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Running title: EmbR2 inhibits PknH autokinase activity

Synopsis

EmbR is a transcriptional regulator which is phosphorylated by the cognate mycobacterial serine/threonine protein kinase PknH. Recent studies demonstrated that PknH-dependent phosphorylation of EmbR enhances its DNA-binding activity and activates transcription of the *embCAB* genes encoding arabinosyltransferases which participate in arabinan biosynthesis. Herein, we identified a genomic region of 4425-bp, that is present in *Mycobacterium tuberculosis* CDC1551, but absent from *M. tuberculosis* H37Rv, comprising the *MT3428* gene, which is homologous to *embR*. Homology modelling of the *MT3428* gene product illustrated its close relationship (56% identity) to EmbR, and was hence termed EmbR2. In marked contrast to EmbR, EmbR2 was not phosphorylated by PknH, although it is a substrate of other *M. tuberculosis* kinases, including PknE and PknF. Tryptophan fluorescence emission of EmbR2 was monitored in the presence of three different PknH-derived phosphopeptides and demonstrated that EmbR2 binds to at least two of the threonine sites known to undergo autophosphorylation in PknH. We observed that the capacity of EmbR2 to physically interact with PknH without being phosphorylated was a result of EmbR2-mediated inhibition of kinase activity: incubation of PknH with increasing concentrations of EmbR2 led to a dose-response inhibition of the autokinase activity, similarly to O6-cyclohexylmethylguanine, a known inhibitor of eukaryotic cyclin-dependent kinases. Moreover, EmbR2 inhibited PknH-dependent phosphorylation of EmbR in a dose-dependent manner. Together, these results suggest that EmbR2 is a regulator of PknH activation, thus directly participating in the control of the PknH/EmbR pair and potentially in mycobacterial physiology/virulence of *M. tuberculosis* CDC1551.

Keywords: *Mycobacterium*, phosphorylation, Ser/Thr protein kinase, PknH, EmbR, EmbR2, inhibitor, FHA domain, structural model

Introduction

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 and their regulation. Based on their genomic context, many of the 11 STPKs from *Mycobacterium tuberculosis* (*M. tb*) were proposed to participate in regulation of diverse physiological functions [1]. An increasing body of evidence indicates that the STPKs play important roles in the physiology and virulence of *M. tb* and are potential therapeutic targets for future drug development against tuberculosis [2]. Recent studies have provided evidence that at least two kinases, PknG and PknH, play a role in the virulence of *M. tb* [3-5]. PknG contributes to the survival of mycobacteria within macrophages by preventing fusion of phagosomes with lysosomes [5]. In contrast, it was demonstrated that a PknH mutant of *M. tb* is hypervirulent in infected mice and it was proposed that PknH regulates intracellular bacterial growth particularly during the chronic phase [4].

Since EmbR (Rv1267c) and the sensor-like Ser/Thr kinase PknH (Rv1268c) are adjacent in the *M. tuberculosis* genome, we first investigated whether EmbR may represent an endogenous substrate of PknH. We found that EmbR was phosphorylated by PknH *in vitro,* and that the interaction with the kinase was mediated by EmbR's C-terminal FHA (ForkHead Associated) domain [6]. FHA domains are small protein modules capable of mediating protein-protein interactions through pThr recognition, and often participate in protein-protein interactions in STPK-dependent signal transduction pathways [7]. Truncation of the EmbR FHA domain or point mutations altering conserved residues in the phosphothreonine-binding pocket dramatically diminished PknH-mediated threonine phosphorylation of EmbR [6]. Elucidation of the three-dimensional structure of EmbR revealed that, in addition to the FHA domain, it possesses two major domains, an N-terminal winged helix-turn-helix DNA-binding domain followed by a central bacterial transcription activation (BTA) domain, suggesting that EmbR may act as a transcriptional regulator in *M. tb* [8]. This was recently supported by the fact that phosphorylation of EmbR enhances its DNA binding activity towards promoter regions of *embCAB* genes [9]. The corresponding gene products are known to participate in the biosynthesis of arabinogalactan and lipoarabinomannan which are critical components of the mycobacterial cell wall [10-12]. The PknH-mediated increase in the transcription of *embAB* also alters resistance to ethambutol, a frontline antituberculosis drug known to target *embAB* [10, 11]. These results along with the *in vivo* up-regulation of PknH inside host macrophages [13] suggest

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a functionally relevant signalling mechanism involving the PknH-EmbR-Emb*CAB*. In addition, Sharma *et al*. demonstrated that the Ser/Thr protein kinases PknA and PknB also regulate the signalling pathway leading to EmbR phosphorylation [9].

In a related actinomycete, *Corynebacterium glutamicum*, phosphoproteome studies experimentally determined 120 unique Ser/Thr-phosphorylated proteins that are phosphorylated by only four STPKs [14]. In support of these results, using a bioinformatic approach, 40 potential PknH substrates were identified (including EmbR) in *M. tb*, and two of the newly predicted substrates were experimentally shown to be phosphorylated by PknH *in vitro* [15]. These two substrates, which lack an FHA domain, are Rv0681, a TetR-class transcription factor, and DacB1, a penicillin-binding protein [15]. The presence of a large number of FHA and non-FHA-containing substrates of PknH in *M. tb* suggests a possible participation of this kinase in numerous signalling pathways. Since PknH appears as a key determinant of pathogen virulence [4], it is very likely that further characterization of the various endogenous substrates and regulatory mechanisms will increase our understanding of the physiology and pathogenicity of *M. tb*. One possible mechanism of regulating the phosphosignalling responses is to control the rate of phosphorylation of both the kinases and the substrates, which can be achieved *in vitro* and *in vivo* by Ser/Thr phosphatases. To date, only one Ser/Thr-phosphatase, termed PstP, has been found in the *M. tb* genome mediating dephosphorylation of kinases [16] as well as substrates [17]. However, it is unlikely that PstP represents the only factor that controls the phosphorylation status of all eleven STPKs and all substrates *in vivo*. We reasoned that additional mechanisms of controlling/regulating the phosphorylation status of PknH may exist. In *Streptomyces coelicolor* A3(2), the KbpA protein which is involved in actinorhodin production has been demonstrated to bind to the STPK AfsK and to inhibit its autophosphorylation activity at serine and threonine residues [18]. KbpA also inhibited phosphorylation of AfsR, which is the substrate of AfsK, presumably because KbpA inhibited the conversion from the inactive nonphosphorylated form of AfsK to the active phosphorylated form [18].

In light of this study, we extended our previous work on the characterization of the PknH/EmbR pair by identifying and characterizing a protein from *M. tuberculosis* CDC1551 that interferes specifically with the autokinase activity of PknH, subsequently inhibiting PknH-dependent phosphorylation of EmbR. This protein, termed EmbR2, closely related to EmbR, is capable of interacting with PknH, but cannot be phosphorylated by this kinase. We predict that this protein would represent a bait for PknH and may represent a novel mechanism of regulation of the PknH/EmbR couple.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

Overproduction of EmbR2 was performed in *Escherichia coli* strain C41(DE3) [19]. *E. coli* TOP-10 (Invitrogen) was used to propagate plasmids in cloning experiments. All strains were grown and maintained at 37 °C in LB medium supplemented with either 25 μg/ml kanamycin or 100 μg/ml ampicillin, when required. Plasmids pET28a and pCR-BluntII-TOPO were purchased from Novagen and Invitrogen, respectively.

Expression and purification of GST-tagged Ser/Thr protein kinases from *M. tuberculosis*

Plasmids designed to express the eight catalytic domains of Ser/Thr protein kinases (PknA, PknB, PknD, PknE, PknF, PknH, PknK, and PknL) fused to glutathione-*S*-transferase (GST) were constructed by PCR amplification using *M. tuberculosis* H37Rv genomic DNA as a template as described previously [17]. Kinases were purified using glutathione-Sepharose 4B matrix as reported earlier [17].

Expression and purification of a His$_6$-tagged EmbR and EmbR2

Construction of pET28a-*embR* expression vector was used to overproduce EmbR in C41(DE3) *E. coli* cells as reported earlier [6]. The *embR2* gene (MT3428), with appropriate sites at both ends, was synthesized by PCR amplification using *M. tuberculosis* CDC1551 genomic DNA as a template and the following primers: embR2-up 5'-CC CAT ATG GCC CGA AAC GAA CTC CG-3' (*Nde*I site underlined) and embR2-lo 5'-GGA TCC TAA CGG CCC GAT TCG TG-3' (*Bam*HI underlined). This 1156-bp DNA fragment was directly ligated into the pCR-BluntII-TOPO, yielding the pCRII-BluntII-*embR2* construct. The *embR2* insert was then extracted and purified following restriction of pCRII-BluntII-*embR2* with *Nde*I and *Bam*HI, and subsequently ligated into the *Nde*I/*Bam*HI-restricted pET28a, thus giving rise to pET28a-*embR2*, which was used to transform *E. coli* C41(DE3) cells. Cultures were incubated at 37 °C under 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 30 °C. Cells were harvested by centrifugation at 6,000×*g* for 10 min, washed in 10 ml of buffer A (50 mM Tris/HCl pH7.5; 150 mM NaCl; 10% glycerol; 1 mM β-mercaptoethanol; 1 mM EDTA; 10 mM imidazole) containing DNAse, RNAse and a protease inhibitor cocktail (Roche). Bacteria were then disrupted in a French pressure cell and the resulting extract was centrifuged for 30 min at 27,000 × *g* at 4°C. The supernatant was

collected and incubated for 2 hours at 4°C on a rotating wheel with Ni-NTA agarose (Qiagen) previously equilibrated with buffer A. After extensive washing with buffer A, bound proteins were eluted with elution buffer (50 mM Tris/HCl pH7.5; 150 mM NaCl; 10% glycerol; 1 mM β-mercaptoethanol; 1 mM EDTA; 300 mM imidazole). Pure EmbR2 fractions were pooled, dialyzed against 50 mM Tris/HCl pH7.5, 150 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA to remove imidazole and stored at –20°C until further use.

Site-directed mutagenesis

Site-directed mutagenesis was carried out based on PCR amplification. A first set of PCR amplifications was generated using pET28a-*embR2* as a template along with the primer pair, #428 (5'-GCC CTG TGG ACG CAA CTC ATC ACC GCA TAC TAT-3') and # 431 (5'- TAT *GGA TCC* CTA ACG GCC CGA TTC GTG CGG -3') to generate *embR2*-R204T/A208T. This PCR product was then used as a primer in a second round of PCR amplification in combination with primer #430 (5'- TAA TAG CT *CAT ATG* GCC CGA AAC GAA CTC CGG TTC-3') to generate the entire 1146-bp *embR2* gene. This DNA fragment, containing the appropriate sites at both ends, was restricted by *Nde*I and *Bam*HI (sites italicized) and ligated in the *Nde*I/*Bam*HI-restricted pET28a, generating pET28a-*embR2*-R204T/A208T. The nucleotide changes in the different primers to allow specific amino acid replacement are underlined. Mutations were verified by DNA sequencing. The His-tagged EmbR2 mutant protein was overexpressed and purified as described above.

In vitro kinase assay

In vitro phosphorylation of about 1 µg (15 pmol) of each STPK 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-^{33}P]ATP. Phosphorylation of EmbR or EmbR2 was performed with 3 µg (66 pmol) of EmbR or EmbR2 in 20 µl of buffer P with 200 µCi/ml [μ-^{33}P]ATP and 250 ng of kinase for 15 min at 37°C. *In vitro* inhibition assays were carried out with 0.5 to 3 µg of EmbR2 (0.55 µM to 3.3 µM) or with 5 to 100 µM of O6-cyclohexylmethylguanine. The reaction was stopped by addition of an equal volume of 2X sample buffer and the mixture was heated at 100°C for 5 min. After electrophoresis, gels were soaked in 16% TCA for 10 min at 90°C, dried and exposed to films. Intensity of the radioactivity was measured with a Molecular Dynamics Typhoon phosphoimager.

Phosphopeptide binding assay

Intrinsic Trp-fluorescence emission was measured between 300 and 400 nm (scan speed 100 nm/min), using a Perkin Elmer Luminescence Spectrometer LS 50B, exciting the sample at a wavelength of 295 nm. Spectra were recorded for each peptide aliquot (1 µl) added to 400 µl of a 5 µM solution of EmbR2, until reaching a final peptide concentration of 0.5 mM (11 additions). A plot of fluorescence intensity at 330 nm vs. peptide concentration (3 independent experiments) resulted in a binding isotherm that was fitted to a one-site saturation model (SigmaPlot, Systat Software Inc.).

Analysis of the phosphoamino acid content of proteins

Phosphoamino acid analysis of the EmbR or EmbR2 proteins labelled *in vitro* with either PknE, PknF or PknH was performed as previously described [20].

Homology model of EmbR2

A homology model of the molecular structure of Mt-CDC1551 EmbR2 was derived based on the crystal structure of EmbR bound to the phosphopeptide SLEVpTEADT [8]. Models were obtained from several web-based modelling servers, including Swiss-Model [21], Geno3D [22], and 3D-JIGSAW [23]. The homology models were analyzed in comparison to the chain A complex of the phosphopeptide-bound structure of EmbR (pdb entry 2FF4, [8]).

Synthesis of O6-cyclohexylmethylguanine

The target sample was prepared by the method of Arris *et al.* [24]. CHCl$_3$:MeOH (20:1) was used for purification *via* silica gel column chromatography yielding a single spot on thin layer chromatography: white solid, yield 66%, mp 202-204 ^{o}C (dec.). ^{1}H NMR (300 MHz, Me$_2$SO-d_6) d 12.38 ppm (bs, 1H, NH), 7.79 (s, 1H, H-8), 6.21 (bs, 2H, NH_2), 4.20 (d, 2H, -OCH_2-, J = 6.1Hz), 182-162 (m, 6H, cyclohexyl H's), 1.32-0.97 (m, 5H, cyclohexyl H's). FABMS *m/z*: 248 (M+H)$^+$. HRMS (ESI) calcd for (M+H)$^+$ (C$_{12}$H$_{17}$N$_5$O) 248.15059, found 248.15083.

Results and Discussion

Identification of EmbR2 in *M. tuberculosis* CDC1551

In this study, we extended our previous studies by deciphering a new mechanism of regulation of phosphorylation of EmbR by PknH. To this end, we first explored the genome of *M. tb* strain CDC1551 which, in contrast to the laboratory strain *M. tb* H37Rv, is involved in a recent cluster of tuberculosis cases and is known to be transmissible and virulent in humans [25]. The CDC1551 strain appears to be highly infectious in humans, is comparable in virulence to strain H37Rv in animal models [26], and has greater immunoreactivity than H37Rv and other clinical strains due to increased induction of TNF-α, IL-6, IL-10, and IL-12 [27]. We identified a genomic region of 4425-bp that is present in CDC1551, but absent from *M. tb* strain H37Rv, comprising the *MT3428* gene [28] (Figure 1A). The insertion sequence *IS*6110 is the principal epidemiological marker for *M. tb*. A number of the insertions and deletions were associated with this insertion sequence, suggesting a role for this element in genome plasticity [29]. The presence in H37Rv of *IS*6110 lacking the 3- to 4-bp direct repeat directly adjacent to the deleted region relative to strain CDC1551 indicates that this deletion was associated with a possible mechanism of homologous recombination between nearby *IS*6110 elements [30]. We also found the *MT3428* gene in other mycobacterial species, including *M. bovis* and *M. bovis* BCG (data not shown). MT3428, annotated as a putative transcriptional regulator, is highly homologous to EmbR. Indeed, the amino acid sequences of *M. tb* H37Rv EmbR and *M. tb* CDC1551 MT3428 (termed EmbR2) align with 55% sequence identity over 381 residues (alignment using ClustalW, Figure 1B). The alignment is almost free from insertions and deletions. A single-residue gap in the FHA domain of EmbR2 maps to residue 300 (EmbR), while an N-terminal 5-residue deletion is located within the first eight residues in the sequence. In contrast to *embR,* which is genetically linked to *pknH* [6, 31], *embR2* is found to be genetically distant from *pknH*.

We have generated homology models of EmbR2 based on our recently determined high resolution X-ray crystal structure of EmbR [8]. This seemed justified based on the high level of sequence identity and the very good resolution of the template structures (PDB: 2FEZ 2.0 Å, 2FF4 1.9 Å). Algorithm-specific bias of the modeling outcome was minimized by generating models using a range of modeling servers. As expected, all models display a conserved backbone conformation for the N-terminal DNA-binding, the central BTA and the C-terminal FHA domain (Figure 1C). The models only diverge slightly around residue 294, where the FHA domain of EmbR2 lacks a residue relative to EmbR. This model clearly confirms the characterization of

EmbR2 as a structural homologue of EmbR. We, thus, addressed the question whether cross-regulation/interaction between these two proteins with PknH would occur.

EmbR2 is not phosphorylated by PknH

We first investigated whether EmbR2 represents a substrate of PknH. To this end, PknH was expressed as a GST fusion [6], whereas EmbR and EmbR2 were expressed and purified as His-tag fusions. When incubated with purified EmbR in the presence of $[\gamma-^{33}P]ATP$, PknH was able to phosphorylate EmbR, consistent with our previous results (Figure 2) [6]. In stark contrast, EmbR2 was not phosphorylated by PknH *in vitro* (Figure 2). This was rather unexpected given the high degree of sequence and structural conservation between these two proteins, an identical domain organisation and invariance of three key residues in the FHA domain (Arg312, Ser326 and Asn348) that are required for recognition of EmbR by PknH [6].

In their attempt to identify endogenous substrates of the PknH kinase, Zheng *et al.* searched the *M. tb* genome database for open reading frames containing sequences similar to the motif TQLGT which encompasses a site of autophosphorylation in the activation loop of PknH, reasoning that the autophosphorylation and kinase activity may target similar amino acid motifs [15]. This allowed the identification of 40 putative substrates, including EmbR (containing a TQLIT motif), Rv0681 (containing a TQLGT motif) and DacB1 (containing a TQIGT motif). Substitution of the first Thr residue in this motif by an Ala residue completely abolished Rv0681 phosphorylation by PknH, whereas in the case of EmbR and DacB1 phosphorylation by PknH was significantly reduced compared to the wild-type proteins [15]. EmbR2 contains a RQLIA motif instead of the TQLIT motif present in EmbR (Figure 1B). To investigate the possibility that absence of phosphorylation of EmbR2 by PknH correlates with the lack of the TQLIT motif, both the Arg204 and Ala208 were simultaneously replaced by two Thr residues to restore a genuine TQLIT motif within EmbR2. However, when tested in the presence of $[\gamma-^{33}P]ATP$, PknH still failed to phosphorylate the EmbR2-R204T/A208T mutant (Figure 2). This indicates that the lack of a TQLGT motif in EmbR2 is not responsible for the absence of phosphorylation by PknH.

STPK-mediated phosphorylation of EmbR and EmbR2

Overlapping substrate specificity of different *M. tb* kinases has been reported earlier [17, 32, 33]. This prompted us to investigate whether EmbR2 could be phosphorylated by other *M. tb* STPKs. EmbR2 was found to be phosphorylated by PknE and PknF (Figure 3A). Whereas PknF phoshorylates EmbR and EmbR2 equally well, PknE preferentially phosphorylates EmbR over

EmbR2. In marked contrast, PknH is unable to phosphorylate EmbR2, but phosphorylates EmbR, similarly to PknE/F. This suggests different levels of substrate specificity of the different STPKs. A corollary, arising from these experiments is that EmbR and EmbR2, as they interact with multiple STPKs, may be regulated by multiple signals. Although a complex network of interactions between EmbR/EmbR2 and the different STPKs may exist, it remains to be established whether this STPK cross-talk occurs *in vivo.*

We next analysed the nature of the phosphorylated amino acid residues in EmbR/EmbR2 after *in vitro* phosphorylation with PknE, PknF or PknH. Following incubation with the kinases and $[\gamma\text{-}^{33}P]$ATP, labeled proteins were separated by SDS-PAGE, excised, and subjected to acid hydrolysis. Figure 3B shows that, in agreement with our previous results [6], EmbR was phosphorylated by PknH only on threonine residues. However, analysis of the phosphoamino acid content of PknE-phosphorylated EmbR revealed that phosphorylation occurs on both threonine and serine residues, whereas PknF phosphorylated EmbR only on serine residues. These unexpected results prompted us to explore the phosphoamino content of the different Pkn/EmbR2 couples. Whereas EmbR2 was preferentially phosphorylated at threonine residues in the presence of PknE (with minor amounts of phosphoserine being detected), it was mainly phosphorylated at serine residues in the presence of PknF. As for EmbR, these results show that the nature of the phosphorylation sites is kinase-dependent. Furthermore, comparison of the phosphoamino profiles of EmbR and EmbR2 in relation with the different kinases also revealed that the nature of the phosphorylation sites is also dependent of the substrate. PknF phosphorylates EmbR strictly at serines, whereas PknF phosphorylates EmbR2 at both serines and threonines.

Physical interaction between EmbR2 and PknH-derived phosphopeptides

PknH was shown to possess autokinase activity on nine Ser and Thr residues [6, 34] and one of the Thr sites located within the kinase activation loop, Thr170, is required for PknH-mediated phosphorylation of EmbR [6]. As EmbR2 contains three Trp residues, we assessed ligand-binding by intrinsic Trp fluorescence. Intensity of Trp fluorescence emission at 330 nm was monitored as a function of concentration of three different PknH-derived phosphopeptides (DEKLpTQLGT containing the pThr170, TQLGpTAVGT containing the pThr174, DSAGpTLVSS containing the pThr222) as well as using a non-specific phosphopeptide derived from the high-affinity ligand of the FHA domain of the yeast Rad53p check-point kinase [7] (SLEVpTEADT). Changes of the emission intensity at 330 nm with increasing peptide concentrations reflect ligand binding, and the equilibrium dissociation constant Kd can be

derived by fitting the resulting binding isotherm (Table 1). Each of the phosphopeptides bound with an equilibrium dissociation constant (K_d) in the order of 15 μM. This is in marked contrast to binding data obtained with EmbR, where only the Rad53p-derived peptide caused a measurable change in the emission signal, indicating a K_d of about 175 μM [8]. Therefore, these results suggest that EmbR2 binds to at least two of the threonine sites known to undergo autophosphorylation in PknH [34].

Overall, the absence of PknH-mediated phosphorylation of EmbR2 despite low-μM interaction of the latter with at least three likely docking sites on the kinase, lead us to hypothesise that EmbR2 may not act as a substrate of PknH but rather as an inhibitor.

Selective inhibitory effect O6-cyclohexylmethylguanine towards *M. tb* kinases

Cyclin-dependent kinases are increasingly recognized as important targets for therapeutic intervention in various proliferative disease states, including cancer [35]. O6-cyclohexylmethylguanine has been reported as a newly discovered ATP-competitive purine-based inhibitor of CDK1/cyclin B1 and CDK2/cyclin A3, the O6 substituent occupying the kinase ribose binding sites [24, 36]. Notably, screening of a small library of approximately 5,000 compounds from Southern Research through the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (www.taacf.org) identified a small number of 6-alkylthio- and O6-alkyl-purines as well as 2,4-disubstituted- and 2,4,6-trisubstituted pyrimidines that were relatively active versus *M. tb* H37Rv *in vitro* [37]. A cheminformatic search of the scaffolds suggested that these simple analogs may be ATP-competitive inhibitors of signaling kinases in *M. tb*. Recent publications support that these purine and pyrimidine scaffolds show broad and excellent antitubercular activity [38-40], and, in certain cases, antibacterial and enzymatic activity versus ATP-requiring bacterial kinases. Preliminary screening of several active samples from the TAACF *in vitro* screen identified the reported ATP-competitive CDK inhibitor O6-cyclohexylmethylguanine as a potential inhibitor of PknH (data not shown). Thus, we tested the capacity of O6-cyclohexylmethylguanine (Figure 4A) to interfere with the autokinase activity of eight *M. tb* STPKs *in vitro*. The catalytic domains of the kinases were purified as GST-fusion proteins [17] and incubated individually with [γ-^{33}P]ATP in the absence or presence of 50 μM O6-cyclohexylmethylguanine. Figure 4A shows that this compound selectively inhibited the various kinases. Whereas the drug did not appreciably alter the activity of PknA, PknB, PknF, PknK or PknL, it exhibited a potent inhibitory effect on PknH and PknE, and completely abrogated autophosphorylation activity of PknD. Comparative sequence analysis allowed

grouping of the eleven *M. tb* kinases into three distinct homology clusters (PknA/B/L, PknF/I/J and PknD/E/H), while the two soluble enzymes (PknG and PknK) stand apart from all the others [2, 41]. Our results clearly indicate that O6-cyclohexylmethylguanine exhibits specific activity against the third group of kinases only, supporting the notion that PknD/E/H belong to the same clade. It also demonstrates the feasibility to design inhibitors that will target specifically one clade rather than another. This selectivity is of particular interest since signalling elements, including STPK, may represent a new class of attractive targets, and the expertise in designing such inhibitors for eukaryotic STPKs represent an intense research area [42] and could be exploited for the development of new antitubercular drugs.

Figure 4B (left panels) shows that O6-cyclohexylmethylguanine inhibited PknH autokinase activity in a dose-dependent fashion, with an IC_{50} value of around 5 μM. This prompted us to examine whether EmbR2 alters the autophosphorylation activity of PknH by incubating the kinase with increasing concentrations of EmbR2 in the presence of [γ-^{33}P]ATP. Figure 4B (right panels) demonstrated inhibition of the autokinase activity of PknH in a dose-responsive manner, similarly to O6-cyclohexylmethylguanine. The IC_{50} value of EmbR2 was around 1.1 μM (1 μg of EmbR2 in the conditions used), thus indicating that a 5-fold excess of EmbR2 at a molar level was sufficient to inhibit 50% of the kinase activity of PknH.

EmbR2 inhibits PknH-dependent phosphorylation of EmbR

It is known that activation of kinases often depends on regulatory processes involving phosphorylation/dephosphorylation through an autocatalytic mechanism. For instance, the activation loop is a major control element of an active/inactive conformational switch in numerous kinases [43], and the conformation of the activation loop often depends on the phosphorylation state [44]. Based on structural studies, it is thought that the activation loop controls both the accessibility to the catalytic site and the binding of the substrate. Combining on-target dephosphorylation/MALDI-TOF MS and LC-ESI/MS/MS has recently allowed the identification of nine phosphorylation sites in PknH [34] resulting from autophosphorylation, a prerequisite to its kinase activity. Since EmbR2 inhibits autophosphorylation of PknH, PknH-dependent phosphorylation of EmbR could be dependent on the presence of EmbR2. Figure 5 clearly shows a dose-dependent inhibition of EmbR phosphorylation with increasing concentrations of EmbR2. However, when EmbR2 was replaced by an unrelated protein, for instance GST, there was no inhibitory effect on EmbR phosphorylation, thus demonstrating that the inhibitory effect was specific to EmbR2. These results suggest that, in the reaction mixture

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containing PknH and EmbR, EmbR2 inhibited the phosphorylation of EmbR by PknH, presumably because EmbR2 prevents the conversion from the inactive non-phosphorylated form of PknH to the active phosphorylated form. It is noteworthy that a similar mechanism of regulation has been reported in *S. coelicolor* A3(2), in which the KbpA protein binds to and inhibits autophosphorylation of AfsK as well as phosphorylation of AfsR, the substrate of AfsK [18].

Concluding remarks

The cell wall of pathogenic mycobacteria undergoes continuous changes in the course of growth, intramacrophagic survival or during infection in response to environmental changes [45] and many of the environmental stimuli are communicated within mycobacteria by sensor kinases present on the mycobacterial membrane [1]. PknH is unique to pathogenic mycobacteria, which is induced on infection of macrophages with *M. tb* [13]. The *embCAB* operon encoding arabinosyltransferases was identified as the cellular target of EmbR in *M. tb* [9]. PknH-dependent phosphorylation of EmbR enhances the DNA binding activity of EmbR towards promoter regions of *embCAB* genes. The consequent enhanced transcription of *embC* results in a higher ratio of lipoarabinomannan (LAM) to lipomannan (LM) [9], which are two major cell wall lipoglycans. These complex molecules are important determinants in the mycobacterial physiology and in the modulation of the host immune system during disease progression [46]. Furthermore, PknH expression modestly alters ethambutol resistance by virtue of its ability to increase the transcription of the *embA* and *embB* genes [9]. Together, this establishes that *embCAB* activation is controlled by EmbR phosphorylation and connects the LAM/LM ratio with the phosphorylation status of PknH. However, little is known with respect to the regulation processes controlling PknH activation. Although this could be achieved (at least partially) by phosphorylation/dephosphorylation *via* the action of the Ser/Thr phosphatase PstP [16, 17], this study also suggests that EmbR2 could be a key regulator of PknH activation. We propose that PknH and EmbR form a pair participating in arabinan metabolism that can also be controlled by EmbR2, providing a novel mechanism of regulating phosphorylation of EmbR *via* PknH. In summary, this study describes an additional level of regulation of mycobacterial STPKs, which may explain how a limited number of kinases participate in many complex signalling pathways. It has been proposed that the PknH-mediated signalling pathways contribute to the regulation of bacillary load during the infection process [4]. Upon infection in mice, the *M. tb* H37Rv Δ*pknH*

mutant survived to a higher load in the mouse organs, especially during the chronic stage of infection, indicating that PknH plays a role in regulating bacillary load to facilitate adaptation to the host environment during the infection process. In this context, *in vivo* expression of EmbR2 in *M. tb* CDC1551 or in other pathogenic species such as *M. bovis* (which possesses an identical *embR2* gene), could inhibit PknH activation, and consequently increase the virulence phenotype of these strains. Whether EmbR2 plays an *in vivo* inhibitory role in a negative feedback system in the PknH/EmbR regulatory pathway and contributes to survival and hypervirulence in pathogenic mycobacteria remains to be determined .

Acknowledgments

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Figure legends

Figure 1. (A) Organization of the region of insertion containing the *embR2* locus in *M. tuberculosis* CDC1551 relative to strain H37Rv. The thick line represents the 4.4-kb insertion region. The coordinates of the insertion region and those of the *embR2* gene are indicated, according to [28]. Arrows represent the direction of transcription. The *embR2* gene is shown in grey. **(B) Sequence alignment of EmbR from the strain H37Rv and EmbR2 from the strain CDC1551.** Identical residues are shown in black. Arrowheads indicate the position of the three highly conserved FHA residues (Arg312, Ser326 and Asn348 with regard to the EmbR sequence). **(C) Superposition of the Cα trace of EmbR (Yellow, PDB: 2FF4) with those of the homology models of EmbR2 (cyan – SWISSMODEL, magenta – 3DJIGSAW, blue – GENO3D).** Shown in gray sticks is the phosphopeptide bound to the FHA domain of EmbR. Inset: region of the 1-residue deletion in the FHA domain, leading to discrepancies in backbone conformation of the different homology models.

Figure 2. *In vitro* phosphorylation of EmbR and EmbR2 by PknH. His-tagged fusion proteins were purified from *E. coli* C41(DE3) carrying either pET28a-*embR*, pET28a-*embR2* or pET28a-*embR2(R204T/A208T)*. Purified GST-PknH was incubated with 3 μg of either purified His-tagged EmbR, EmbR2 or EmbR2-R204T/A208T in the presence of [γ-^{33}P]ATP for 15 min at 37°C. Samples were separated by SDS-PAGE and detected by Coomassie blue staining (upper panels) and then exposed overnight to a film and visualized by autoradiography (lower panels). Positions of PknH, EmbR and EmbR2 are indicated.

Figure 3. *In vitro* phosphorylation of EmbR and EmbR2 by PknE and PknF. (A) Purified GST-PknE and GST-PknF were incubated with either His-tagged EmbR (left panels) or His-tagged-EmbR2 (right panels) with [γ-^{33}P]ATP. Samples were separated by SDS-PAGE and visualized following Coomassie blue staninig (upper panels) or autoradiography after exposure to a film (lower panels). **(B) Phosphoamino acid content of the Pkn-phosphorylated EmbR/EmbR2.** EmbR and EmbR2 were phosphorylated *in vitro* in presence of either GST-PknE, GST-PknF or GST-PknH and [γ-^{33}P]ATP, 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.

Figure 4. Dose-response inhibition of PknH autokinase activity by O6-cyclohexylmethylguanine or EmbR2. (A) O6-cyclohexylmethylguanine inhibits PknD, PknE and PknH activity. Autophosphorylation inhibition by O6-cyclohexylmethylguanine was evaluated against eight out of the eleven *M. tuberculosis* STPKs by incubating the kinases with [γ-^{33}P]ATP for 20 min at 37°C in the absence (-) or presence (+) of 50 μM of inhibitor. Samples were separated by SDS-PAGE and visualized by autoradiography. **(B)** Purified GST-PknH (0.3 μg) were incubated with increasing concentrations of either O6-cyclohexylmethylguanine (up to 100 μM) or EmbR2 (up to 3 μg) in the presence of [γ-^{33}P]ATP for 20 min at 37°C. Samples were separated by SDS-PAGE. The upper panels correspond to the Coomassie blue-stained gels, showing the presence of comparable amounts of PknH in each assay, the middle panels correspond to the corresponding autoradiograms, whereas the lower panels reflect densitometric analysis. In the case of EmbR2, each bar represent the mean of values from three independent experiments.
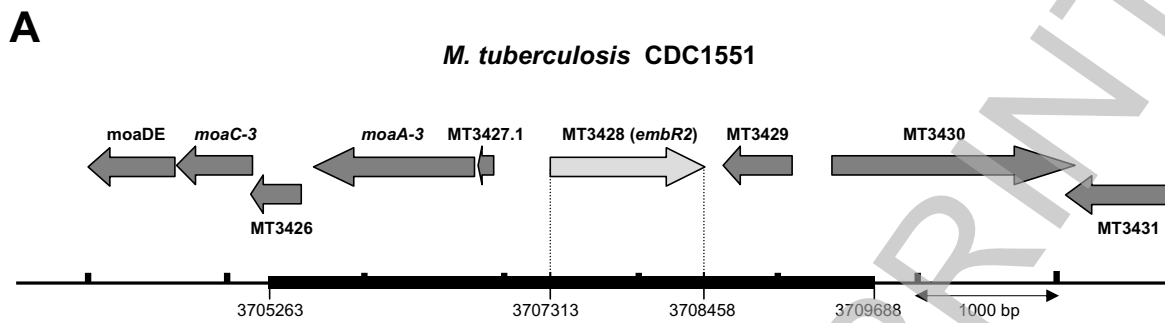
Figure 5. PknH-dependent phosphorylation of EmbR is inhibited by EmbR2. Equal amounts of purified GST-PknH (0.3 μg) and EmbR (1 μg) were incubated with increasing concentrations (up to 3 μg) of EmbR2 with [γ-^{33}P]ATP for 20 min at 37°C. Samples were separated by SDS-PAGE and the effect of EmbR2 on PknH and EmbR phosphorylation was visualized by autoradiography. The upper panels represent the corresponding autoradiograms, whereas the lower panels reflect densitometric analysis. In a control experiment, EmbR2 was replaced by GST.

Table 1 : Phosphopeptide binding of EmbR2. Intensity of Trp fluorescence emission at 330 nm was evaluated as a function of concentration of three different PknH-derived phosphopeptides (DEKLpTQLGT containing the pThr170, TQLGpTAVGT containing the pThr174, and DSAGpTLVSS containing the pThr222) as well as the Rad53p-ligand (SLEVpTEADT). The K_d values are as indicated.
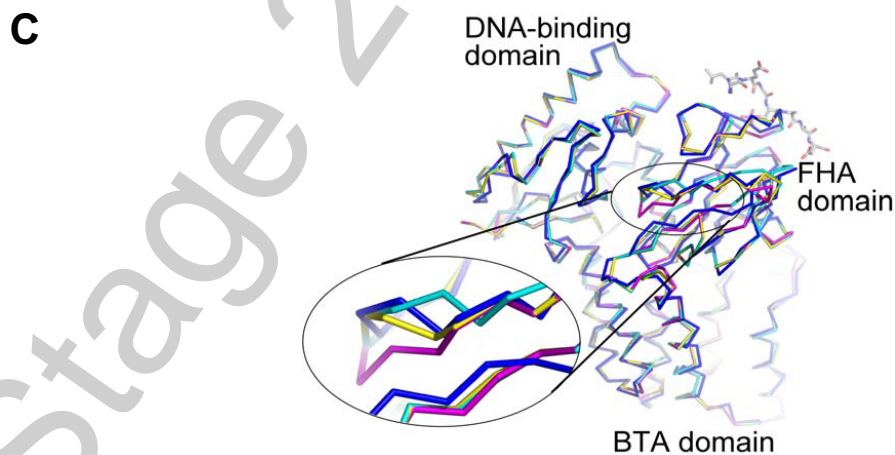
	Phosphopeptide sequence	K_d (in µM)
PknH	DEKLpTQLGT	18.3 ± 4.4
	TQLGpTAVGT	21.1 ± 4.1
	DSAGpTLVSS	11.9 ± 1.7
Rad53p	SLEVpTEADT	17.1 ± 1.4

21

Figure 1

A

M. tuberculosis CDC1551

moaDE *moaC-3* MT3427.1 MT3428 (*embR2*) MT3429 MT3430

MT3426

MT3431

3705263 3707313 3708458 3709688 1000 bp

B

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EmbR_MtubH37Rv      1 MAGSATVEKRLDFGLLGPLQMTIDGTPVPSGTPKQRAVIAMLVINRNRPVGVDALITALW
EmbR2_MtubCDC1551   1 MAR.....NELRFGILGPLEISAGFRSLPLGTPKQRAVLATLIIHRNRPVGIDSLIDAAW

EmbR_MtubH37Rv     61 EEWPPSGARASIHSYVSNLRKLLGGAGIDPRVVLAAAPPGYRLSIPDNTCDLGRFVAEKT
EmbR2_MtubCDC1551  56 EQDRPEGSRATVYTYVSNLRRLVSTTGADSHSLLASAPPGYRLAVADNQYDVARFLSQRS

EmbR_MtubH37Rv    121 AGVHAAAAGRFEQASRHLSAALREWRGPVLDDLRDFQFVEPFATALVEDKVLAHTAKAEA
EmbR2_MtubCDC1551 116 AGLRAAAAGSFEQASDHLSAALAEWRGPVLDDLREFSFVTRLANSLVEDKIIAHTALAEA

EmbR_MtubH37Rv    181 EIACGRASAVIAELEALTFEHPYREPLWTQLITAYYLSDRQSDALGAYRRVKTTILADDLG
EmbR2_MtubCDC1551 176 EIACGRADSVISELEELILEHPYHEALWRQLIAAYYVSERQSDALDAYRRLKTSLAEDLG

EmbR_MtubH37Rv    241 IDPGPTLRALNERILRQQPLDAKKSAKTTAAGTVTVLDQRTMASGQQAVAYLHDIASGRG
EmbR2_MtubCDC1551 236 VDPGPKVRTLYEQVLRQQALDTRVVVQAAAGDIIRALEHSPGMIDRSPRAAIRDAAGHR.

EmbR_MtubH37Rv    301 YPLQAAATRIGRLHDNDIVLDSANVSRHHAVIVDTGTNYVINDLRSSNGVHVQHERIRSA
EmbR2_MtubCDC1551 295 SPLGRLPLRIGRSKSNDMVLPDCKVSPYHAVIVNTGESFMITDLRSVNGVYVRGRRIATT

EmbR_MtubH37Rv    361 VTLNDGDHIRICDHFFTFQISAGTHGGT
EmbR2_MtubCDC1551 355 ATLNDGDHIRIGDHELTFEVIPHESGR.
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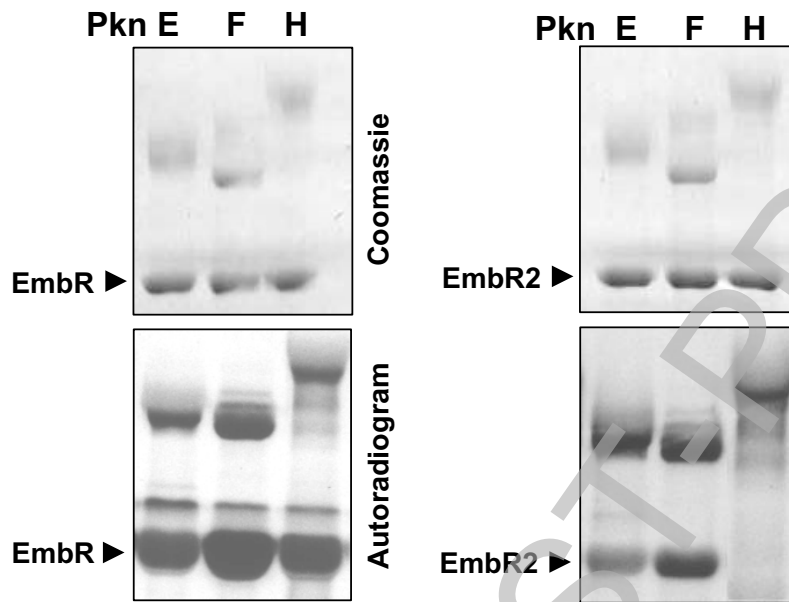
C

DNA-binding domain

FHA domain

BTA domain

Figure 2

Figure 3

Figure 4

A

O6-cyclohexylmethylguanine

O6-cyclohexylmethylguanine

B

O6-cyclohexylmethylguanine

EmbR2

Figure 5