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The *Mycobacterium tuberculosis* transcriptional repressor EthR is negatively regulated by Serine/Threonine phosphorylation

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ABSTRACT

Recent efforts have underlined the role of Serine/Threonine Protein Kinases (STPKs) in growth, pathogenesis and cell wall metabolism in mycobacteria. Herein, we demonstrated that the *Mycobacterium tuberculosis* EthR, a transcriptional repressor that regulates the activation process of the antitubercular drug ethionamide (ETH) is a specific substrate of the mycobacterial kinase PknF. ETH is a prodrug that must undergo bioactivation by the monooxygenease EthA to exert its antimycobacterial activity and previous studies reported that EthR represses transcription of *ethA* by binding to the *ethA-ethR* intergenic region. Mass spectrometry analyses and site-directed mutagenesis identified a set of four phosphoacceptors, namely Thr2, Thr3, Ser4 and Ser7. This was further supported by the complete loss of PknF-dependent phosphorylation of a phosphoablative EthR mutant protein. Importantly, a phosphomimetic version of EthR, in which all phosphosites were replaced by Asp residues, exhibited markedly decreased DNA-binding activity compared with the wild-type protein. Together, these findings are the first demonstration of EthR phosphorylation and indicate that phosphorylation negatively affects its DNA-binding activity, which may impact ETH resistance levels in *M. tb*.

1. Introduction

One of the major sources of sickness and death worldwide is the global tuberculosis (TB) epidemic. *Mycobacterium tuberculosis* (*M. tb*) has a complex life style comprising different environments and developmental stages. The success of *M. tb* results from its remarkable capacity to survive within the infected host, where it can persist for several decades. Additionally, TB incidence has been dramatically increased by the co-infection with the human immunodeficiency virus, which also contributed to the emergence of multidrug resistant tuberculosis (MDR-TB), defined as resistant to the first-line agents isoniazid (INH) and rifampicin (RIF) [1]. To treat MDR-TB, less active and often more toxic second-line compounds are used, including ethionamide (ETH) [2]. ETH is a structural analogue of INH and it has been previously shown that both compounds target the same enzyme, the enoyl-acyl carrier protein reductase InhA of the type II fatty acid synthase, resulting in the inhibition of mycolic acids synthesis [3]. Many of the current TB chemotherapeutics, including ETH, are prodrugs that required to be activated by specific mycobacterial enzymes to achieve their antimycobacterial activities. The biochemical conversion of ETH is mediated by the monooxygenase EthA [4,5]. When the prodrug activator, EthA [5], is overexpressed, the sensitivity of the corresponding prodrugs has been observed to be steadily increasing (Fig. 1A). Conversely, mutations within *ethA* are responsible of high resistance levels against ETH, thus limiting the antimycobacterial potency of the drug.

The expression of *ethA* is under the control of a transcriptional regulator, encoded by the neighboring gene *ethR* [6]. Overexpression of *ethR* leads to ETH resistance whereas inactivation of *ethR* results in ETH hypersensitivity [5]. EthR is a member of the TetR/CamR repressor family that shares highly homologous N-terminal DNA-binding domains [7] and its cooperative octamerization on a 55-bp operator located in the *ethA-ethR* intergenic region, leads to *ethA* transcription [6] (Fig. 1A). Thereby, EthR is responsible for the poor activation process of ETH by EthA, contributing to an intrinsic level of resistance to ETH.

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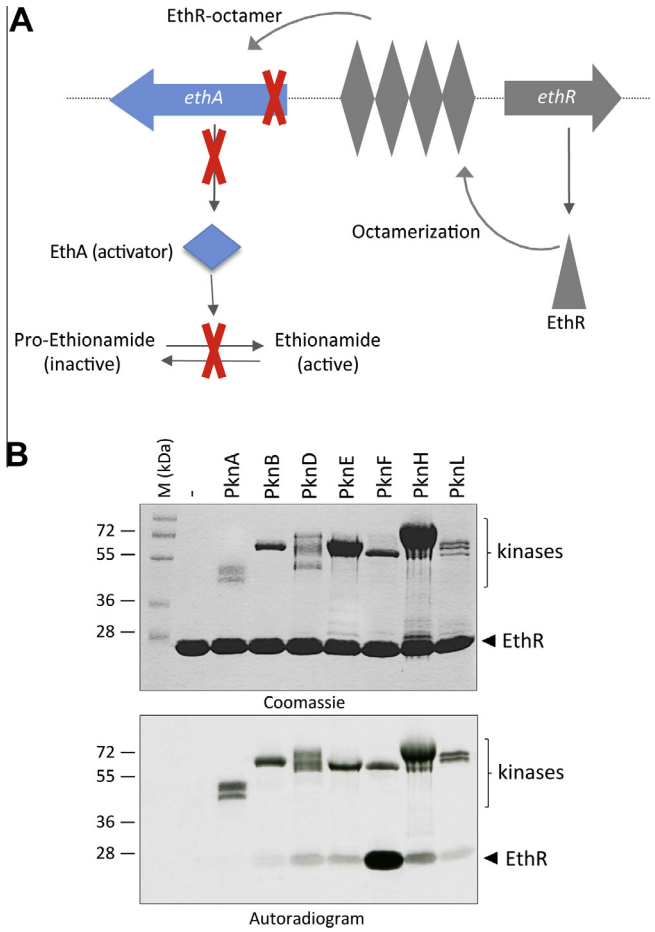

Fig. 1. (A) Schematic representation of EthR regulatory role. (B) *In vitro* phosphorylation of EthR by multiple STPKs. Purified His-tagged EthR was individually incubated with several *M. tb* STPKs purified as GST-tag fusion proteins in the presence of [γ-^{33}P]-ATP. Samples were separated by SDS–PAGE and stained with Coomassie Blue (upper panel) and visualized by autoradiography after overnight exposure to a film (lower panel).

Reversible protein phosphorylation is a key mechanism by which environmental signals are transmitted to cause changes in protein expression or activity in both eukaryotes and prokaryotes. The *M. tb* genome contains eleven Ser/Thr Protein Kinases (STPKs) [8,9] and most are being investigated for their physiological roles and potential application for future drug development to combat TB [10]. This significant number of STPKs suggests that phosphorylation may influence a wide range of biological functions, such as adaptation to various environmental conditions like stress, cell wall synthesis, cell division and pathogenicity [9,11,12]. Consequently, identification and investigation of the biochemical and physiological role of STPK substrates involved in regulating the metabolism of antitubercular drugs may add new insights into our understanding of the complex signalling networks operating in *M. tb* and may open new avenues for future chemotherapeutic developments. As a first step towards this goal, we addressed whether regulation of the activation process of ETH may be influenced by STPK-dependent regulatory mechanisms.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Strains used for cloning and expression of recombinant proteins were *Escherichia coli* 10G and BL21(DE3)Star (Table S1). Both were grown at 37 °C in LB medium with 100 μg/ml ampicillin or 100 μg/ml spectinomycin, when required.

2.2. Cloning, expression, and purification of EthR and mutant proteins

The *ethR* gene (Rv3855) was amplified by PCR using *M. tb* H37Rv chromosomal DNA as a template and primers containing a *Nde*I or a *Bam*HI restriction site listed in Table S2. Amplified products were cloned into the pETPhos vector [13], a variant of pET15b (Novagen) that includes the replacement of the thrombin site coding sequence with a tobacco etch virus (TEV) protease site and with a N-terminal His-tag free of Ser/Thr/Tyr residues, generating pETPhos_*ethR*. Deletion of the first ten *ethR* residues was generated by PCR using pETPhos_*ethR* as a template and primers containing a *Nde*I and *Bam*HI restriction site listed in Table S2, generating pETPhos_*ethR_ΔNterm* plasmid. pETPhos_*ethR* derivatives harbouring either single or multiple Thr to Ala substitutions or multiple Thr to Asp substitutions at T2, T3, S4 and S7 within *ethR* were generated by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). This resulted in pETPhos_*ethR_T2A*, pETPhos_*ethR_T3A*, pETPhos_*ethR_S4A*, pETPhos_*ethR_S7A*, pETPhos_*ethR_T2A/T3A/S4A/S7A* (referred to as pETPhos_*ethR_Ala*) and pETPhos_*ethR_T2D/T3D/S4D/S7D* (referred to as pETPhos_*ethR_Asp*), respectively (Table S1). A duet strategy was used to generate hyper-phosphorylated EthR protein as described previously [14]. Briefly, the *ethR* gene was cloned into the pCDFDuet-1 vector carrying the sequence encoding the PknF kinase domain using primers listed in Table S2, to generate pDuet_*ethR*, subsequently transformed into *E. coli* BL21(DE3)Star. Overexpression and purification of the recombinant proteins were performed as described previously [13].

2.3. In vitro kinase assay

In vitro phosphorylation was performed with 4 μg of EthR or derivatives in 20 μl of buffer P (25 mM Tris–HCl, pH 7.0; 1 mM DTT; 5 mM $MgCl_2$; 1 mM EDTA; 50 mM ATP) with 200 μCi ml^{-1} [γ-^{33}P]-ATP corresponding to 65 nM (PerkinElmerPerkinElmer, 3000 Ci/mmol), and 0.6–4.2 μg of kinase in order to obtain for each specific kinase its optimal autophosphorylation activity for 30 min at 37 °C. Cloning, expression and purification of the seven recombinant GST-tagged STPKs from *M. tb* were described previously [15].

2.4. Mass spectrometry analysis

Purified His-tagged hyper-phosphorylated EthR from the *E. coli* strain carrying pDuet_*ethR* and co-expressing the PknF kinase, was subjected to mass spectrometry without further treatment. Subsequent mass spectrometry analyses were performed as previously reported [14,16].

2.5. Electrophoretic mobility shift assays (EMSA)

The DNA probes for EMSA were generated by PCR using *M. tb* H37Rv chromosomal DNA as a template, which encompasses a 55-bp operator located in the *ethA-ethR* (*ethA-R*) intergenic region, with respective primers pairs listed in Table S2 as already described [6]. The 5′ ends of the double-stranded PCR products were labelled using [γ-^{32}P]-ATP and T4 polynucleotide kinase. A typical assay mixture contained in 20 μl: 10 mM Tris–HCl, pH 7.5; 50 mM NaCl; 1 mM EDTA; 1 mM dithiothreitol (DTT); 0.1 μg of nonspecific competitor (polydI-dC); 5% (v/v) glycerol; radioactive DNA probe (2000 cpm ml^{-1}) and increasing amounts (0, 0.5, 1, 2, 3 μg) of purified EthR. After 30 min of incubation at room temperature, 20 μl of this mixture were loaded onto a native 4% (w/v) polyacrylamide TBE Ready Gel (Bio-Rad) and electrophoresed in 1% TBE (v/v) buffer

for 1 h at 100 V cm^{-1}. Radioactive species were detected by autoradiography using direct exposure to films.

2.6. Immunoblotting analysis

The purified recombinant proteins derived from pETPhos_*ethR*, pDuet_*ethR*, and pDuet_*ethR_Ala* were used for immunoblotting using anti-phosphothreonine or anti-phosphoserine antibodies according to the manufacturer's instructions (Invitrogen) and revealed using secondary antibodies labeled with IRDye 800CW infrared dyes (LiCOR).

2.7. Biacore

Real-time analysis of molecular interactions between EthR wild-type or variants and the *ethA* promoter region was conducted on a BiacoreTM 2000 apparatus (GE Healthcare). A 106-bp fragment overlapping the *ethA-R* intergenic region was obtained using H37Rv chromosomal DNA as template and primers pair O270/O271 listed in Table S2. Immobilization of the DNA was performed on a streptavidin-coupled CM5 Sensor Chip using the standard protocol provided with the Amine Coupling Kit (BiacoreTM). Briefly, streptavidin was injected at 500 ng/ml in 10 mM sodium acetate (pH 3.5) for 12 min at a flow rate of 10 ml/min. The biotinylated DNA (approximately 69 kDa) was injected through each flow cell at 200 ng/ml to reach a 205 Resonance Unit (RU) stable fixation to streptavidin. Integrity and quantity of fixed DNA was controlled with a 120 s injection of 1.6 mM calf histone H1 (Sigma) at 10 ml/min. Binding of EthR to the DNA was carried out at 25 °C in 0.05 M NaH_2PO_4 (pH 7.15), 0.25 M NaCl, 0.002 M $MgCl_2$, 0.05 M KCl and 0.001 M DTT at a flow rate of 20 µl/min. The protein was injected at 0.3 µM (concentration of the monomer) in the running buffer until equilibrium was reached. The Sensor Chip was regenerated by a 60 s injection of 0.03% SDS. Final curves presented are representative of experiments repeated a minimum of three times and were obtained by subtraction of the signal corresponding to a control flow cell functionalized with a biotinylated double stranded 113-bp long irrelevant DNA fragment (+14 to +127 fragment of the *E. coli bla* gene PCR-amplified from pUC18 using primers pair O343/O344 listed in Table S2).

3. Results and discussion

3.1. PknF-mediated phosphorylation of EthR

Several transcriptional regulators have recently been shown to be regulated by Ser/Thr phosphorylation [16,17], prompting us to examine whether EthR might be regulated *via* phosphorylation. Therefore, STPKs of *M. tb* (PknA, PknB, PknD, PknE, PknF, PknH, or PknL) were expressed as GST fusions and purified from *E. coli* as described previously [15]. Recombinant EthR was expressed and purified from *E. coli* BL21(DE3)Star harbouring the pETPhos_*ethR*. Interestingly, when the different STPKs were incubated in the presence of EthR and [γ-^{33}P]-ATP, phosphorylation of EthR was clearly observed with PknF (Fig. 1B). As expected, no radioactive signal was detected when kinases were omitted from the reaction. These results indicate that EthR is a specific substrate and interacts with PknF *in vitro*.

3.2. Identification of the EthR phosphorylation sites

In order to identify the nature and position of the phosphorylation sites, a purified phosphorylated isoform of EthR was prepared using the duet system, allowing to co-express both PknF and EthR, and directly analyzed by mass spectrometry after tryptic digestion, following a method previously reported [14]. The MS/MS spectra not only confirmed the occurrence of phosphate groups, but also identified four phosphorylation sites at Thr2, Thr3, Ser4 and Ser7 (Fig. S1).

Definitive identification and localization of the phosphorylation sites was next achieved by site-directed mutagenesis to introduce either single or quadruple mutations (Ser/Thr to Ala replacements) in EthR, to prevent specific phosphorylation. The different mutants were expressed using the pETPhos constructs, purified as His-tagged proteins and individually analyzed following incubation with [γ-^{33}P]-ATP and PknF. The mixtures were then separated by SDS–PAGE and analyzed by autoradiography. The phosphorylation status was not significantly modified in single mutants (Fig. 2A) whereas complete abrogation of the phosphorylation signal was evidenced in both the quadruple EthR_Ala and the EthR_ΔNterm mutants (lacking the first ten residues). This confirms the proper identification of the sites in the N-terminal region of EthR.

To further address the relevance of *in vitro* phosphorylation, the phosphoablative *ethR* allele was cloned into the pCDFDuet-1 vector [14], which co-expresses PknF. The purified EthR_Ala was assessed for phosphorylation *in vivo* by Western blotting using anti-phosphothreonine or anti-phosphoserine antibodies. First, the specificity of the antibodies for the phosphorylated EthR isoform was demonstrated using the protein purified from *E. coli* co-expressing PknF together with EthR (Fig. 2B) corresponding to pDuet_*ethR*. As anticipated, pDuet_*ethR_Ala*, used as a source to produce the EthR_T2A/T3A/S4A/S7A mutant in conjunction with PknF, failed to react with the antiphosphothreonine or antiphosphoserine antibodies, further confirming that these phosphosites are unique in EthR.

3.3. Localization of the phosphoacceptors

TetR type regulators, including EthR, are characterized by an N-terminus domain responsible for their binding to their DNA operator. Interpro assigns proteins to the TetR family based on PROSITE signature PS01081, PRINTS motif PR00455, and Pfam Hidden Markov Model (HMM) profile PF00440. These signatures emphasize the presence of a well organize Helix-Turn-Helix (HTH) domain often composed of 3 α-helix stretches with α-helix 1 stabilizing α-helices 2 and 3 which are directly involved in the protein-DNA contacts. In most of known TetR type of regulator, a N-terminus stretch of generally unstructured amino acid is predicted to precede the HTH DNA binding domain. In the case of TetR, this region was clearly shown to be important for the binding of the repressor to its DNA operator. Interestingly, the four phosphorylation sites are located in the N-terminal extension of EthR, and could therefore, modify the DNA-binding activity of EthR. To the best of our knowledge, there are so far no reports concerning the possible impact of post-translational modification of this region on the DNA binding properties of members of this family. Unfortunately, in the crystal structure of *M. tb* EthR [18], the first 21 residues are not defined, so are supposedly very mobile. Due to the lack of structural information, the phosphoacceptors could not be defined on the three-dimensional structure and hence their contribution in EthR regulation cannot be predicted. Nevertheless, several recent studies reported that *Corynebacterium glutamicum* OdhI or *M. tb* GarA proteins possess disordered N-terminal extensions which, following phosphorylation by STPK, undergo major conformational changes characterized by the binding of the phosphorylated and ordered N-terminal part of the proteins to their respective C-terminal FHA domain, and consequently inhibiting them [19,20].

Moreover, multiple sequence alignments of EthR orthologs from various mycobacterial species indicated that the four putative phosphorylation sites are conserved in *M. tb* and *Mycobacterium*

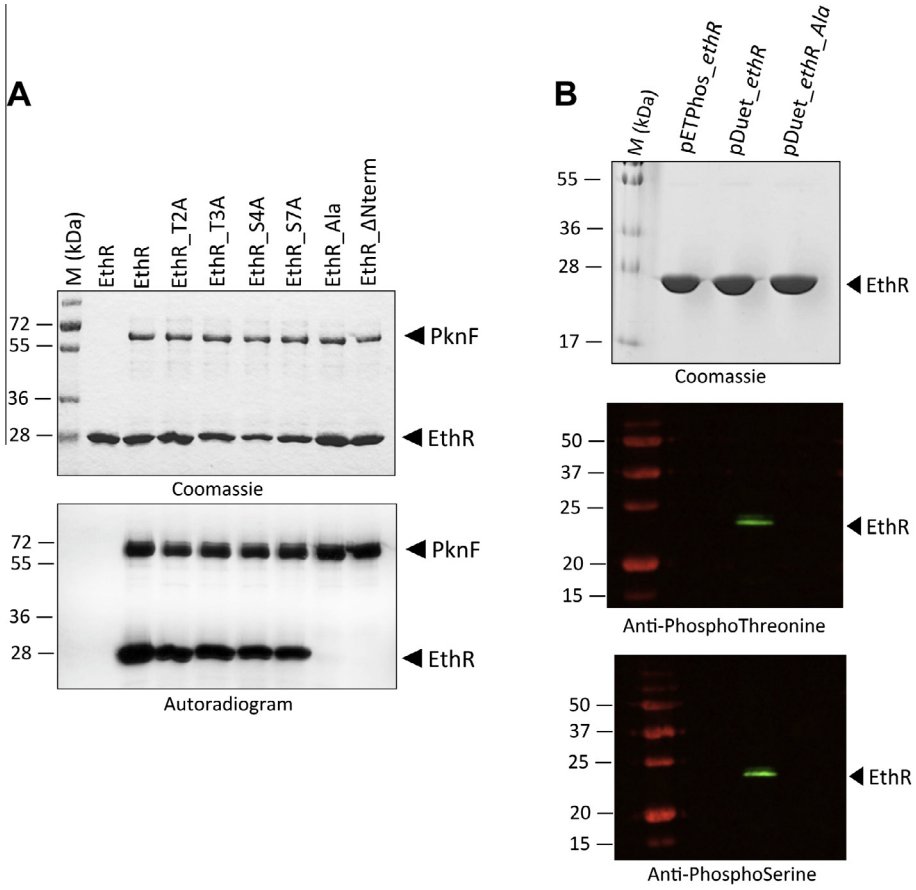

Fig. 2. (A) Phosphorylation of EthR variants with Ala-substituted phosphosites. Purified EthR wild-type (EthR), single mutants (EthR_T2A, EthR_T3A, EthR_S4A, EthR_S7A), EthR phosphoablative mutant (EthR_Ala) and EthR_ΔNterm mutant were incubated with PknF and [γ-^{33}P]-ATP, separated by SDS–PAGE, stained with Coomassie Blue (upper panel) and visualized by autoradiography (lower panel). (B) Phosphorylation status of EthR or EthR_Ala in a PknF co-expressing *E. coli* strain.

bovis, whereas not all the sites are conserved in other orthologs. A full conservation of this potential regulatory segment may be restricted to the *M. tb* complex (Fig. 3A).

3.4. Phosphorylation decreases DNA-EthR affinity

It was previously shown that EthR represses *ethA* transcription by cooperative binding on the *ethA* promoter [6]. In addition, the localization of the phosphorylation sites suggested that the phosphorylation of EthR might influence the DNA-binding activity of this protein. In addition, it was previously shown that EthR represses *ethA* transcription by cooperative binding on the *ethA* promoter [6]. This prompted us to investigate whether phosphorylation of EthR could alter its DNA binding property, by using two complementary approaches. First, we analyzed and compared the ability of the non-phosphorylated (EthR) and the hyper-phosphorylated form (EthR-P) to bind to the *ethA-ethR* intergenic region, known to be a target of EthR [6]. EMSA revealed that the unphosphorylated isoform binds to the *ethA* promoter probe in a dose-dependent manner (Fig. 3B) as reported previously [6]. In contrast, the phosphorylated isoforms (EthR_Asp and EthR-P) showed a dramatic reduction of mobility shift (Fig. 3B), supporting the view that phosphorylation negatively affects the ability of EthR to bind to its DNA target. As anticipated, both the EthR_Ala phosphoablative and the EthR_ΔNterm mutants retained a DNA-binding activity similarly to the non-phosphorylated protein (Fig. 3B).

The second approach was aimed to determine and compare the DNA binding activities of the different EthR variants using surface plasmon resonance (Fig. 3C). The double-stranded target DNA corresponding to the operator of EthR was immobilized onto a CM5 Sensor Chip. The binding activity of the various forms of EthR to the chip was measured by injecting a fix dose of EthR in the BIAcore® at a flow rate of 20 µl/min for 3 min. Our results indicate that both phosphomimetic and phosphorylated EthR proteins, EthR_Asp and EthR-P respectively, have lost between 40% and 50% of their binding activity compare to the wild-type protein. In contrast, EthR_ΔN term has lost only 18% of its activity, similarly to EthR_Ala. Overall, these results are in agreement with EMSA and confirm that, at least *in vitro*, phosphorylation of EthR negatively affects its DNA binding activity.

It is noteworthy that these effects were not due to a tertiary or quaternary structure change due to the introduction of the mutations, as shown by the trypsinolysis kinetic profiles, equivalent for the wild type and for the mutated or phosphorylated EthR proteins (Fig. S2). This was also confirmed by X-ray crystallography demonstrating that the tridimentionnal structure of the protein portion located between residues 21 and 214 is virtually identical in EthR (pdb 1U9N) and in EthR_Asp, as revealed by the 0.3 Å average backbone root-mean-square distance (RMSD) between the two structures (data not shown, Table S3). The absence of electron density corresponding to the first amino acids (1–21), harboring the phosphorylation sites, revealed that this region is either unstructured or highly mobile, while the DNA binding region formed by the HTH domain appears perfectly structured. This confirms that the phosphomimetic amino-acid replacement in the proximal N-terminal portion may impact on the DNA binding affinity of

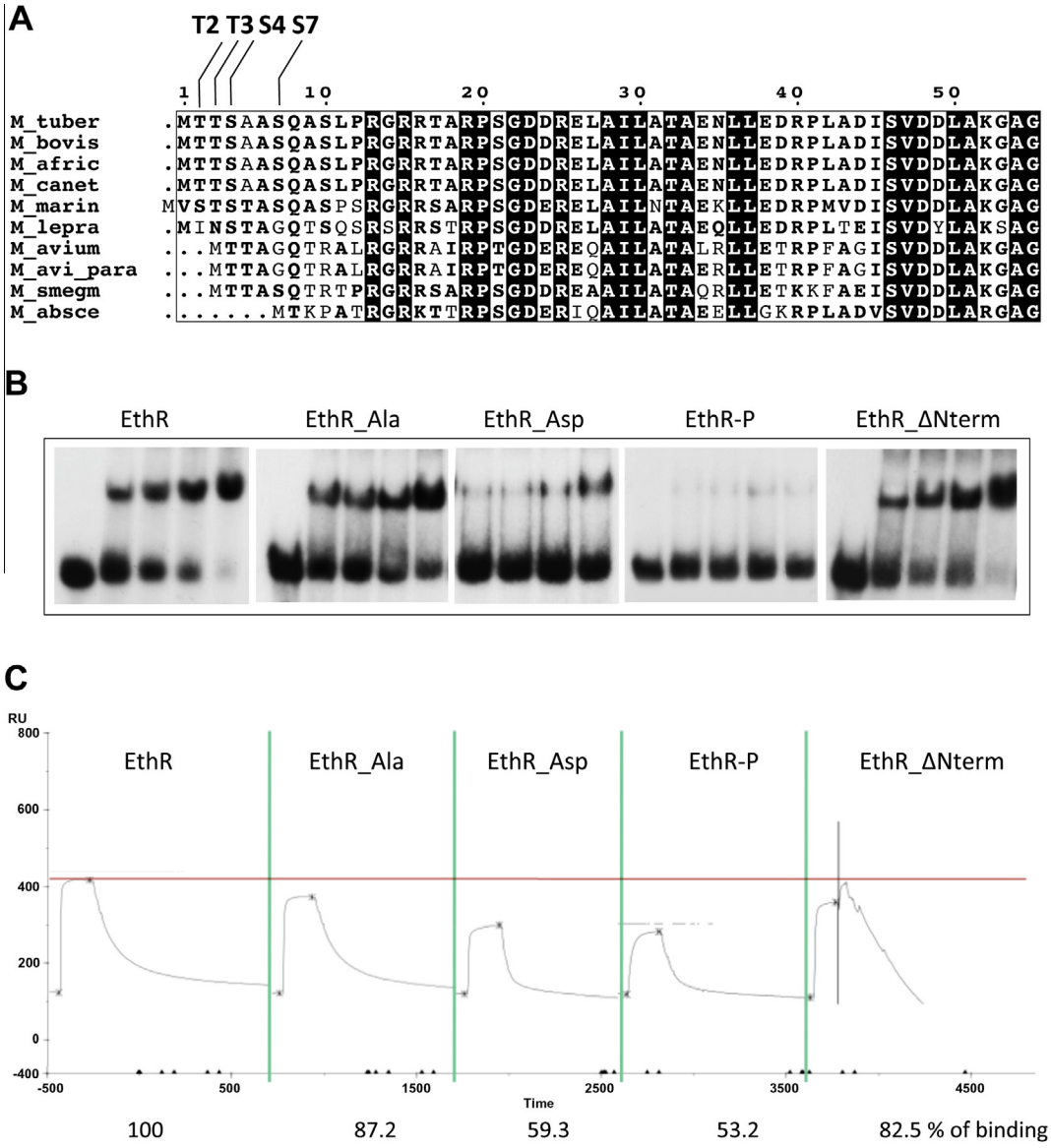

Fig. 3. (A) Multiple sequence alignment of EthR N-terminal region from mycobacteria. Sequence alignment of the *M. tb* EthR homologues in *M. bovis*, *M. africanum*, *M. canetti*, *M. marinum*, *M. leprae*, *M. avium*, *M. avium paratuberculosis*, *M. smegmatis*, and *M. abscessus*. Residues conserved in all species are presented in black boxes. The phosphorylated sites of *M. tb* EthR and their positions are indicated. (B) Phosphorylation-dependent binding of EthR mutants to the upstream region of *ethA* gene. The 191 bp [γ-^{32}]-ATP labeled probe (2000 cpm ml^{-1}) was incubated with an increasing amount of purified His-tagged EthR derivatives corresponding to 0, 0.5, 1, 2, and 3 μg. After incubation, EthR-DNA complexes and free DNA were separated by non-denaturing polyacrylamide gel electrophoresis and subjected to autoradiography. (C) Interaction between EthR-variants and the DNA intergenic region of *ethA-ethR* measured by Surface Plasmon Resonance. Increasing concentrations of protein were injected on a sensor chip functionalized with the DNA region, until saturation of the signal. Percentages refer to the binding capacities of EthR-variants in comparison with EthR taken as the reference (100%).

the protein without affecting the integrity of the DNA-binding domain of the regulator, but most likely through fine-tuned electrostatic influence in this region.

3.5. Phosphorylation increases the BDM41906 inhibitor effect

Transcriptional control by TetR-type regulators is based on the competitive binding to their DNA operator on one hand, and to specific ligands on the other hand. Binding to one of these two substrates precludes binding to the other. Since phosphorylation affects the binding of EthR to its target, it appears important to determine whether this phenomenon may also influence the activity of EthR ligands. In a recent study, a series of 1,2,4-oxadiazole compounds highly potent for the inhibition of the DNA binding properties of EthR were developed [21]. Herein, we compared the DNA binding activity of EthR and EthR-P in the presence of the oxadiazole BDM41906. As demonstrated above, EthR-P shows a reduced affinity for its DNA operator compared to EthR. It was then essential for this experiment to use concentrations of EthR isoforms that gives equivalent SPR response at saturation. Fig. 4A shows that injection of 500 nM EthR or 750 nM EthR-P gave an equivalent plateau-signal at around 80 RU. We next compared the capacity of BDM41906 to inhibit both isoforms of EthR at these relative concentrations. Fig. 4B shows that 300 nM BDM41906 are sufficient to affect the binding capacity of EthR-P (at 750 nM), and 1 mM completely abolished this binding. In contrast, 300 nM BDM41906 failed to inhibit EthR, and significant residual binding activity of EthR was still observed with 1 mM BDM41906. It can be inferred that the efficacy of BDM41906 to inhibit the binding of EthR to its DNA operator is improved when EthR is phosphor- ylated.

In conclusion, we provide here the first evidence of a post-translational modification of the *M. tb* transcriptional repressor

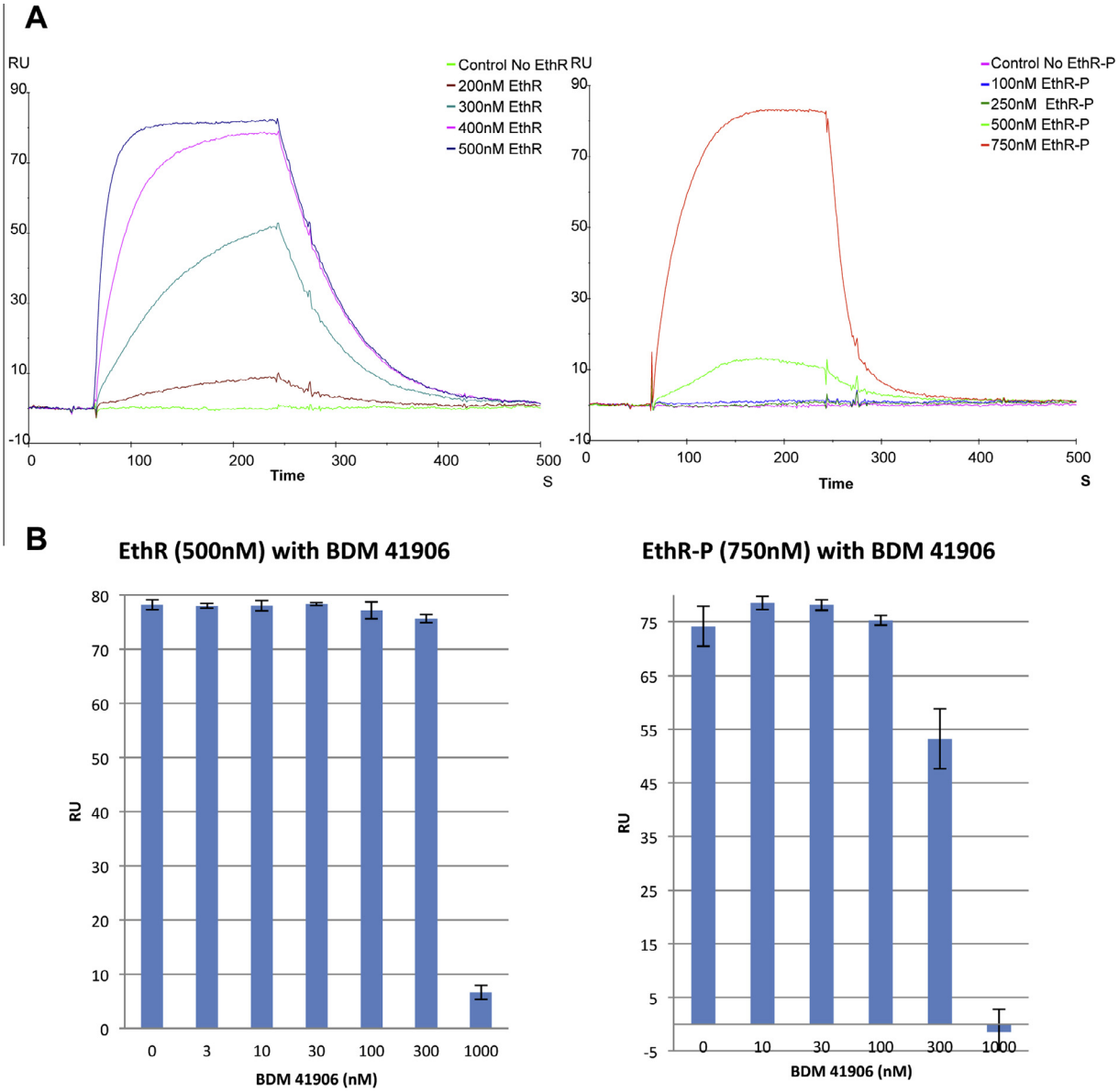

Fig. 4. Impact of the phosphorylation of EthR on its ligand-mediated DNA-binding inhibition. (A) Calibration of the binding activity of EthR and EthR-P on its DNA operator. Increasing concentrations of EthR and EthR-P were used to reach an equivalent response in SPR. (B) Effect of ligand BDM41906 concentration on the DNA binding activity of EthR and EthR-P.

EthR. This phosphorylation process was shown to occur strictly at the extreme N-terminus portion of the protein, known to interact specifically with DNA. Herein, we demonstrated that phosphorylation modifies the DNA binding activity of EthR at a dual level. First, it impairs the affinity of the *apo* form of EthR for its DNA operator, probably by electrostatic interference. Unexpectedly, it also enhances the inhibitory effect of the EthR specific BDM41906 ligand whose binding pocket is structurally independent and distant from the DNA binding site. These results strongly suggest that phosphorylation of EthR may play a fine-tuning regulatory role in controlling the expression of *ethA* in *M. tb*. Although this remains to be established experimentally, it is anticipated that STPK-mediated phosphorylation of EthR may impact the susceptibility/resistance profiles of clinical strains against ETH.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.074.

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