An FHA Phosphoprotein Recognition Domain Mediates Protein EmbR Phosphorylation by PknH, a Ser/Thr Protein Kinase from *Mycobacterium tuberculosis*†

Virginie Molle,‡,⊥ Laurent Kremer,§,⊥ Christine Girard-Blanc,‡ Gurdyal S. Besra,|| Alain J. Cozzone,‡ and Jean-François Prost*,‡

Institut de Biologie et Chimie des Protéines, Université de Lyon, Centre National de la Recherche Scientifique, Lyon, and Laboratoire des Mécanismes Moléculaires de la Pathogénie Microbienne, INSERM U447, Institut Pasteur de Lille/IBL, Lille, France, and School of Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K

Received July 3, 2003; Revised Manuscript Received October 24, 2003

ABSTRACT: In bacteria, regulatory phosphorylation of proteins at serine and/or threonine residues by Ser/Thr protein kinase (STPK) is an emerging theme in prokaryotic signaling, particularly since the prediction of the occurrence of several STPKs from genome sequencing and sequence surveys. Here we show that protein PknH possesses an autokinase activity and belongs to the large STPK family found in *Mycobacterium tuberculosis*. Evidence is presented that PknH can also phosphorylate EmbR, a protein suspected to modulate the level of arabinosyltransferase activity involved in arabinan biosynthesis of arabinogalactan, a key molecule of the mycobacterial cell wall. Interestingly, EmbR possesses an FHA (forkhead-associated) domain, a newly described phosphoprotein recognition domain, which plays an essential role in PknH−EmbR interaction and phosphorylation of EmbR by PknH. It is demonstrated that mutation of each of three particular residues of this FHA domain, Arg312, Ser326, and Asn348, totally abolishes the PknH-mediated phosphorylation of EmbR, thus highlighting the critical role of this domain in the direct interaction between EmbR and PknH.

For many years after the discovery of protein phosphorylation, catalyzed namely by serine/threonine protein kinases (*25*), the prevailing view was that these enzymes were present only in eukaryotes. However, the occurrence of similar kinases was later recognized in prokaryotes as well (*3*, *22*, *29*, *31*, *34*, *37*, *55*). Since then, evidence has been provided that STPKs[1] may play a regulatory role in signal transduction pathways (*6*, *51*), just like another well-known family of bacterial kinases, the sensor histidine kinases, which are key enzymes of the so-called "two-component systems" (*17*, *42*). In the case of *Mycobacterium tuberculosis*, the theoretical analysis of the genome sequence has predicted the presence of 11 different STPKs (*3*, *10*). Among these, only PknA, PknB, PknD, PknE, PknF, and PknG have been shown so far to catalyze autophosphorylation, and none of them have been yet demonstrated to phosphorylate endogenous proteins (*4*, *9*, *24*, *36*, *43*). Interestingly, a recent in silico analysis has indicated that several genes encoding STPKs seem to be critical for *M. tuberculosis* survival (*28*).

In this work, special attention has been paid to the PknH kinase, which had not been biochemically characterized previously. This kinase is predicted to consist of 626 amino acids, with a putative single trans-membrane-spanning helix located between amino acids L_{404} and I_{426}, dividing the protein into an N-terminal cytosolic domain and a C-terminal periplasmic domain. The analysis of the amino acid sequence of the protein indicates that the N-terminal region contains all of the 11 consensus catalytic subdomains of the Hanks-type kinase (*20*) which are required for a functional kinase activity. In addition, analysis of the *M. tuberculosis* genome database has revealed that the *pknH* gene (Rv1266c) is located downstream of the *embR* gene (Rv1267c), encoding a putative transcriptional activator belonging to the OmpR-like family (*32*, *33*, *45*). In *Mycobacterium avium*, EmbR modulates the level of arabinosyltransferase activity involved in the arabinan biosynthesis (*8*). Mycobacterial arabinan is a complex homopolymer consisting of D-arabinofuranosyl residues present in two forms in the cell wall. It can be found as part of the heteropolysaccharide arabinogalactan (AG) that is essential to the structural integrity of the cell wall and as part of lipoarabinomannan (LAM), a potent immunomodu-

† This work was supported by grants from the Ministère de la Recherche (Contract FNS 2000 Microbiologie), the Fondation pour la Recherche Médicale, the Société Ezus-Lyon 1 (Contract 482.022), the Institut Universitaire de France, the Medical Research Council (U.K.), and the INSERM.

* To whom correspondence should be addressed. Phone: 00 33 (0)4 72 72 26 72. Fax: 00 33 (0)4 72 72 26 01. E-mail: jf.prost@ibcp.fr.

‡ Université de Lyon.

§ Institut Pasteur de Lille/IBL.

|| The University of Birmingham.

⊥ These authors contributed equally to this work.

[1] Abbreviations: STPK, serine/threonine protein kinase; EMB, ethambutol; FHA, forkhead-associated domain; LB, Luria−Bertani medium; GST, glutathione *S*-transferase; IPTG, isopropyl 1-thio-*β*-galactopyranoside; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid disodium salt; DNase I, deoxyribonuclease I.; RNase A, ribonuclease A; PBS, phosphate-buffered saline; DTT, 1,4-dithio-D,L-threitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; SARP, *Streptomyces coelicolor* antibiotic regulatory protein; HTH, helix−turn−helix; DPA, dolichol phosphoarabinose; BAD, bacterial activation domain.

10.1021/bi035150b CCC: $25.00
Published on Web 11/26/2003

Table 1: Bacterial Strains and Plasmids Used in This Study

strain or plasmid	genotype or description	ref or source
DH5	*supE*44 *lac*U169(80*lacZ*M15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi-I relA*1	18
BL21λ(DE3)	B F^- *dcm ompT hsdS*($r_B^-m_B^-$) *gal* (DE3)	47
pET28a	*E. coli* expression vector generating His_6 fusion proteins for overexpression	Novagen
pET19b	*E. coli* expression vector generating His_6 fusion proteins for overexpression	Novagen
pGEX-KT	*E. coli* expression generating GST-fusion proteins for overexpression	Amersham
pGEX(M)	pGEX with a 321-bp *Eco*RI/*Bam*HI fragment from pET19b introducing a *Hin*dIII site in the pGEX polylinker	this study
pET28−*embR*	pET28a with a 1167-bp *Bam*HI/*Hin*dIII fragment encoding the entire EmbR protein	this study
pET28−$embR_{R312A}$	pET28a with a 1167-bp *Bam*HI/*Hin*dIII fragment encoding the entire EmbR protein, mutated on R312	this study
pET28−$embR_{S326A}$	pET28a with a 1167-bp *Bam*HI/*Hin*dIII fragment encoding the entire EmbR protein, mutated on S326	this study
pET28−$embR_{N348A}$	pET28a with a 1167-bp *Bam*HI/*Hin*dIII fragment encoding the entire EmbR protein, mutated on N348	this study
pET28−$embR_{1-921}$	pET28a with a 921-bp *Bam*HI/*Hin*dIII fragment encoding the N-terminal domain $EmbR_{1-307}$ deleted of the FHA domain	this study
pGEX−$pknH_{1-1203}$	PGEX-KT with a 1203-bp *Bam*HI/*Bam*HI fragment encoding the cytoplasmic domain of $PknH_{1-401}$	this study
pGEX(M)−$pknH_{1-1203}$-K45M	PGEX(M) with a 1198-bp *Bam*HI/*Hin*dIII fragment encoding the cytoplasmic domain of $PknH_{1-401}$, mutated on K45	this study
pGEX(M)−$pknH_{1-1203}$-T170A	PGEX(M) with a 1198-bp *Bam*HI/*Hin*dIII fragment encoding the cytoplasmic domain of $PknH_{1-401}$, mutated on T170	this study

lator and virulence factor. It has been suggested that EmbR regulates the transcription of the *emb* operon in *M. avium*, consisting of the *embA* and *embB* genes (*8*). Several studies have shown that the membrane-associated arabinosyltransferases are targets for ethambutol (EMB), a widely used antitubercular drug (*30*, *35*). It has been further demonstrated that proteins EmbA and EmbB are the targets of EMB in *M. avium*, since the inhibition of arabinan synthesis leads to the accumulation of mycolic acids and to cell death (*8*). In *M. tuberculosis*, three contiguous genes, designated, respectively, as *embC*, *embA*, and *embB*, encode arabinosyltransferases and represent targets for EMB (*49*). Furthermore, numerous studies have identified mutations in the *embCAB* locus that are found only in EMB-resistant isolates of *M. tuberculosis*, with the most commonly affected amino acid being Met306 in EmbB (*46*). Molecular genetic analysis of nucleotide polymorphisms associated with EMB resistance in several human isolates of *M. tuberculosis* has revealed punctual substitutions within EmbR (*44*), which suggests a possible link between EMB resistance and EmbR activity. The comparison of sequences using several alignment tools and 3D models has shown that (i) EmbR is homologous to several activator proteins involved in antibiotic production in *S. coelicolor*, such as DnrI and AfsR, (ii) EmbR appears to be genetically and functionally related to the winged-helix family of transcriptional regulators, among which the *Escherichia coli* regulatory protein OmpR is the reference model, and (iii) the putative DNA-binding domain of EmbR is located within the N-terminal region, as observed for AfsR. The DNA-binding activity of AfsR, which appears to be the protein most closely related to EmbR, is modulated by phosphorylation on serine and threonine residues. This regulation is catalyzed by AfsK, a eukaryotic-like STPK, which possesses strong homology with PknH (*34*, *51*).

Considering the homology of the PknH/EmbR couple with the AfsK/AfsR pair, as well as the adjacent chromosomal positions of the *pknH* and *embR* genes, it seemed of interest to study the putative cross-regulation/interactions between these two proteins. In this work, we describe, first, the overproduction and molecular characterization of the cytosolic kinase domain of PknH and its juxtamembrane linker. We also bring evidence that PknH is capable of autophosphorylation on serine and threonine residues, which confirms its autokinase activity. On the other hand, the EmbR protein is shown to undergo direct phosphorylation by PknH, which represents the first example of an endogenous protein substrate modified by a serine/threonine kinase in *M. tuberculosis*. In addition, we demonstrate that the phosphorylation of EmbR by PknH relies on an FHA (forkhead-associated) domain. Furthermore, the amino acid residues of the EmbR FHA domain involved in this reaction have been identified through the generation of FHA-domain-targeted mutants defective in PknH phosphorylation.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions. Strains, plasmids, and primers used in this study are listed in Tables 1 and 2. Overproduction of EmbR and PknH was performed in *E. coli* strain BL21(DE3) (*47*). *E. coli* DH5 strain (*18*) was used to propagate plasmids in cloning experiments. All strains were grown and maintained in LB medium at 37 °C. When required, media were supplemented with either 50 μg/mL ampicillin or 25 μg/mL kanamycin.

DNA Manipulations. Plasmids were purified by using a Qiaprep Purification Kit (Qiagen). All restriction enzymes, T4 DNA ligase, and the Klenow fragment were used as recommended by the manufacturer (Promega). PCR amplifications were performed using either the Pfu polymerase or the HotStar DNA polymerase, purchased from Promega and Qiagen, respectively. PCR products and plasmid DNA fragments were purified using the QiaexII kit (Qiagen). Oligonucleotides were provided by Sigma-Genosys Ltd. or Eurogentec (Liège, Belgium). Transformation of *E. coli* cells was performed following the method described by Dagert and Erhlich (1979). DNA sequencing was carried out by Genome-Express Corp. The nucleotide sequence of all synthesized and mutated genes was verified to ensure error-free amplifications and proper base replacements. DNA sequences were analyzed by the DNAid computer program

Table 2: Primers Used in This Study

primer[a]	5′ to 3′ sequence[b,c]
63 (−)	TAT*GGATCC*ACGGGTTGGTTTTGCGCGGGGTCTG
64 (+)	TAT*GGATCC*GCACAGGACTCGCGGGTGGG
77 (+)	TAT*GGATCC*ATGGCTGGTAGCGCGACAGTGGAGAAGCGG
88 (−)	TATA*AGCTT*GAGTTGGTTTTGCGCGGGGTCTG
91 (−)	TATA*AGCTT*CTACGTGCCGCCATGCGTCCCCGCG
105 (+)	CGGATCGGG**G**GTCTGCATGACAACG
108 (−)	GCCTAACTGGG**C**CAGTTTCTCGTC
109 (+)	GCGCCAACGTC**GC**CCGCCACCACGCC
110 (−)	CGCGGTCATCAGC**A**TGACGGCGACGG
111 (+)	CGATCGTCC**GC**CGGCGTGCATGTG
115 (−)	TATA*AGCTT*CTACGCGGCTTGCAGTGGGTAGCCGC

[a] 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 bold.

(*11*). Blast searches (*2*) and sequence alignments (*5*, *50*) were performed by using our laboratory site server accessible via the World Wide Web (http//:www.ibcp.fr).

Construction of His$_6$-Tagged EmbR Expression Plasmids and GST-Tagged PknH Cytoplasmic Domain Expression Plasmid. The 1167-bp *embR* gene fragment, with appropriate sites at both ends, was synthesized by PCR amplification using *M. tuberculosis* H37Rv genomic DNA as a template and primer pair, 77/91 (Table 2). This DNA fragment was restricted by *Bam*HI and *Hin*dIII and ligated into vector pET28a digested with the same enzymes, thus yielding pET28−*embR* (Table 1). To construct the 924-bp *embR* gene fragment deleted from the FHA domain, PCR amplification was carried out by using pET28−*embR* as a template and primer pair, 77/115 (Table 2). The amplified fragment was then restricted by *Bam*HI and *Hin*dIII enzymes and ligated into pET28a vector previously digested with the same enzymes, yielding pET28−*embR*$_{1-921}$ (Table 1).

A plasmid designed to express the cytoplasmic domain of PknH fused with GST was constructed by PCR amplification using *M. tuberculosis* H37Rv genomic DNA as a template and primer pair, 63/64 (Table 2). The 1198-bp amplified fragment was restricted by *Bam*HI and ligated into pGEX-KT vector previously digested with the same enzyme, thus yielding pGEX−*pknH*$_{1-1203}$ (Table 1).

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out on the basis of PCR amplification. The strategy consisted in creating substitutions in EmbR and PknH$_{1-401}$ proteins. Concerning EmbR single mutants, a first set of PCR amplifications was performed using pET28−*embR* as a template (Table 1) along with the primer pairs 105/91, 109/91, and 111/91 (Table 2) to generate *embR*$_{R312A}$, *embR*$_{S326A}$, and *embR*$_{N348A}$ DNA primers, respectively. These primers were then used in a second round of PCR amplification in combination with primer 77, to generate the entire 1167-bp *embR* gene. These different DNA fragments, containing the appropriate sites at both ends, were restricted by *Bam*HI and *Hin*dIII and ligated with pET28a vector previously digested with the same enzymes. The resulting plasmids were termed pET28−*embR*$_{R312A}$, pET28−*embR*$_{S326A}$, and pET28−*embR*$_{N348A}$, respectively (Table 1). Concerning the core FHA domain deletion, PCR amplifications were performed using pET28−*embR* as a template (Table 1) with the primer pair 77/115 (Table 2). The DNA fragment synthesized was hydrolyzed by *Bam*HI/*Hin*dIII and ligated into pET28a vector digested with the same enzymes. The resulting plasmid was termed pET28−*embR*$_{1-921}$ (Table 1).

Concerning PknH$_{1-401}$ mutants, a similar procedure was applied using pGEX−*pknH*$_{1-1203}$ as a template (Table 1) with primer pairs 64/110 and 64/108 to generate *pknH*$_{1-1203}$-K45M and *pknH*$_{1-1203}$-T170A DNA primers, respectively (Table 2). These primers were then used in a second set of PCR amplifications, in combination with primer 88, thus generating the entire *pknH*$_{1-1203}$ fragment. The two different DNA fragments synthesized, which contained the appropriate sites at both ends, were restricted with *Bam*HI/*Hin*dIII and ligated with pGEX(M) vector previously digested with the same enzymes. The resulting plasmids were termed pGEX(M)−*pknH*$_{1-1203}$-K45M and pGEX(M)−*pknH*$_{1-1203}$-T170A, respectively (Table 1).

Overproduction of the EmbR His$_6$-Tagged and PknH$_{1-401}$ GST-Tagged Fusion Proteins. E. coli BL21 (DE3) cells were transformed with the pET28a vector derivatives expressing the wild-type or mutated EmbR protein, and with the pGEX-(M) derivatives expressing the wild-type or mutated GST−PknH$_{1-401}$ protein (Table 1). Recombinant *E. coli* strains harboring the pET28a derivatives were used to inoculate 100 mL of LB medium supplemented with kanamycin, 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 25 °C, with shaking. Recombinant strains harboring the pGEX(M) derivatives 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−PknH Cytoplasmic Domain and Related Mutant Proteins. Cells were harvested by centrifugation at 6000*g* for 10 min, washed in 10 mL of buffer A (50 mM Tris−HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM aprotinin), and centrifuged again under the same conditions. The cell pellet was resuspended in buffer A containing DNase I and RNase A at a final concentration of 5 μg/mL each, 10 μM leupeptin, and 6 μM pepstatin. Cells were disrupted in a French pressure cell at 16000 lb/in.^{2} (psi). The resulting suspension was centrifuged at 4 °C for 30 min at 30000*g*. The supernatant was incubated for 5 h with glutathione−Sepharose 4B matrix (Pharmacia Biotech), suitable for purification of GST fusion proteins. The protein−resin complex was packed into a column for washing and elution. The column was washed with 50 mL of PBS. Protein elution was carried out with buffer B (50 mM Tris−HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM DTT) containing 15 mM

glutathione. Eluted fractions were analyzed by SDS−PAGE (*27*). After dialysis against buffer C (40 mM Tris−HCl, pH 7.9, 200 mM NaCl, 0.2 mM DTT, 0.2 mM EDTA, 10% glycerol), pure fractions were pooled and tested for in vitro phosphorylation in the presence of PknH. All mutant proteins were purified under the same conditions, and their identity was checked by mass spectrometry (MALDI-TOF).

Purification of Wild-Type EmbR and Related Mutant Proteins. Cells were harvested by centrifugation at 6000*g* for 10 min and washed in 10 mL of buffer D (50 mM phosphate, pH 8.0, 3 M NaCl, 20 mM imidazole) containing DNAse and RNAse. Bacteria were disrupted in a French pressure cell, and the resulting extract was centrifuged for 20 min at 30000*g* at 4 °C. The supernatant was collected and applied onto a Ni^{2+}-NTA agarose column (Pharmacia) previously equilibrated with breakage buffer. The column was extensively washed with phosphate buffer E (50 mM phosphate, pH 7.4, 0.3 M NaCl, 50 mM imidazole) and eluted with a stepwise gradient of imidazole (50−250 mM). Fractions of 1 mL were collected, and the presence of EmbR was detected by 10% SDS−PAGE. Fractions containing pure EmbR were pooled, dialyzed against buffer C (40 mM Tris−HCl, pH 7.9, 200 mM NaCl, 0.2 mM DTT, 0.2 mM EDTA, 10% glycerol), and stored at −20 °C until further use. Each mutant protein was purified in the same conditions.

In Vitro Kinase Assay. In vitro phosphorylation of about 1 μg (15 pmol) of $PknH_{1-401}$ was carried out for 15 min at 37 °C in a reaction mixture (20 μL) containing buffer P (25 mM Tris−HCl, pH 7.0, 1 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA) with 200 μCi/mL [γ-^{32}P]ATP. To assess the initial PknH phosphorylation rate, 1 μg (15 pmol) of PknH was pretreated by alkaline phosphatase (Roche) before incubation with [γ-^{32}P]ATP. Phosphorylation of Mtb−EmbR by $PknH_{1-401}$ was performed with 2 μg (45 pmol) of EmbR in 20 μL of buffer P with 200 μCi/mL [γ-^{32}P]ATP and 250 ng of $PknH_{1-401}$ for 15 min at 37 °C. The reaction was stopped by addition of an equal volume of 2× sample buffer, and the mixture was heated at 100 °C for 5 min. One-dimensional gel electrophoresis was performed as described by Laemmli (*27*). After electrophoresis, gels were soaked in 16% TCA for 10 min at 90 °C, and dried. Radioactive proteins were visualized by autoradiography using direct exposure to films. When needed, radioactivity was measured with a Molecular Dynamics Typhoon phosphoimager.

Analysis of the Phosphoamino Acid Content of Proteins. Protein samples ($PknH_{1-401}$ or EmbR) were separated by one-dimensional gel electrophoresis and electroblotted onto an Immobilon poly(vinylidene difluoride) (PVDF) membrane. Phosphorylated proteins bound to the membrane fraction were detected by autoradiography. The ^{32}P-labeled protein bands were excised from the Immobilon blot and hydrolyzed in 6 M HCl for 1 h at 110 °C. The acid-stable phosphoamino acids thus liberated were separated by electrophoresis in the first dimension at pH 1.9 (800 V·h) in 7.8% acetic acid and 2.5% formic acid, followed by ascending chromatography in the second dimension in 2-methyl-1-propanol/formic acid/water (8:3:4). After migration, radioactive molecules were detected by autoradiography. Authentic phosphoserine, phosphothreonine, and phosphotyrosine were run in parallel and visualized by staining with ninhydrin.

PknH−EmbR Interaction Assay. The soluble fraction (5 μg of protein) containing His−EmbR (wild-type or the truncated form of EmbR) was incubated with 10 μg of either prephosphorylated GST−PknH or the mutated form of PknH, PknH-K45M, at 25 °C for 4 h in 1 mL of PBS buffer. The binding assay was performed overnight with glutathione−Sepharose 4B matrix (Pharmacia Biotech), suitable for purification of GST fusion proteins. The protein−resin complex was packed into a 1.5 mL Eppendorf tube and washed five times with 1 mL of PBS each. The proteins thus retained were eluted with buffer B (50 mM Tris−HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM DTT) containing 15 mM glutathione. Eluted fractions were precipitated with TCA, resuspended in 30 μL of sodium dodecyl sulfate loading buffer, and boiled for 3 min. Gel electrophoresis (15 μL out of 30 μL of protein solution) was carried out according to Laemmli (*27*) in a 12.5% SDS−polyacrylamide gel, and proteins were electroblotted on an Immobilon-P membrane (Millipore). Then, INDIA HisProbe-HRP (SuperSignal West HisProbe Kit, Pierce Chemicals), a nickel-activated derivative of horseradish peroxidase (HRP), was used to directly detect the blotted recombinant poly-histidine-tagged EmbR fusion protein. The SuperSignal kit (Pierce) and BioMax film (Kodak) were used for protein visualization. As a control, 5 μg of EmbR was incubated either with 10 μg of GST or alone in PBS buffer.

RESULTS

Expression and Purification of the Cytosolic Domain of PknH ($PknH_{1-401}$). The truncated *pknH* gene (Rv1266) encoding the cytoplasmic domain of the PknH protein (residues 1−401) was synthesized by PCR amplification using genomic DNA from *M. tuberculosis* H37Rv. The amplified product was cloned into plasmid pGEX-KT to yield pGEX−$pknH_{1-1203}$ and used to transform *E. coli*. Analysis of the GST−chimeric protein by SDS−PAGE revealed that the wild-type enzyme was expressed in a soluble form, and migrated as a diffuse band with the predicted molecular mass of approximately 70 kDa. Since this diffuse migration had already been reported for other phosphorylated STPKs (*9*, *37*), it was therefore attributed to autophosphorylation of PknH. However, the high proline content of the protein (Figure 1A) might also account for this diffuse band.

PknH Autophosphorylates in vitro on Serine and Threonine Residues. PknH is predicted to contain 626 amino acids, and its N-terminal region contains all the essential amino acids and sequence subdomains that are characteristic of the Hank family of eukaryotic-like protein kinases (Figure 1A). These include the central core of the catalytic loop, consisting of subdomain VI (corresponding to Arg138−Asn144) and the invariant residue K45 in the consensus motif within subdomain II, which is usually involved in the phosphotransfer reaction and also required for the autophosphorylating activity of eukaryotic-like STPK (*19*, *20*, *40*, *54*). To investigate whether PknH harbored serine/threonine protein kinase activity, $PknH_{1-401}$was incubated with [γ-^{32}P]ATP as a phosphate donor. As shown in Figure 1B (lane 1), wild-type PknH incorporated radioactive phosphate from [γ-^{32}P]-ATP, and gave rise to a radioactive signal at about 70 kDa, a size similar to that observed for $PknH_{1-401}$. This result indicated that PknH is capable of autolabeling, indicative of an autophosphorylating activity. Confirmation that this activity resulted from an autophosphorylation of $PknH_{1-401}$

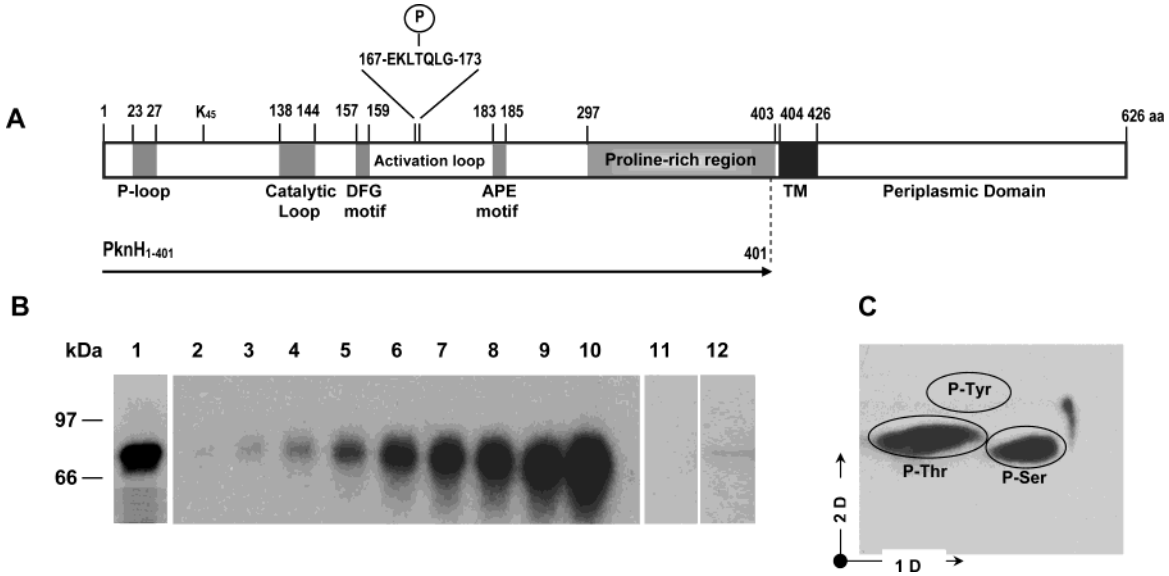

FIGURE 1: Structural domains of PknH and phosphorylation site of the Ser/Thr kinase domain. (A) Schematic presentation of the PknH domains. *M. tuberculosis pknH* encodes a predicted transmembrane protein with a single transmembrane helix connecting a periplasmic C-terminal domain to a cytosolic N-terminal domain with the typical Hanks motif of the STPK family. (B) The intracellular domain of PknH, GST−PknH$_{1-401}$, and PknH$_{1-401}$ site-directed mutants PknH$_{1-401}$-K45M and PknH$_{1-401}$-T170A were overproduced and purified on glutathione−Sepharose 4B matrix. Incubation of GST−PknH$_{1-401}$ (5 pmol) was performed with [γ-^{32}P]ATP for 15 min (lane 1). A kinetic analysis was performed for 30 s (lane 2), 1 min (lane 3), 2 min (lane 4), 4 min (lane 5), 6 min (lane 6), 8 min (lane 7), 10 min (lane 8), 12 min (lane 9), and 15 min (lane 10). Incubation of GST−PknH$_{1-401}$-K45M (lane 11) or GST−PknH$_{1-401}$-T170A (lane 12) was performed with [γ-^{32}P]ATP for 10 min. Proteins were analyzed by SDS−PAGE, and radioactive bands were revealed by autoradiography. (C) Phosphoamino acid content of GST−PknH$_{1-401}$. GST−PknH$_{1-401}$ labeled with [γ-^{32}P]ATP was analyzed by SDS−PAGE, electroblotted onto an Immobilon PVDF membrane, excised, and hydrolyzed in acid. The phosphoamino acids thus liberated were separated by electrophoresis in the first dimension (1D) and ascending chromatography in the second dimension (2D). After migration, radioactive molecules were detected by autoradiography. Authentic phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) were run in parallel as internal standard controls, and visualized by ninhydrin staining.

was obtained by introducing a specific mutation of the conserved Lys45 residue present in subdomain II into PknH by site-directed mutagenesis. Namely, this Lys45 was substituted by a Met residue, and the mutated form of PknH, PknH$_{1-401}$-K45M, was purified from *E. coli* and then tested for autophosphorylation in the presence of [γ-^{32}P]ATP. As expected, no radioactive signal could be detected (Figure 1B, lane 11), thus showing that PknH expressed autophosphorylating activity. A kinetic analysis of PknH phosphorylation was carried out to assess the initial PknH phosphorylation rate (Figure 1B, lanes 2−10). Incorporation of γ-phosphate occurred rapidly, reaching about 50% of its maximum rate within 8 min of reaction. This autokinase activity was dependent on bivalent cations such as Mg^{2+}, as its autophosphorylation was enhanced in the presence of 2−10 mM Mg^{2+}, and abolished by addition of 20 mM EDTA (data not shown).

To investigate which amino acid residues were phosphorylated in PknH, phosphoamino acid analysis was performed on recombinant proteins. Proteins were labeled with [γ-^{32}P]-ATP in vitro as described above, separated by SDS−PAGE, excised, and subjected to acid hydrolysis. Under these conditions, only acid-resistant phosphoamino acids were analyzed since other phosphorylated compounds, such as phosphohistidine, phosphoarginine, or phosphoaspartate, are known to be acid-labile. Analysis of the autoradiogram presented in Figure 1C indicated that PknH was phosphorylated on both serine and threonine residues, but not on tyrosine.

Thr170 within the Activation Loop Is Required for Catalytic Activation. A key feature of several protein kinases is that they are phosphorylated on residue(s) located within a particular segment in the center of the kinase domain, which is termed the "activation loop". When mapped on the recent crystal structure of PknB (*40*, *54*), this segment could be defined as the region spanning from the conserved sequence DFG to the APE domain, corresponding to residues 157−185 in PknH (Figure 1A). Interestingly, this segment includes Thr170, corresponding to Thr171 in PknB, one of the four phosphorylated residues described as a phosphorylation site in the activation loop of PknB (*54*). The presence of such a residue in PknH supports the concept that phosphorylation of the activation loop plays a regulatory role. To test the possible link between this residue and intraphosphorylation of PknH, Thr170 was mutated to an Ala residue. When incubated with [γ-^{32}P]ATP in the same conditions as above, PknH$_{1-401}$-T170A was unable to generate a radioactive signal (Figure 1A, lane 12). This result strongly argued for Thr170 being a phosphorylation site of PknH, and suggested the existence of a cooperative effect between the phosphorylation of Thr170 and autophosphorylation on other residue(s) of PknH.

Biochemical Characterization of M. tuberculosis EmbR. Analysis of the protein primary structure deduced from the *M. tuberculosis* genome indicated that EmbR possesses a strong homology with proteins AfsR and DnrI from *Streptomyces coelicolor*, which both belong to the OmpR class of transcriptional regulators, and is a member of the SARP family (*52*). This family consists of regulatory proteins that activate transcription of essential genes for the biosynthesis of secondary metabolites. Three-dimensional models of the N-terminal domain of EmbR, ranging from residue 1 to

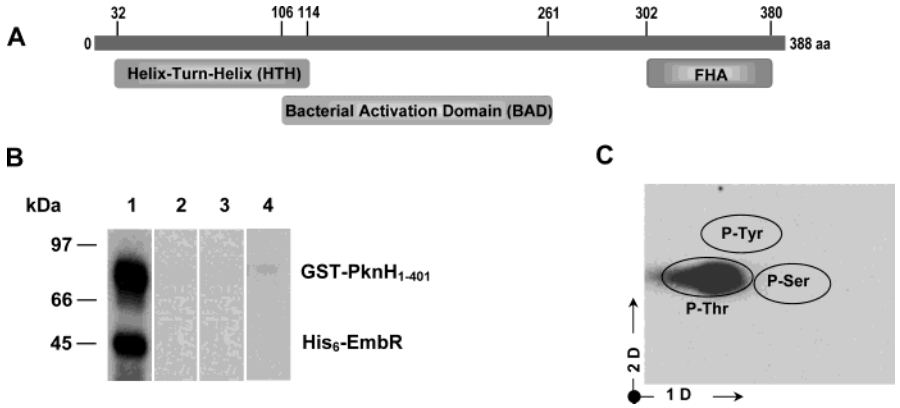

FIGURE 2: Structural domains of EmbR and phosphorylation of EmbR by $PknH_{1-401}$. (A) Schematic presentation of EmbR from *M. tuberculosis*. EmbR encodes a probable transcriptional regulator protein with an N-terminal DNA-binding winged-helix−turn−helix domain, followed by a bacterial transcriptional activator domain (BAD) found in the DnrI/RedD/AfsR family of regulators. The EmbR C-terminus region presents a typical forkhead-associated domain found in eukaryotic and prokaryotic proteins. (B) In vitro phosphorylation of His_6−EmbR by GST−$PknH_{1-401}$. His_6−EmbR was overproduced and purified on a Ni^{2+}-immobilized matrix. The following proteins and protein fragments were incubated with [γ-^{32}P]ATP: His_6−EmbR and GST−$PknH_{1-401}$ (lane 1), His_6−EmbR alone (lane 2), His_6−EmbR with GST−$PknH_{1-401}$-K45M (lane 3), His_6–EmbR with GST−$PknH_{1-401}$-T170A (lane 4). Proteins were separated by SDS−PAGE, and radioactive bands were revealed by autoradiography. (C) Phosphoamino acid content of His_6−EmbR.

residue 114 (Figure 2A), exhibit structural homologies with the DNA-binding domain architecture of the winged-helix family of transcriptional activators (data not shown). This domain consists of a central three-helical bundle containing the helix−turn−helix (HTH) variant separated by a relatively long loop. Considering that (i) EmbR is suspected to regulate the transcription of the *emb* operon, whose gene products participate in arabinan synthesis and represent the primary target of ethambutol (*8*, *49*), (ii) EmbR shares strong homology with AfsR, a phosphorylated transcriptional regulator, and (iii) *pknH* and *embR* are adjacent genes in the *M. tuberculosis* genome (*10*), we tested the ability of PknH to phosphorylate EmbR. For this, an expression system was used to allow overproduction and purification of EmbR. The *embR* gene was cloned and expressed under the control of the T7 promoter in the *E. coli* expression vector pET28a. Analysis of the purified recombinant protein by SDS−PAGE revealed that the wild-type protein, fused to a His tag, was expressed in a soluble form with the predicted molecular mass of 45 kDa.

EmbR Is a Substrate of PknH in Vitro. The ability of PknH to phosphorylate EmbR was examined via an in vitro phosphorylation assay. Purified PknH was added to the reaction mixture in the presence of [γ-^{32}P]ATP and purified EmbR. The reaction products were separated by SDS−PAGE, and labeled proteins were identified by autoradiography. As shown in Figure 2B (lane 1), PknH could phosphorylate EmbR, whereas EmbR alone was unable to incorporate γ-^{32}P (Figure 2B, lane 2), thus confirming that EmbR was a substrate of PknH and possessed no autokinase activity. The optimal conditions for the phosphorylation of EmbR by PknH were examined at intervals of 0.5 pH unit, from pH 5 to pH 11, using various phosphorylation buffers (data not shown). Optimal phosphorylation activity of EmbR by PknH occurred at neutral pH in buffer P (25 mM Tris−HCl, pH 7.0, 1 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA). To analyze the link between intraphosphorylation of PknH and phosphorylation of EmbR, EmbR was incubated with $PknH_{1-401}$-K45M or $PknH_{1-401}$-T170A, which were both unable to autophosphorylate. Purified $PknH_{1-401}$-K45M or $PknH_{1-401}$-T170A was incubated in a mixture containing EmbR. The reaction products were separated by SDS−PAGE, and labeled proteins were identified by autoradiography. As shown in Figure 2B (lanes 3 and 4), neither $PknH_{1-401}$-K45M nor $PknH_{1-401}$-T170A was able to generate a radioactive signal that would correspond to a phosphorylated form of EmbR. Therefore, these results suggested that PknH must be phosphorylated prior to EmbR phosphorylation.

EmbR Is Phosphorylated Specifically on Threonine. To investigate which amino acid residues were phosphorylated by PknH, we analyzed the phosphoamino acid content of phosphorylated EmbR. The protein was labeled with [γ-^{32}P]-ATP in vitro as described above, separated by SDS−PAGE, excised, and subjected to acid hydrolysis. Figure 2C shows that EmbR was phosphorylated only on threonine. Thus, PknH appears to be an STPK, capable of autophosphorylation on both serine and threonine residues (Figure 1C) and able to phosphorylate EmbR specifically on threonine.

The FHA Domain Is Required for EmbR Phosphorylation and PknH−EmbR Interaction. Previous in silico analysis indicated that the C-terminal region of EmbR possesses an FHA domain (*21*), a modular phosphopeptide recognition domain with a striking specificity for a phosphothreonine-containing epitope (*14*). This domain was originally described in eukaryotes, and a vast majority of FHA-containing proteins were found to be associated with proteins involved in numerous processes including intracellular signal transduction, control of transcription, DNA repair, and cell cycle progression (*1*, *7*, *53*). By contrast, in prokaryotes, there was no report of experimental characterization of the physiological role or identification of the binding partner(s) of any bacterial FHA domain, despite the prediction of such a phosphopeptide recognition domain in several Gram-negative and Gram-positive genomes, including *M. tuberculosis* (*41*). The presence of an FHA domain in EmbR strongly suggested a role in protein−protein interaction and, more precisely, an interaction of EmbR with a protein phosphorylated on a threonine residue. In connection with our previous studies, we suspected that the EmbR FHA domain could mediate specific interaction with PknH. To check whether this phosphopeptide recognition motif was involved in protein−

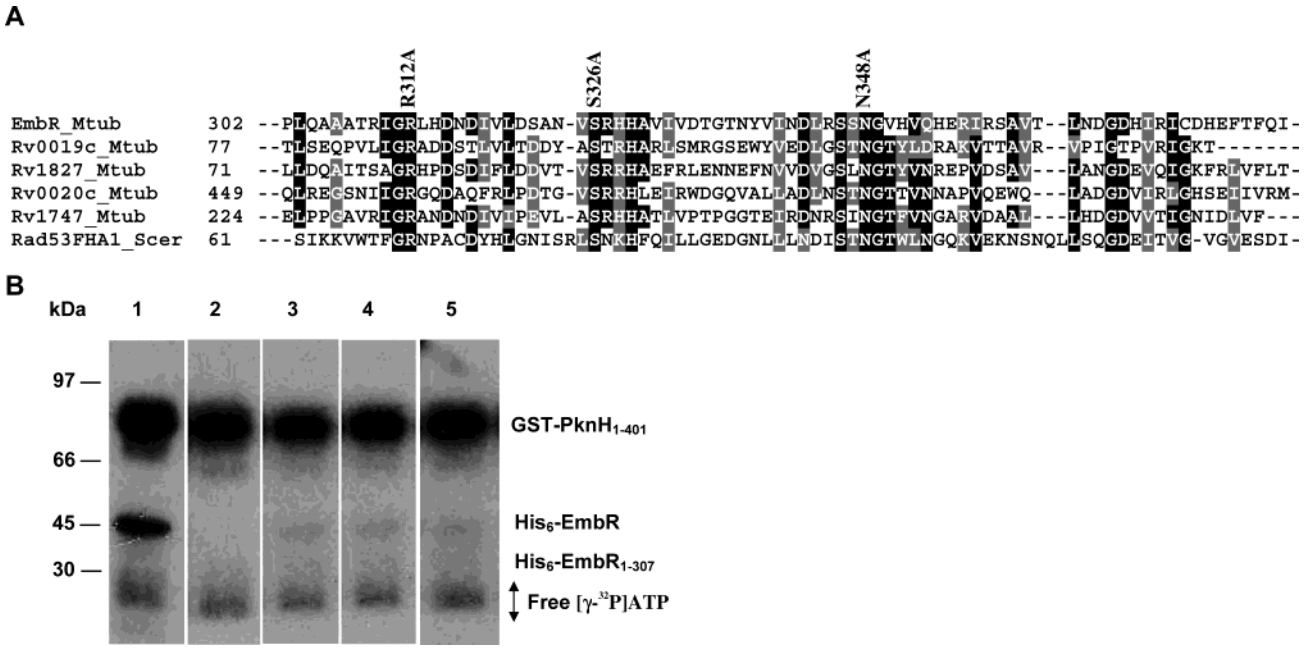

FIGURE 3: Phosphorylation of EmbR by PknH. (A) Sequence alignment of FHA-domain-containing proteins. Sequences were aligned using the ClustalW program. Alignments were shaded using the BOXSHADE server. Sequence designations and NCBI GI numbers are as follows: EmbR from *M. tuberculosis*, GI 15608407; Rv0019c from *M. tuberculosis*, GI 15607161; Rv1827 from *M. tuberculosis*, GI 15608964; Rv0020c from *M. tuberculosis*, GI 15607162; Rv1747 from *M. tuberculosis*, GI 15608885; Rad53 FHA1:FHA domains from *S. cerevisiae*, GI 134835. (B) In vitro phosphorylation of His_6−EmbR mutants by GST−$PknH_{1-401}$. His_6−EmbR, His_6−EmbR-R312A, His_6−EmbR-S326A, His_6−EmbR-N348A, and His_6−$EmbR_{1-307}$ were overproduced and purified on a Ni^{2+}-immobilized matrix. Incubation of GST−$PknH_{1-401}$ was performed in the presence of [γ-^{32}P]ATP for 15 min with His_6–EmbR (lane 1), His_6−$EmbR_{1-307}$ (lane 2), His_6−EmbR-R312A (lane 3), His_6−EmbR-S326A (lane 4), or His_6−EmbR-N348A (lane 5). Proteins were analyzed by SDS−PAGE, and radioactive bands were revealed by autoradiography.

protein interaction between EmbR and PknH through a phosphorylation process, the FHA domain was either deleted or mutagenized at crucial positions (Figure 3A). Deletion of the core FHA domain was performed by truncating the 81 residues corresponding to the C-terminal region of EmbR, yielding the $EmbR_{1-307}$ mutant. The punctual mutations consisted of Ala substitutions of the conserved Arg312, Ser326, and Asn348 of EmbR corresponding, respectively, to Arg70, Ser85, and Asn107 of FHA1 in Rad53 (Figure 3A). In the Rad53 FHA1 domain (*14*), these three highly conserved residues play a critical role in the direct binding of the phosphopeptide, either by contacting the phosphopeptide backbone (Arg312 and Asn348) or via a phosphothreonine residue through an extensive network of hydrogen bonding (Arg312 and Ser326) (*14*).

To test the hypothesis that the EmbR FHA domain could mediate the specific interaction between PknH and its substrate, both phosphorylation and interaction assays were performed with either wild-type or mutated EmbR. PknH catalyzed the phosphorylation of wild-type EmbR (Figure 3B, lane 1) as indicated by the incorporation of radioactive phosphate. By contrast, deletion of the core FHA domain abolished the phosphorylation of EmbR by PknH, as indicated by the absence of ^{32}P incorporation in $EmbR_{1-307}$ (Figure 3B, lane 2). Similarly, PknH failed to phosphorylate EmbR-R312A, EmbR-S326A, and EmbR-N348A mutants, as shown by the low incorporation of radioactivity (less than 5% compared to that of wild-type EmbR) (Figure 3B, lanes 3−5). Therefore, these results indicated that EmbR FHA domain mutants were defective in phosphorylation by PknH. Together, these data demonstrated that EmbR phosphorylation by PknH is mediated by the FHA domain of EmbR.

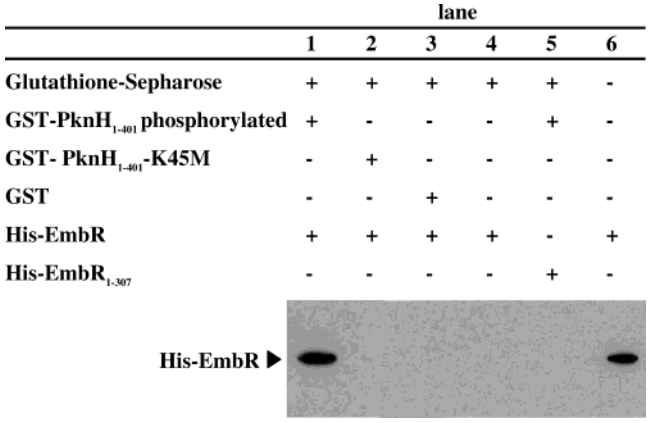

FIGURE 4: Physical interaction between $PknH_{1-401}$ and EmbR. The soluble fraction containing either wild-type His−EmbR (lanes 1 and 2) or truncated $EmbR_{1-307}$ (lane 5) was incubated with either prephosphorylated GST−PknH (lanes 1 and 5) or PknH-K45M (lane 2) at 25 °C for 4 h in 1 mL of PBS buffer. The binding assay was performed overnight with glutathione−Sepharose 4B matrix (Pharmacia Biotech), suitable for purification of GST fusion proteins (lanes 1−5). The proteins thus retained were eluted, precipitated, and resuspended in 30 μL of SDS loading buffer. Gel electrophoresis was carried out in a 12.5% SDS−polyacrylamide gel, and proteins were electroblotted onto an Immobilon-P membrane (Millipore). INDIA HisProbe-HRP was used to directly detect the blotted recombinant poly-histidine-tagged EmbR fusion protein (lower part). As a control, EmbR was incubated with gluthatione−Sepharose either with GST (lane 3) or alone (lane 4) in PBS buffer. The presence of either a protein or gluthatione−Sepharose is indicated by "+" and the absence by "−".

On the other hand, the physical association of EmbR with PknH and the role of PknH autophosphorylation on this interaction were examined by using a pull-down assay. The

recombinant poly-histidine-tagged EmbR fusion protein was incubated with either the phosphorylated protein fragment $PknH_{1-401}$ (Figure 4, lane 1) or the unphosphorylated mutant form $PknH_{1-401}$-K45M (Figure 4, lane 2), each fused to the GST tag. As a control, the soluble fraction containing EmbR was incubated in glutathione−Sepharose either with GST (Figure 4, lane 3) or alone (Figure 4, lane 4). GST complexes were pulled down with gluthatione−Sepharose, separated by SDS−PAGE, and transferred onto a PVDF membrane before detection of His−EmbR. Figure 4 indicated that, in this assay, His−EmbR was recovered in the soluble fraction eluted from gluthatione−Sepharose when it was preincubated with phosphorylated $PknH_{1-401}$ (lane 1), but was not recovered when it was preincubated with the unphosphorylated form of the kinase fragment (lane 2). Control experiments showed, in addition, that no recovery of His−EmbR occurred when it was incubated either alone (lane 4) or in the presence of GST (lane 3). It therefore appeared that the complex was formed only via the phosphorylated form of PknH. Moreover, to confirm that this interaction required the FHA domain of EmbR, the same type of experiment was carried out with the FHA-truncated form of EmbR (His−$EmbR_{1-307}$ mutant) (Figure 4, lane 5). The absence of recovery of EmbR in this assay (lane 5) indicated, by comparison with lane 1, that the FHA domain of EmbR is required for this interaction.

DISCUSSION

Modification of proteins by phosphorylation on a limited number of amino acid residues is a general mechanism used to control protein activities in both eukaryotic and prokaryotic cells. In bacteria, namely, the presence of several serine/threonine or tyrosine kinases suggests a central role of protein phosphorylation in regulating various biological functions, ranging from environmental adaptative responses to bacterial pathogenicity. Regulatory devices involving STPKs and phosphatases, or tyrosine kinases and tyrosine phosphatases, represent an emerging theme in prokaryotic signaling cascades (*16*, *22*, *31*, *37*−*39*). The main result of this study is the characterization of PknH, a new member of the large STPK family in *M. tuberculosis*, and the demonstration of a relationship between its autokinase activity and its phosphorylating activity toward protein EmbR, which occurs through an FHA domain.

PknH Is a Eukaryotic-like Ser/Thr Protein Kinase. On the basis of the hydropathic profile of its amino acid sequence, it was predicted that PknH would comprise two distinct domains: a cytosolic N-terminal domain harboring all 11 consensus catalytic subdomains of the Hanks-type kinase (*20*), and a periplasmic C-terminal domain separated from the N-terminal domain by a single transmembrane helix. The topology of PknH is consistent with that of several eukaryotic and prokaryotic receptors, especially PknB, the only prokaryotic STPK whose X-ray crystal structure is currently available (*40*, *54*). In vitro phosphorylation assays, based on the incorporation of exogenous radiolabeled phosphate by $PknH_{1-401}$, demonstrate the autocatalytic activity of PknH. In addition, substitution of the invariant subdomain II lysine residue of PknH is accompanied by a loss of the kinase activity, which confirms that (i) this amino acid is essential for catalyzing the phosphorylation reaction, in agreement with previous reports (*20*, *37*), and (ii) the phosphorylation of the cytosolic fragment is due to the intrinsic kinase activity of PknH, rather than to any other kinase activity. Moreover, phosphoamino acid analysis of PknH confirms that PknH is phosphorylated exclusively on serine and threonine residues. The activating conformational changes observed in eukaryotic STPKs are related to the activation loop, which possesses the capacity to undergo large conformational changes while the enzyme switches between inactive and active states (*23*). By comparison with several other STPKs, we have identified the presence of a phosphorylatable residue, Thr170, in the activation loop of PknH, suggesting that it may play a central role in the catalytic activity of the enzyme. Suppression of PknH autophosphorylation by Thr170 mutagenesis strongly suggests that phosphorylation on an amino acid residue is required for PknH autophosphorylation at other sites (Figure 4). As described for several eukaryotic STPKs, this autocatalytic event promotes allosteric changes that are needed for full kinase activation. Additional experiments, including mutagenesis analysis based on the three-dimensional model of PknH and/or structural resolution of the protein, are required to better understand the mechanism of action, and to characterize the phosphorylation residues in PknH.

PknH Phosphorylates EmbR in an FHA-Dependent Event. Although six different STPKs have been described in *M. tuberculosis*, the nature of the corresponding substrates and the biological role of these enzymes have not been determined yet. In this study, we have shown that incubation of $PknH_{1-401}$ with EmbR and [γ-^{32}P]ATP yields a phosphorylated form of EmbR. This result indicates that EmbR is a direct phosphorylation target of the PknH kinase. Moreover, we have demonstrated that the phosphorylation of EmbR by PknH proceeds through a cooperative two-step mechanism. First, PknH is phosphorylated in an autophosphorylation reaction, occurring at position Thr170, which significantly increases the protein kinase activity. Then, the activated kinase phosphorylates EmbR via an interphosphorylation reaction (Figure 4). Referring to eukaryotic systems, a large number of STPKs are known to catalyze autophosphorylation in an intramolecular process generally modulated by regulatory ligands, which allow rapid switching of numerous cellular functions. A similar situation can be envisaged for prokaryotes, namely, for PknH, which would behave like a "eukaryotic-like" STPK receptor, capable of transducing a signal(s) through EmbR phosphorylation. In eukaryotic organisms, the formation of such complex networks of interacting proteins involves conserved protein modules or domains that regulate signal transduction by mediating protein−protein interaction. Among these, the FHA domains mediate protein−protein interaction via a modular phosphopeptide recognition domain with a striking specificity for a phosphothreonine-containing epitope (*13*, *14*). The presence of an FHA domain in EmbR suggests that this module may mediate EmbR−PknH interaction in connection with signal transduction. The finding that EmbR interacts only with the phosphorylated form of PknH and that this interaction is abolished by deletion of the FHA domain of EmbR demonstrates that this phosphopeptide recognition motif is involved in protein−protein interaction between the two partners. Moreover, the observation that phosphorylation of EmbR by PknH is abolished by deletion, or by several punctual mutations present in this module, shows that the interaction between these two proteins through the FHA

domain is essential for EmbR phosphorylation by PknH, and that residues Arg312, Ser326, and Asn348 participate in the binding to the phosphothreonine-containing peptide of PknH. In *Saccharomyces cerevisiae*, such an interaction between the FHA-containing protein Dun1 and the Rad53 kinase has been previously described (*7*). On this basis, we propose that the recruitment of EmbR depends on interaction between its FHA domain and the phosphorylated form of PknH (Figure 4). In these conditions, the PknH/EmbR pair would represent a classical signal transduction network, which would thus be described for the first time in bacteria. Still, it cannot be ruled out that other Pkns of *M. tuberculosis* would also phosphorylate and associate with EmbR.

FHA domain in M. tuberculosis. Although genome databases allow prediction of several proteins with putative FHA domains in various bacteria (six proteins in *M. tuberculosis*), no experimental characterization of the physiological role and identification of the binding partner(s) of any bacterial FHA domain has been reported yet. Although regulatory phosphorylation/dephosphorylation of serine/threonine residues by STPKs and phosphatases is an emerging theme in prokaryotic signaling cascades, only two identified substrates for STPKs have been previously described. In *S. coelicolor*, the protein AfsR is the substrate of AfsK (*34*, *51*), and in *Myxococcus xanthus*, a 6-phosphofructokinase is phosphorylated by Pkn4 (*39*). The characterization of an FHA domain in a protein−protein interaction between an STPK and its substrate in *M. tuberculosis* opens new perspectives to decipher the molecular mechanisms involved in mycobacterial physiology and pathogenicity. This mode of interaction also provides a framework for future investigation of the function of certain proteins in different bacteria such as *Pseudomonas aeruginosa*, *M. xanthus*, or *Vibrio cholerae*, in which several FHA-domain-containing proteins have been predicted by in silico analysis (*41*). Three of the six putative FHA-domain-containing proteins described in *M. tuberculosis* (Figure 3A) are encoded by specific genes (Rv0019c, Rv0020c, Rv1747) that belong to gene clusters coding for STPKs (*10*) (*41*). Thus, Rv0019c and Rv0020c, which are located within the *pknA/pknB* gene cluster, represent valuable candidates for phosphorylation by the corresponding two kinases. Similarly, Rv1747, presumably coding for an ABC transporter, is adjacent to the *pknF* gene, and may represent a possible target for the STPK PknF. Current work is in progress in our laboratory to characterize these different putative substrates and the related kinases.

Potential Role of the PknH−EmbR Interaction in Arabinan Biosynthesis. EmbR is a member of the *S. coelicolor* SARP family (*52*), known to regulate the genes involved in the biosynthesis of secondary metabolites. Consistently, EmbR has been proposed to influence the expression level of the *embAB* operon, known to somehow participate in arabinan synthesis in *M. avium* (*8*). Indeed, membranes from *Mycobacterium smegmatis* cells carrying the *M. avium embAB* and *embR* genes retain significantly more arabinosyltransferase activity than cells harboring only *embAB*, when treated with a similar amount of ethambutol (EMB) (*8*). EMB is a synthetic compound that represents a major antitubercular drug often used for the treatment of patients infected with multi-drug-resistant *M. tuberculosis* strains (*26*), which acts by inhibiting the biogenesis of arabinogalactan biosynthesis from arabinan (*12*, *35*, *48*). Whereas, in *M. avium*, *embR* is located immediately upstream of *embAB*, the *embR* gene of *M. tuberculosis* is not located upstream of the *embCAB* operon but lies elsewhere in the genome (*10*, *49*). Recent work suggests that proteins EmbA and EmbB participate in the formation of the terminal hexaarabinofuranosyl motif in arabinogalactan (*15*), whereas EmbC is more likely involved in lipoarabinomannan biosynthesis by translocating the arabinan polymer across the plasma membrane (*56*). Sequence analysis of the *embCAB* locus in EMB-resistant *M. tuberculosis* clinical isolates confirmed the unique association of substitutions in amino acid residue 306 of EmbB with the EMB resistance (*46*). However, other mutations have also been found in the *embB*, *embA*, *embC*, and *embR* genes (*44*, *46*).

Therefore, the present data suggest that the phosphorylation of EmbR by PknH may play a pivotal role in the transcriptional regulation of the *embCAB* operon in *M. tuberculosis*. Whether the PknH/EmbR pair participates in the regulation of the arabinosyltransferase-encoded genes and in the EMB resistance requires further investigation. Still, one can anticipate that, since arabinogalactan represents an essential mycobacterial cell wall component for mycobacterial growth and represents a key target for the development of new antitubercular chemotherapies, the inhibition of PknH activity may also represent an attractive way to investigate.

REFERENCES

1. Ahn, J. Y., Li, X., Davis, H. L., and Canman, C. E. (2002) *J. Biol. Chem. 277*, 19389−19395.
2. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res. 25*, 3389−3402.
3. Av-Gay, Y., and Everett, M. (2000) *Trends Microbiol. 8*, 238−244.
4. Av-Gay, Y., Jamil, S., and Drews, S. J. (1999) *Infect. Immun. 67*, 5676−5682.
5. Bairoch, A., Bucher, P., and Hofmann, K. (1997) *Nucleic Acids Res. 25*, 217−221.
6. Bakal, C. J., and Davies, J. E. (2000) *Trends Cell Biol. 10*, 32−38.
7. Bashkirov, V. I., Bashkirova, E. V., Haghnazari, E., and Heyer, W. D. (2003) *Mol. Cell. Biol. 23*, 1441−1452.
8. Belanger, A. E., Besra, G. S., Ford, M. E., Mikusova, K., Belisle, J. T., Brennan, P. J., and Inamine, J. M. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 11919−11924.
9. Chaba, R., Raje, M., and Chakraborti, P. K. (2002) *Eur. J. Biochem. 269*, 1078−1085.
10. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., III, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Barrell, B. G., and et al. (1998) *Nature 393*, 537−544.
11. Dardel, F., and Bensoussan, P. (1988) *Comput. Appl. Biosci. 4*, 483−486.
12. Deng, L., Mikusova, K., Robuck, K. G., Scherman, M., Brennan, P. J., and McNeil, M. R. (1995) *Antimicrob. Agents Chemother. 39*, 694−701.
13. Durocher, D., and Jackson, S. P. (2002) *FEBS Lett. 513*, 58−66.
14. Durocher, D., Taylor, I. A., Sarbassova, D., Haire, L. F., Westcott, S. L., Jackson, S. P., Smerdon, S. J., and Yaffe, M. B. (2000) *Mol. Cell 6*, 1169−1182.
15. Escuyer, V. E., Lety, M. A., Torrelles, J. B., Khoo, K. H., Tang, J. B., Rithner, C. D., Frehel, C., McNeil, M. R., Brennan, P. J., and Chatterjee, D. (2001) *J. Biol. Chem. 276*, 48854−48862.
16. Grangeasse, C., Doublet, P., and Cozzone, A. J. (2002) *J. Biol. Chem. 277*, 7127−7135.
17. Gross, R., Arico, B., and Rappuoli, R. (1989) *Mol. Microbiol. 3*, 1661−1667.
18. Hanahan, D. (1983) *J. Mol. Biol. 166*, 557−580.
19. Hanks, S. K., and Hunter, T. (1995) *FASEB J. 9*, 576−596.

20. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) *Science 241*, 42−52.
21. Hofmann, K., and Bucher, P. (1995) *Trends Biochem. Sci. 20*, 347−349.
22. Inouye, S., Jain, R., Ueki, T., Nariya, H., Xu, C. Y., Hsu, M. Y., Fernandez-Luque, B. A., Munoz-Dorado, J., Farez-Vidal, E., and Inouye, M. (2000) *Microb. Comp. Genomics 5*, 103−120.
23. Johnson, L. N., Noble, M. E., and Owen, D. J. (1996) *Cell 85*, 149−158.
24. Koul, A., Choidas, A., Tyagi, A. K., Drlica, K., Singh, Y., and Ullrich, A. (2001) *Microbiology 147*, 2307−2314.
25. Krebs, E. G., and Fischer, E. H. (1989) *Biochim. Biophys. Acta 1000*, 302−309.
26. Kremer, L. S., and Besra, G. S. (2002) *Expert Opin. Invest. Drugs 11*, 1033−1049.
27. Laemmli, U. K. (1970) *Nature 227*, 680−685.
28. Lamichhane, G., Zignol, M., Blades, N. J., Geiman, D. E., Dougherty, A., Grosset, J., Broman, K. W., and Bishai, W. R. (2003) *Proc. Natl. Acad. Sci. U.S.A. 100*, 7213−7218.
29. Leonard, C. J., Aravind, L., and Koonin, E. V. (1998) *Genome Res. 8*, 1038−1047.
30. Maddry, J. A., Suling, W. J., and Reynolds, R. C. (1996) *Res. Microbiol. 147*, 106−112.
31. Madec, E., Laszkiewicz, A., Iwanicki, A., Obuchowski, M., and Seror, S. (2002) *Mol. Microbiol. 46*, 571−586.
32. Martinez-Hackert, E., and Stock, A. M. (1997) *Structure 5*, 109−124.
33. Martinez-Hackert, E., and Stock, A. M. (1997) *J. Mol. Biol. 269*, 301−312.
34. Matsumoto, A., Hong, S. K., Ishizuka, H., Horinouchi, S., and Beppu, T. (1994) *Gene 146*, 47−56.
35. Mikusova, K., Slayden, R. A., Besra, G. S., and Brennan, P. J. (1995) *Antimicrob. Agents Chemother. 39*, 2484−2489.
36. Molle, V., Girard-Blanc, C., Kremer, L., Doublet, P., Cozzone, A. J., and Prost, J. F. (2003) *Biochem. Biophys. Res. Commun. 308*, 820−825.
37. Motley, S. T., and Lory, S. (1999) *Infect. Immun. 67*, 5386−5394.
38. Nadvornik, R., Vomastek, T., Janecek, J., Technikova, Z., and Branny, P. (1999) *J. Bacteriol. 181*, 15−23.
39. Nariya, H., and Inouye, S. (2002) *Mol. Microbiol. 46*, 1353−1366.
40. Ortiz-Lombardia, M., Pompeo, F., Boitel, B., and Alzari, P. M. (2003) *J. Biol. Chem. 278*, 13094−13100.
41. Pallen, M., Chaudhuri, R., and Khan, A. (2002) *Trends Microbiol. 10*, 556−563.
42. Parkinson, J. S. (1993) *Cell 73*, 857−871.
43. Peirs, P., De Wit, L., Braibant, M., Huygen, K., and Content, J. (1997) *Eur. J. Biochem. 244*, 604−612.
44. Ramaswamy, S. V., Amin, A. G., Goksel, S., Stager, C. E., Dou, S. J., El Sahly, H., Moghazeh, S. L., Kreiswirth, B. N., and Musser, J. M. (2000) *Antimicrob. Agents Chemother. 44*, 326−336.
45. Sheldon, P., Busarow, S., and Hutchinson, C. (2002) *Mol. Microbiol. 44*, 449−460.
46. Sreevatsan, S., Stockbauer, K. E., Pan, X., Kreiswirth, B. N., Moghazeh, S. L., Jacobs, W. R., Jr., Telenti, A., and Musser, J. M. (1997) *Antimicrob. Agents Chemother. 41*, 1677−1681.
47. Studier, F. W., and Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113−130.
48. Takayama, K., and Kilburn, J. O. (1989) *Antimicrob. Agents Chemother. 33*, 1493−1499.
49. Telenti, A., Philipp, W. J., Sreevatsan, S., Bernasconi, C., Stockbauer, K. E., Wieles, B., Musser, J. M., and Jacobs, W. R., Jr. (1997) *Nat. Med. 3*, 567−570.
50. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res. 22*, 4673−4680.
51. Umeyama, T., Lee, P. C., and Horinouchi, S. (2002) *Appl. Microbiol. Biotechnol. 59*, 419−425.
52. Wietzorrek, A., and Bibb, M. (1997) *Mol. Microbiol. 25*, 1181−1184.
53. Xu, X., Tsvetkov, L. M., and Stern, D. F. (2002) *Mol. Cell. Biol. 22*, 4419−4432.
54. Young, T. A., Delagoutte, B., Endrizzi, J. A., Falick, A. M., and Alber, T. (2003) *Nat. Struct. Biol. 10*, 168−174.
55. Zhang, C. C., and Libs, L. (1998) *Mol. Gen. Genet. 258*, 26−33.
56. Zhang, N., Torrelles, J., McNeil, M. R., Escuyer, V., Khoo, K., Brennan, P. J., and Chatterjee, D. (2003) *Mol. Microbiol. 48*, 875−888.

BI035150B