberculosis Phosphatase PstP Dephosphorylates KasA—M. tuberculosis has only one gene (pstP) encoding a transmem-
brane Ser/Thr phosphatase (12, 18). PstP efficiently dephosphorylates a variety of phosphorylated proteins, including PknA and PknB (17, 18) as well as other M. tuberculosis STPK domains (42). Dephosphorylation of STPKs simultaneously abolishes binding sites for substrates containing FHA domains (22) and substantially reduces protein kinase activity (18). Here, we addressed the question whether PstP was active on in vivo phosphorylated KasA, as might be expected if the phosphorylation activity is regulated and dependent on a reversible phosphorylation mechanism. Therefore, the cytoplasmic region of PstP (residues 1–298), containing the phosphatase catalytic core, was expressed as a His-tagged protein. The recombinant His-tagged PstP-(1–298) was produced as a soluble protein in E. coli, purified (Fig. 5D, right panel) and incubated with phosphorylated KasA isolated from M. bovis BCG. Two-dimensional gel analysis revealed a single spot migrating toward the basic pH and corresponding to an unphosphorylated state, suggesting that PstP-(1–298) dephosphorylated mono- and di-phosphorylated KasA (Fig. 5B). To our knowledge, this is the first time that PstP has been shown to dephosphorylate a mycobacterial STPK-phosphorylated substrate, suggesting that PstP exerts its phosphatase activity not only on kinases but also on the kinase substrates. These results strongly suggest that phosphorylation of KasA is reversible in vivo, where the phosphatase PstP may play a key regulatory role.

Phosphorylation Differentially Modulates KasA and KasB Condensing Activities—KasA and KasB have been previously shown to be β-ketoacyl-AcpM synthases of the mycobacterial FAS-II system (6, 10). FAS-II is responsible for the production of the long-chain fatty acid that is subsequently modified to form the meromycolate chain of mycolic acids. To date, there is very little information as to whether mycolic acid biosynthesis...
can be regulated post-translationally. We therefore sought to investigate the regulatory implications of the phosphorylation of FAS-II condensing enzymes. Kinetic analysis was carried out to investigate the effect of phosphorylation on substrate affinity and turnover of KasA and KasB. The effect of phosphorylation on KasA activity was analyzed using the recombinant purified protein from *E. coli* or from *M. bovis* BCG as sources of non-phosphorylated or phosphorylated KasA, respectively, and by varying both C₁₆-AcpM and malonyl-AcpM substrate concentrations (Fig. 6, A and B). The apparent $K_m$ values for substrates C₁₆-AcpM and malonyl-AcpM were about 18 μM and 12 μM, respectively, and did not change on phosphorylation, indicating that phosphorylation does not affect binding of these substrates. However, the apparent $V_{max}$ was consistently reduced by ~3–4-fold on phosphorylation, leading to a reduction in the rate of the condensation reaction. We also compared these parameters for unphosphorylated versus phosphorylated KasB (Fig. 6, C and D). In contrast to KasA, we found that phosphorylation consistently increased the condensation activity of KasB. Although the apparent $K_m$ values for C₁₆-AcpM remained unchanged following phosphorylation (about 12 μM), the apparent $K_m$ of malonyl-AcpM was reduced by 3-fold to 10 μM. These unexpected results demonstrate that phosphorylation affects KasA and KasB differently and suggests that they might be phosphorylated at different sites, despite their strong overall homology. Alternatively, structural variations around a conserved phosphorylation site might produce this differential effect.
Based on in vitro assays and drug inhibition studies, it has been proposed that KasA is involved in the initial elongation of meromycolic acid precursors that are further extended by KasB (6, 43). This hypothesis has recently been supported by in vivo studies in both kasA and kasB mutants. Null mutants of kasB, but not kasA, could be generated in Mycobacterium smegmatis, suggesting that, unlike kasB, kasA is essential (44). It was also found that conditional depletion of KasA in M. smegmatis leads to the loss of mycolic acid biosynthesis prior to cell lysis (44). A kasB mutant of Mycobacterium marinum showed an impaired growth within infected macrophages, although KasB was not required for normal growth in broth medium, indicating a critical role of KasB in intracellular survival (4). Moreover, the meromycolates were shortened by 2–4 carbon units in the kasB mutant and this defect was localized to the proximal portion of the meromycolate chain (4). Therefore, the importance for pathogenic mycobacteria to produce full-length mycolates for intracellular survival and virulence, one can postulate that KasA and KasB activities are being differentially regulated.

Because kasA and kasB are adjacent genes and belong to the same operon, one would predict that differential regulation proceeds at a post-translational rather than a transcriptional level. Our results suggest that phosphorylation is the post-translational modification that reduces the activity of KasA and enhances that of KasB. The differential effect of phosphorylation of KasA and KasB, two highly similar enzymes sharing the same enzymatic activity but with different substrates specificities, is rather unexpected. This is even more surprising given the fact that both enzymes share a similar phosphorylation profile in vivo, and are presumably phosphorylated at the same conserved threonine residues. This may represent an unusual mechanism of regulation where damping KasA may allow time to produce immature mycolates. Conversely, boosting KasB activity may ensure that mycobacteria produce only the full-length mycolates required for virulence and intracellular survival.

Among the few substrates of M. tuberculosis STPKs identified so far mitFabD, KasA and KasB are the only ones to which a clear enzymatic function has been assigned. Our work suggests that phosphorylation of these enzymes plays a role in the control of the FAS-II pathway and paves the way for studies dedicated to the regulation of mycolic acid biosynthesis. The differential expression of the mycobacterial kinases in response to stress conditions may directly affect the phosphorylation profile of these substrates, and as a consequence modulate mycolic acid biosynthesis in order to promote adaptation to environmental changes. In an elegant study, Veyron-Churlet et al. (28) have proposed an attractive model based on the interaction between the various FAS-II components, in which different specialized FAS-II complexes are interconnected. In particular, this model predicts the occurrence of two FAS-II systems involved in the elongation step: the “elongation-1 FAS-II” (E1-FAS-II) formed by a core and KasA, capable of elongating acyl-AcpM originating from FAS-I (or an initiation-FAS-II complex); the longer chain of acyl-AcpM products would then be channeled into the “elongation-2 FAS-II” (E2-FAS-II), comprising the core and KasB that would complete meromycolate synthesis. Thus, our results support a model in which STPK-dependent phosphorylation can induce either positive or negative signaling to the E1-FAS-II and E2-FAS-II complexes (Fig. 7). Further work is needed to understand how environmental changes, including those encountered within the infected macrophages, affect this regulation mechanism. Moreover, it was recently demonstrated that GroEL1 modulates mycolates synthesis during biofilm formation and physically associates with KasA (45). A ΔgroEL1 mutant of M. smegmatis, that is defective in biofilm formation also showed a marked reduction in KasA and KasB levels. Therefore, whether GroEL1 interacts specifically with phosphorylated KasA or KasB rather than the non-phosphorylated proteins deserves to be investigated. It may be reasonable to speculate that GroEL1 plays a regulatory role in phosphorylation of these proteins by the kinases. Another perspective of this work is the opening of a new field of investigation for future
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drug development against tuberculosis by interfering with these regulatory processes, such as by selective inhibition of FAS phosphorylation. This notion is supported by the fact that specific inhibitors of protein kinases have been successfully developed for therapeutic usage against a variety of diseases (46), and a specific inhibitor of PknG was capable to inhibit growth of M. tuberculosis inside macrophages (47). This work provides a new framework for future investigation of FAS-II regulation, not only in mycobacteria but also in apicomplexan parasites and in plants, which also possess a FAS-II system.

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