The mycobacterial SenX3–RegX3 two-component system consists of the SenX3 sensor histidine kinase and its cognate RegX3 response regulator. This system is a phosphorelay-based regulatory system involved in sensing environmental Pi levels and induction of genes required for Pi acquisition under Pi-limiting conditions. Here we demonstrate that overexpression of the kinase domain of Mycobacterium tuberculosis PknB (PknB-KD\(^{\text{MtB}}\)) inhibits the transcriptional activity of RegX3 of both \textit{M. tuberculosis} and \textit{Mycobacterium smegmatis} (RegX3\(^{\text{MtB}}\) and RegX3\(^{\text{Ms}}\), respectively). Mass spectrometry results, along with those of \textit{in vitro} phosphorylation and complementation analyses, revealed that PknB kinase activity inhibits the transcriptional activity of RegX3\(^{\text{MtB}}\) through phosphorylation events at Thr-100, Thr-191, and Thr-217. Electrophoretic mobility shift assays disclosed that phosphorylation of Thr-191 and Thr-217 abolishes the DNA-binding ability of RegX3\(^{\text{MtB}}\) and that Thr-100 phosphorylation likely prevents RegX3\(^{\text{MtB}}\) from being activated through conformational changes induced by SenX3-mediated phosphorylation. We propose that the convergence of the PknB and SenX3-RegX3 signaling pathways might enable mycobacteria to integrate environmental Pi signals with the cellular replication state to adjust gene expression in response to Pi availability.

The adaptation and survival of \textit{Mycobacterium tuberculosis} (\textit{MtB})\(^2\) under hostile host environments require exquisite regulation of gene expression in response to changing environments (1, 2). Phosphorelay through proteins is a major mechanism by which environmental signals are transmitted to elicit appropriate adaptive responses (3, 4). Two-component systems (TCSs), which constitute the primary regulatory systems utilizing phosphorelay in prokaryotes, consist of sensory histidine kinases (HKs) and their cognate response regulators (RRs). In response to a specific ligand or environmental signal, an HK is autophosphorylated on a conserved histidine residue. The phosphoryl group is subsequently transferred from the HK to an aspartate residue conserved in the N-terminal receiver domain of the partner RR to activate the RR effector domain. Because most RRs contain the helix–turn–helix DNA-binding motif as an effector domain, the activated RRs normally serve as active transcription factors to regulate gene expression (5–10). \textit{MtB} possesses 11 paired TCSs and five orphan RRs (11–13). Of 11 paired TCSs, the SenX3-RegX3 TCS has been suggested to play an important role mainly in the adaptation of mycobacteria to Pi-limiting conditions (14, 15). Other roles have also been suggested with regard to virulence (16–19), persister formation (20), sensing of diatomic gases (O\(_2\), NO, and CO) (21), and membrane vesicle biogenesis (22). When the level of Pi falls below a certain threshold value in the environment, the SenX3 HK is activated to phosphorylate the RegX3 RR. The activated RegX3 RR positively regulates expression of many genes, including those implicated in the acquisition of Pi under Pi-limiting conditions, such as the \textit{pstSCAB} operon encoding a high-affinity phosphate-specific ABC transporter (Pst) and the \textit{phoA} gene encoding alkaline phosphatase (14, 23, 24). Although the mechanism by which the SenX3 HK senses Pi availability has not yet been fully elucidated, SenX3 appears not to sense Pi levels by itself. The kinase/phosphatase activity of SenX3 has been suggested to be regulated by the functional state of the Pst uptake system with the assistance of other auxiliary proteins (PhoU in \textit{Mycobacterium smegmatis} and PhoY in \textit{MtB}). This suggestion was made on the basis of the finding that inactivation of either Pst transporter or PhoU (PhoY) by mutation brings about constitutive activation of the SenX3–RegX3 TCS and constitutive expression of the RegX3 regulon independent of Pi availability (20, 24, 25). According to the suggested model, the Pst transporter generates an inhibitory signal under Pi-replete conditions that is transmitted by PhoU (PhoY) to the SenX3 HK. The inhibitory signal shifts the equilibrium of SenX3 activity from the kinase mode to the phosphatase mode, resulting in repression of the RegX3 regulon. The PhoU and PhoY proteins have been suggested to function as adaptor proteins between the Pst transporter and SenX3 through their interactions between the Per–ARNT–Sim (PAS) domain of SenX3 and the PstB ATPase subunit of the Pst system (26–28).
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Under P_limiting conditions, the inhibitory signal is diminished or removed, which renders SenX3 active to phosphorylate the RegX3 RR. Intriguingly, a recent study demonstrated that the SenX3 HK is a hemoprotein with a b-type heme accommodated in its PAS domain and that oxidation of the heme from a ferrous to a ferric state enhances SenX3 autokinase activity, whereas binding of NO or CO to the heme leads to inhibition of autokinase activity (21). This finding suggests the possibility that SenX3 can also serve as a sensor kinase for diatomic gases such as O_2, NO, and CO.

Because a eukaryotic-like Ser/Thr protein kinase (STPK) was first characterized in Myxococcus xanthus (29), many STPKs have been identified and characterized in bacteria, and increasing attention has been paid to the importance of STPKs in prokaryotic signaling pathways related to stress responses, development, virulence, regulation of central metabolism, as well as cell division and morphology (30–33). In contrast to HKs that have a strict substrate specificity, STPKs can normally phosphorylate multiple substrates, resulting in pleiotropic responses from a single signal in the signal transduction pathway (31). The genomes of Mtb and M. smegmatis contain 11 and 13 STPK genes, respectively (11, 34). The membrane-bound PknB is one of 11 STPKs in Mtb and is conserved in all mycobacteria (34). It has been shown to be essential for both Mtb and M. smegmatis (35–37) and to be involved in cell elongation, division, peptidoglycan biosynthesis, and regulation of oxygen-dependent cell replication (38–40).

Cross-talk between STPKs and TCSs have been reported to take place in several bacterial regulatory systems (41–50). Recently, we demonstrated that the purified kinase domain of Mtb PknB (PknB-KD_Mtb) robustly phosphorylated six RR s (RegX3, NarL, KdpE, TsrR, DosR/DevR, and MtrA) among 11 paired RRs of Mtb and that overexpression of PknB-KD_Mtb in M. smegmatis led to significant inhibition of DosR (DevR) transcriptional activity by phosphorylating Thr-180 located in the helix–turn–helix motif of DosR (DevR). As an extension of our previous study, here we report that overexpression of PknB-KD_Mtb significantly inhibits the transcriptional activity of RegX3 of Mtb (RegX3_Mtb) by phosphorylating Thr-100, Thr-191, and Thr-217.

Results

Protein–protein interactions between RegX3 and PknB

We demonstrated previously that purified RegX3_Mtb was strongly phosphorylated by purified PknB-KD_Mtb in vitro (50). Because phosphorylation of RegX3_Mtb by PknB_Mtb seems to require protein–protein interactions between them, we determined protein interactions between RegX3_Mtb and PknB_Mtb by yeast two-hybrid (Y2H) assay. PknE (one of 11 STPKs in Mtb) and SenX3 of Mtb (SenX3_Mtb) were included in the experiment as negative and positive controls, respectively (Fig. 1). For the Y2H assay, the regX3_Mtb gene was cloned into the prey vector pGADT7 linker, whereas the gene portions encoding the KDs of PknB_Mtb, PknE_Mtb, and SenX3_Mtb were cloned into the bait vector pGBK7T7. Consistent with a previous report (51), the yeast strain coexpressing RegX3_Mtb and SenX3_Mtb grew well in the absence of histidine (−His), indicating protein–protein interactions between RegX3_Mtb and its cognate SenX3_Mtb HK. Coexpression of PknB_Mtb with RegX3_Mtb led to growth of yeast in the absence of histidine whereas coexpression of PknE_Mtb with RegX3_Mtb did not. As expected, the yeast strains expressing either SenX3_Mtb or PknB_Mtb alone without expression of RegX3_Mtb did not grow on −His medium. Taken together, the Y2H results suggest a possible interaction of RegX3_Mtb with PknB_Mtb.

Overexpression of PknB-KD_Mtb inhibits RegX3 transcriptional activity in vivo

To assess the effect of RegX3 phosphorylation by PknB on the transcriptional activity of RegX3, we overexpressed PknB-KD_Mtb in M. smegmatis and examined the transcriptional activity of RegX3 by determining the expression level of the phoA gene, which encodes alkaline phosphatase and is regulated by the SenX3–RegX3 TCS. Because RegX3_Mtb (MSMEG_0937) shares 93% identity with RegX3_Mtb at the amino acid level (Fig. 1), we employed M. smegmatis overexpressing PknB-KD_Mtb in place of Mtb to examine the overexpression effect of PknB-KD_Mtb on RegX3 transcriptional activity. The gene portion encoding PknB-KD_Mtb was overexpressed from an acetamide-inducible promoter on pMHpKnB that is a derivative of the pMH201 integration vector. Before determining the effect of PknB-KD_Mtb overexpression on the transcriptional activity of RegX3, we examined the overexpression effects of PknB-KD_Mtb on aerobic growth of M. smegmatis under P_replete and P_limiting conditions (Fig. S2). The M. smegmatis control strain with pMH201 grew under P_replete conditions approximately three times faster than the same strain under P_limiting conditions. Although growth of the M. smegmatis strain was severely inhibited by PknB-KD_Mtb expressed from pMHpKnB under both P_replete and P_limiting conditions, the optical density at 600 nm (A_{600}) and colony-forming unit values of the cultures of the M. smegmatis strain with pMHpKnB were increased over time, indicating that overexpression of PknB-KD_Mtb is not lethal to M. smegmatis but significantly inhibits its growth under conditions tested in this study. The expression level of phoA in M. smegmatis was determined using a phoA::lacZ transcriptional fusion plasmid, pNCPchoA. As shown in Fig. 2A, phoA

![Figure 1. Determination of protein–protein interactions between RegX3_Mtb and PknB_Mtb in the Y2H assay. The regX3_Mtb gene was cloned into the pGADT7 linker (encoding the GAL4 activation domain), resulting in pPLRegX3. The gene portions encoding the KDs of Mtb PknB, PknE, and SenX3 were cloned into pGBK7T7 (encoding the GAL4 DNA-binding domain). Yeast strains cotransformed with both pPLRegX3 and pGBK7T7 derivatives were used for the Y2H assay. To distinguish false positive interactions, the empty pGADT7/ linker vector was introduced into the yeast strains containing the pGBK7T7 derivatives in place of pPLRegX3, and the resulting yeast strains were used as negative controls. All yeast strains were spotted onto SD−/Leu−/−Trp plates (+His) and histidine-deficient SD−/Leu−/−Trp/−His plates (−His).](image-url)
expression was strongly induced in the *M. smegmatis* strain with both pNCphoA and pMH201 grown under Pi-limiting conditions compared with the same strain grown under Pi-replete conditions. However, the *M. smegmatis* strain harboring both pNCphoA and pMHPknB showed a significantly decreased level of *phoA* expression under Pi-limiting conditions relative to the control strain with pNCphoA and pMH201 grown under Pi-replete conditions. As expected, virtually no β-gal activity was detected in the *M. smegmatis* strains with the pNC empty vector. The overexpression effect of PknB-KDMtb on expression of *phoA* was also examined at the transcript level using a His tag antibody. *, *p < 0.01.

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**Figure 2. Effect of PknB-KDMtb overexpression on phoA expression in *M. smegmatis***. A, pMH201-derived pMHPknB was employed for overexpression of PknB-KDMtb in *M. smegmatis*. The overexpression effect of PknB-KDMtb on RegX3 transcriptional activity was examined by determining the expression level of *phoA* in *M. smegmatis* strains harboring an *phoA-lacZ* transcriptional fusion plasmid (pNCphoA) and pMHPknB. The expression level of *phoA* was measured by determining β-gal activity in the *M. smegmatis* strains. As controls, the *M. smegmatis* strain with both pNCphoA and pMH201 as well as the *M. smegmatis* strains harboring pNC in place of pNCphoA were included in the experiment. The *M. smegmatis* strains were grown aerobically to an *A*₅₆₀ of 0.4–0.5 in glucose–MOPS medium under Pi-replete or Pi-limiting conditions in the presence of 0.2% (w/v) of acetamide. The expression levels of *phoA*, *pstS*, and *ahpC* in the WT strains of *M. smegmatis* containing pMHPknB or pMH201 were also determined by RT-PCR. RT-PCR of the 16S RNA gene was performed to ensure that same amounts of total RNA were used for RT-PCR. *B*, the expression level of *phoA* was measured by determining β-gal activity in the *M. smegmatis* strains with pMHPknB and pNCphoA. The *M. smegmatis* strains were grown aerobically to an *A*₅₆₀ of 0.4–0.5 in glucose–MOPS medium under Pi-limiting conditions in the presence of increasing concentrations of acetamide (Ace) ranging from 0.001 to 0.2% (w/v). The same strain cultured in the absence of acetamide was included as a control. All values are the averages of the results from either three (A) or five (B) independent determinations, respectively. The error bars indicate standard deviations. Western blot analysis was performed for detection of the expressed His₆-tagged PknB-KDMtb. Cell-free crude extracts (either 20 μg (A) or 30 μg (B)) were separated on SDS-PAGE, followed by Western blot analysis with a His tag antibody.
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In vivo using a ΔregX3 conditional mutant strain of M. smegmatis. The ΔregX3 conditional mutant of M. smegmatis with deletion of its own regX3Ms gene carries the acetamide-inducible regX3Mtb gene on the chromosomal DNA. Therefore, the mutant was expected to exhibit mutant phenotypes in the absence of acetamide, whereas RegX3Mtb was expected to be overexpressed in the mutant in the presence of acetamide. The transcriptional activity of RegX3Mtb was examined by determining the expression level of phoA by RT-PCR and real-time qPCR (Fig. 3). Because the pAZI9018b-derived pAZIP-knB, which was used for overexpression of PknB-KD^Mtb, carries the gene encoding PknB-KD^Mtb which is under the control of an IPTG-inducible promoter, addition of IPTG to the growth medium led to overexpression of PknB-KD^Mtb. When grown under P_l-limiting conditions in the presence of acetamide, the ΔregX3 conditional mutant strain with the empty pAZI9018b vector exhibited a high expression level of phoA expression relative to the mutant strain with the empty pAZI9018b vector grown under the same conditions. When grown under P_l-limiting conditions without acetamide, phoA expression was almost abolished in both the mutant strains because of the lack of RegX3Mtb expression. In contrast to the strong inhibition (~90%) of RegX3Ms transcriptional activity by overexpression of PknB-KD^Mtb in the WT strain of M. smegmatis (Fig. 2A), overexpression of PknB-KD^Mtb in the ΔregX3 conditional mutant of M. smegmatis reduced the transcriptional activity of RegX3Mtb to a lesser extent, which might be attributable to the overexpression effect of RegX3Mtb in the ΔregX3 conditional mutant strain grown in the presence of acetamide. Considering the results shown in Figs. 2 and 3, we suggest that the increased kinase activity of PknB^K40M inhibits the transcriptional activity of both RegX3Mtb and RegX3Ms.

Inhibition of RegX3 transcriptional activity by PknB-KD^Mtb overexpression results from the kinase activity of PknB-KD^Mtb

It is conceivable that the inhibition of RegX3Ms transcriptional activity in M. smegmatis by PknB-KD^Mtb overexpression is a consequence of the sequestration of RegX3Ms by the overexpressed PknB-KD^Mtb through their protein−protein interactions rather than by increased PknB kinase activity. To examine this possibility, we determined the overexpression effect of inactive PknB-KD^Mtb with the K40M mutation (37, 38) on phoA expression in M. smegmatis (Fig. 4). The mutant form of PknB-KD^Mtb was overexpressed using pMHPhKnB_K40M, which has the same construct as pMHPhKnB except for the K40M mutation in pknB. The transcriptional activity of RegX3Ms was determined by measuring the promoter activity of phoA in M. smegmatis strains harboring pNCphoA. When grown under P_l-limiting conditions, the M. smegmatis strain with both pMH201 and pNCphoA exhibited a high expression level of
phoA, whereas overexpression of PknB-KDMtb almost abolished phoA expression in the M. smegmatis strain with pMHP-KnB and pNCphoA, which is consistent with the result shown in Fig. 2A. However, overexpression of the K40M mutant form of PknB-KD

In M. smegmatis grown under Pi-limiting conditions led to merely a slight decrease in phoA expression compared with the control strain with pMH201 grown under the same conditions. Western blot analysis revealed that the similar amounts of the WT and mutant forms of PknB-KD were synthesized in the M. smegmatis strains carrying pMHPknB and pMHPknBK40M, respectively. Taken together, these results unequivocally suggest that the inhibitory effect of PknB-KD overexpression on phoA expression was exerted by the increased PknB kinase activity in M. smegmatis.

Phosphorylation of Thr-100, Thr-191, and Thr-217 leads to inactivation of RegX3 transcriptional activity

To identify the amino acid residues of RegX3Mtb that are phosphorylated by PknB-KD

Figure 5. Effect of T29E, T100E, T151E, T191E, T193E, and T217E mutations on phosphorylation of RegX3Mtb by PknB-KD

0.05 nmol of PknB-KD

was mixed with 0.1 nmol of the WT and mutant forms (T29E, T100E, T151E, T191E, T193E, and T217E) of RegX3Mtb in 20 μl of reaction buffer (20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, and 10 mM MnCl₂). The phosphorylation reactions were started by adding 100 μM cold ATP and 1,000 Ci/mole [γ-32P]ATP, incubated at 30 °C, and terminated at the indicated time points. Thereafter, proteins were resolved by SDS-PAGE. Phosphorylated RegX3Mtb proteins were detected by autoradiography. The duplicated reaction mixtures without addition of ATP were subjected to SDS-PAGE, and the gels were stained by CBB. As loading controls, the stained gels are presented below the autoradiograms to compare the amounts of the purified WT and mutant forms of RegX3Mtb used in the assay. M, molecular weight marker lanes.

Figure 5. Effect of T29E, T100E, T151E, T191E, T193E, and T217E mutations on phosphorylation of RegX3Mtb by PknB-KD

0.05 nmol of PknB-KD

was mixed with 0.1 nmol of the WT and mutant forms (T29E, T100E, T151E, T191E, T193E, and T217E) of RegX3Mtb in 20 μl of reaction buffer (20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, and 10 mM MnCl₂). The phosphorylation reactions were started by adding 100 μM cold ATP and 1,000 Ci/mole [γ-32P]ATP, incubated at 30 °C, and terminated at the indicated time points. Thereafter, proteins were resolved by SDS-PAGE. Phosphorylated RegX3Mtb proteins were detected by autoradiography. The duplicated reaction mixtures without addition of ATP were subjected to SDS-PAGE, and the gels were stained by CBB. As loading controls, the stained gels are presented below the autoradiograms to compare the amounts of the purified WT and mutant forms of RegX3Mtb used in the assay. M, molecular weight marker lanes.
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Figure 6. Effect of T29E, T100E, T151E, T191E, T193E, and T217E mutations on the transcriptional activity of RegX3<sup>Mtb</sup> in vivo. The ΔregX3 conditional mutant strains of <i>M. smegmatis</i> with pNBV1RegX3WT, pNBV1RegX3T29E, pNBV1RegX3T100E, pNBV1RegX3T151E, pNBV1RegX3T191E, pNBV1RegX3T193E, or pNBV1RegX3T217E were used for complementation analysis. The complementation test was performed by determining the expression level of <i>phoA</i> gene expressed in the strains grown under Pi-limiting conditions in the absence (−Ace) or presence of (+Ace) of acetamide, was included in the experiment. RT-PCR for 16S rRNA was conducted to ensure that the same amounts of total RNA were employed for RT-PCR. Protein levels of the WT and mutant forms of RegX3<sup>Mtb</sup> were determined by Western blot analysis with a His tag antibody, and the result is shown below the RT-PCR result.

To determine whether the DNA-binding affinity of RegX3<sup>Mtb</sup> is affected by phosphorylation of Thr-100, Thr-191, and Thr-217, we examined the binding of the corresponding phosphomimetic mutant forms (T100E, T191E, and T217E) of RegX3<sup>Mtb</sup> to DNA fragments containing the upstream regulatory region of <i>phoA</i> by EMSA. As shown in Fig. 7A, more DNA fragments were shifted with increasing concentrations of WT and T100E RegX3<sup>Mtb</sup>. In contrast, almost no band shift was observed for T191E RegX3<sup>Mtb</sup>, and only a marginal band shift occurred for T217E RegX3<sup>Mtb</sup> at high concentrations of the protein. To directly ascertain whether phosphorylation of RegX3<sup>Mtb</sup> influences its DNA binding affinity, we performed EMSA using the RegX3<sup>Mtb</sup> protein subjected to the phosphorylation reactions of RegX3<sup>Mtb</sup> with WT or mutant forms of PknB-KD<sup>Mtb</sup>. When the phosphorylation reaction of RegX3<sup>Mtb</sup> was done without PknB-KD<sup>Mtb</sup>, the DNA fragments with the <i>phoA</i> upstream region were shifted in the EMSA (Fig. 7B). Phosphorylation of RegX3<sup>Mtb</sup> by the active PknB-KD<sup>Mtb</sup> abolished the binding of RegX3<sup>Mtb</sup> to the DNA fragments, whereas RegX3<sup>Mtb</sup> subjected to the phosphorylation reaction with the inactive K40M PknB-KD<sup>Mtb</sup> retained its DNA-binding ability. The WT or mutant form of PknB-KD<sup>Mtb</sup> alone without RegX3<sup>Mtb</sup> did not bind to the DNA fragments. Taken together, the results in Fig. 7 suggest that phosphorylation of RegX3<sup>Mtb</sup> on Thr-191 and Thr-217 by PknB<sup>Mtb</sup> significantly reduces the binding affinity of RegX3<sup>Mtb</sup> for its target DNA sequence whereas phosphorylation of RegX3<sup>Mtb</sup> on Thr-100 by PknB<sup>Mtb</sup> does not.

Discussion

The pknB gene forms an operon with pknA, phpA, rodA, pstP (encoding a metal-dependent Ser/Thr protein phosphatase), and two genes coding for forkhead-associated domain–containing proteins. This genetic locus was found to be conserved near the replication origin in the chromosomes of Acti-
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Figure 7. Binding of the purified WT, T100E, T191E, and T217E mutant forms of RegX3Mtb to the phoA upstream region and the effect of RegX3Mtb phosphorylation by PknB-KD\textsuperscript{Mtb} on the DNA binding affinity of RegX3Mtb. A, 100 fmol each of 123-bp DNA fragments containing a RegX3-binding site upstream of phoA (specific DNA) and 60-bp DNA fragments without a RegX3-binding site (control DNA) were used in an EMSA with various concentrations of purified WT, T100E, T191E, or T217E RegX3\textsuperscript{Mtb}. RegX3\textsuperscript{Mtb}–DNA reaction mixtures were incubated for 20 min at 25 °C. The RegX3\textsuperscript{Mtb}–DNA reaction mixtures were subjected to native PAGE (6% (w/v) acrylamide gel). The gels for the EMSA were stained with SYBR Green staining solution after electrophoresis. The concentrations of WT, T100E, T191E, and T217E RegX3\textsuperscript{Mtb} used in the EMSA are shown above the lanes. B, to phosphorylate RegX3\textsuperscript{Mtb}, 120 pmol of RegX3\textsuperscript{Mtb} was incubated with 60 pmol of WT PknB-KD\textsuperscript{Mtb} in 16 μl of reaction buffer (20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 10 mM MgCl\textsubscript{2}, and 10 mM MnCl\textsubscript{2}) containing 100 μM ATP for 1 h at 30 °C. As a negative control, inactive K40M PknB-KD\textsuperscript{Mtb} was employed instead of WT PknB-KD\textsuperscript{Mtb}. 4 μl of the DNA mixture containing 200 fmol each of specific and control DNA fragments was added to 16 μl of the above phosphorylation reaction mixtures and incubated for 20 min at 25 °C. 10 μl of the DNA-binding reaction mixtures were used for the EMSA, and 10 μl of the remaining reaction mixtures were subjected to SDS-PAGE to see the amounts of RegX3\textsuperscript{Mtb} and PknB-KD\textsuperscript{Mtb} used in the EMSA. The CBB-stained gel used for SDS-PAGE is presented below the EMSA result.

nobacteria, including mycobacteria (34, 55, 56). PknA and PknB have been suggested to be essential STPKs for mycobacteria and to be implicated in signal transduction regulating cell wall synthesis and cell shape (38, 39, 57–60). PknB is composed of the N-terminal KD and the C-terminal extracytoplasmic domain consisting of four penicillin-binding protein and Ser/Thr kinase–associated (PASTA) repeats (61). These kinds of PASTA repeats are also found in PknB-like STPKs of Gram-positive bacteria, including Actinobacteria (62, 63). Several lines of evidence for phosphorylation of RRs by STPKs in mycobacteria have been reported. In 2010, phosphorylation of the DosR (DevR) RR by PknB was first reported in Mtb (43). PknH has demonstrated to phosphorylate DosR (DevR) on Thr-198 and Thr-205 to enhance DosR (DevR) transcriptional activity (44). Recently, we revealed that phosphorylated PknB-KD\textsuperscript{Mtb} strongly phosphorylated six RRs (RegX3, NarL, KdpE, TrcR, DosR/DevR, and MtrA) of Mtb in vitro (45). Based on this finding, we began to study signal convergence between PknB and TCSs in mycobacteria and demonstrated that overexpression of PknB-KD\textsuperscript{Mtb} in M. smegmatis resulted in a significant inhibition of DosR (DevR) transcriptional activity by phosphorylating Thr-180 of DosR (DevR) (50). We chose the SenX3–RegX3 TCS to further study the cross-talk between PknB and TCSs in mycobacteria because well-known reporter genes such as phoA and pstS, which are under strict control of the SenX3–RegX3 TCS, were available to determine the transcriptional activity of RegX3, and the activation condition of the SenX3–RegX3 TCS (Pi-limited condition) was established (14).

We observed that the transcriptional activity of RegX3\textsuperscript{Ms} and RegX3\textsuperscript{Mtb} was reduced with increased expression and activity of PknB-KD\textsuperscript{Mtb} (Figs. 2–4). Furthermore, MS/MS analysis following the in vitro phosphorylation reaction with purified RegX3\textsuperscript{Mtb} and PknB-KD\textsuperscript{Mtb} led to detection of six phosphorylation sites (Thr-29, Thr-100, Thr-151, Thr-191, Thr-193, and Thr-217) on RegX3\textsuperscript{Mtb} (Fig. S4). Because our MS/MS results did not show the phosphorylation extent of the identified Thr residues, we performed an in vitro phosphorylation assay with nonphosphorylatable Thr-to-Glu mutant forms of RegX3\textsuperscript{Mtb} and purified PknB-KD\textsuperscript{Mtb} (Fig. 5), which revealed that Thr-100 and Thr-191 of RegX3\textsuperscript{Mtb} are the major sites of phosphorylation, whereas Thr-217 is the minor phosphorylation site. In addition, the phosphomimetic T100E, T191E, and Thr217E mutant forms of RegX3\textsuperscript{Mtb} were shown to lose transcriptional activity, as judged by the complementation result (Fig. 6). Altogether, these results indicate that PknB kinase activity inhibits the transcriptional activity of RegX3\textsuperscript{Mtb} mainly through phosphorylation of Thr-100 and Thr-191 (Thr-98 and Thr-189 in the case of RegX3\textsuperscript{Ms}), which are located in the receiver domain and the DNA-binding helix–turn–helix motif of RegX3, respectively (Fig. S6). From the location of Thr-191 on RegX3\textsuperscript{Mtb}, it was assumed that phosphorylation of Thr-191 might inhibit the transcriptional activity of RegX3\textsuperscript{Mtb} by perturbing DNA binding of RegX3\textsuperscript{Mtb}. Indeed, our EMSA revealed that the phosphomimetic T191E mutation in RegX3\textsuperscript{Mtb} led to abolishment of the DNA-binding ability of RegX3\textsuperscript{Mtb} (Fig. 7). The location of Thr-217 near the helix–turn–helix motif accounts for abolishment of the transcriptional activity and DNA-binding ability of T217E RegX3\textsuperscript{Mtb}. Despite the lack of its transcriptional activity, the T100E mutant form of RegX3\textsuperscript{Mtb} has been shown to retain the same degree of DNA-binding ability as the WT form of RegX3\textsuperscript{Mtb} (Fig. 7), indicating that the lack of transcriptional activity of T100E RegX3\textsuperscript{Mtb} is not the result of its inability to bind DNA. The RR of the TCS contains six well-conserved residues (three acidic amino acids, one Thr/Ser, one Tyr/Phe, and one Lys) that are functionally important for phosphorylation of the RR and activation of the effector domain through phosphorylation-induced conformational changes (64–66). In the case of RegX3\textsuperscript{Mtb}, six residues correspond to Glu-8, Asp-9, Asp-52, Thr-79, Tyr-98, and Lys-101. The location of Thr-100 between Tyr-98 and Lys-101 implies that the
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T100E mutation (and, therefore, phosphorylation of Thr-100 by PknB) is likely to make it unlikely that RegX3\(^{Mtb}\) is activated through phosphorylation-induced conformational changes.

As mentioned above, six RR s of the 11 paired TCSs found in \(Mtb\) were shown to be robustly phosphorylated by PknB-KD\(^{Mtb}\). Four (RegX3, KdpE, TrcR, and MtrA) of the six RR s are structurally related to the OmpR family of RR s. The Thr residues corresponding to Thr-191 and Thr-217 of RegX3\(^{Mtb}\) are conserved in the four RR s (RegX3, KdpE, TrcR, and MtrA), whereas the Thr-100–corresponding residues are conserved in RegX3, KdpE, and TrcR but not in MtrA (Fig. 6). Given the strong phosphorylation of KdpE, TrcR, and MtrA by PknB-KD\(^{Mtb}\) like a PknB and the conservation of at least two of three Thr residues corresponding to Thr-100, Thr-191, and Thr-217 of RegX3\(^{Mtb}\), as well as the good conservation of amino acid sequences around the Thr residues in the regulatory domain of RegX3\(^{Mtb}\), it is possible that PknB might inhibit the transcriptional activity of KdpE, TrcR, and MtrA as well.

The signal convergence between TCSs and STPKs was also observed in several Gram-positive bacteria besides mycobacteria. In most cases, an STPK phosphorylates an RR to change the transcriptional activity of the RR. The WalR RR of the WalKR TCS in Bacillus subtilis has been reported to be phosphorylated by PrkC (a PknB homolog) both \textit{in vitro} and \textit{in vivo} (49). WalR shares a relatively high sequence similarity (48% identity and 81% similarity) with RegX3\(^{Mtb}\) over the whole protein sequence (Fig. 57). Phosphorylation of WalR on Thr-101, which corresponds to Thr-100 of RegX3\(^{Mtb}\), has been shown to cause changes in WalR transcriptional activity. It is noteworthy that theThr residues within the Thr residues are very well conserved in WalR and RegX3 (Fig. 57), which reinforces phosphorylation of RegX3\(^{Mtb}\) on Thr-100 by PknB. In group A and group B streptococci, phosphorylation of the CovR RR on Thr-65 by a PknB-like STPK (Stk or Stk1) has been shown to inhibit acetyl phosphate–dependent phosphorylation of CovR (41, 48). The RR06 and RitR RR s of Streptococcus pneumoniae have been shown to be phosphorylated by a PknB-like STPK, Stk\(^{P}\) (42, 45). The GraR RR of the GraSR TCS of Staphylococcus aureus has been demonstrated to be phosphorylated on Thr-128, Thr-130, and Thr-149 by Stk1, which resulted in an increase in the DNA-binding affinity of GraR (46). Phosphorylation of the VraR RR of the S. aureus VraSR TCS on Thr-106 and Thr-119 in the receiver domain and on Thr-175 and Thr-178 within the DNA-binding domain by Stk1 has been shown to reduce its DNA binding affinity (47). The observation that several RR s are phosphorylated by multiple kinases, including cognate HK s and STPKs, suggests that signaling through TCSs in bacteria could be more complex than anticipated.

The PASTA repeats of PknB have been demonstrated to serve as binding modules for muropeptides of peptidoglycan, and binding of muropeptides to the PASTA repeats has been suggested to activate PknB kinase activity (67). PknB has been demonstrated to be localized to the septa and poles of the dividing bacterial cell, where higher local concentrations of muropeptides are available because of high rates of peptidoglycan turnover and synthesis (54, 68). These facts, in conjunction with the observation that the cellular abundance of PknB is significantly reduced in the nonreplicating \(Mtb\) cells relative to that in actively dividing cells (69), suggest that the kinase activity of PknB might be much higher in actively dividing mycobacteria than in nonreplicating mycobacteria, which raises the possibility that PknB might play a role in the regulation of gene expression and cellular processes as a sensor kinase that coordinates the replication state with the regulation of intracellular metabolism and gene expression in mycobacteria.

From this viewpoint, we propose a model for the dual control of RegX3 transcriptional activity by SenX3 and PknB. The major sensory kinase regulating the transcriptional activity of RegX3 in response to P\(_i\) availability is the SenX3 HK. Under favorable growth conditions with sufficient P\(_i\), the RegX3 RR is not activated by the SenX3 HK, and the high activity of PknB might further inhibit the residual transcriptional activity of RegX3 by functioning as an auxiliary “switch” or “safety lock” to minimize leaky expression of the RegX3 regulon. Under P\(_i\)-limiting conditions, where RegX3 is activated by SenX3, reduced PknB activity because of inhibited replication of mycobacterial cells might mitigate the inhibitory effect of PknB on RegX3, enabling full activation of RegX3 transcriptional activity.

In conclusion, we found that overexpression of PknB-KD\(^{Mtb}\) inhibits the transcriptional activity of RegX3\(^{Mtb}\) by phosphorylating Thr-100, Thr-191, and Thr-217 (Thr-98, Thr-189, and Thr-215 for RegX3\(^{Ms}\)). Convergence of the PknB and SenX3-RegX3 signaling pathways might enable mycobacteria to integrate two different signals, the environmental P\(_i\) level and the replication state, to regulate gene expression in response to changing P\(_i\) availability.

Experimental procedures

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table S1. Escherichia coli strains were grown in Luria–Bertani medium at 37 °C. \(M.\) smegmatis strains were grown in Middlebrook 7H9 medium (Difco, Detroit, MI) supplemented with 0.2% (w/v) glucose as a carbon source and 0.02% (v/v) Tween 80 as an anticlumping agent at 37 °C. For Pi-limiting and replete growth conditions, MOPS minimal medium (25 mM MOPS (pH 7.2), 25 mM KCl, 10 mM Na\(_2\)SO\(_4\), 20 mM NH\(_4\)Cl, 10 \(\mu\)M FeCl\(_3\), 2 mM MgSO\(_4\), and 0.1 mM CaCl\(_2\)) supplemented with 50 \(\mu\)M and 10 mM K\(_2\)HPO\(_4\), respectively, were used. \(M.\) smegmatis strains were grown aerobically on a gyratory shaker (200 rpm) to an \(A_{600}\) of 0.4–0.5. Ampicillin (100 \(\mu\)g/ml for \(E.\) coli), kanamycin (50 \(\mu\)g/ml for \(E.\) coli and 15 or 30 \(\mu\)g/ml for \(M.\) smegmatis), and hygromycin (200 \(\mu\)g/ml for \(E.\) coli and 50 \(\mu\)g/ml for \(M.\) smegmatis) were added to the growth medium when required. Overexpression of the genes encoding PknB-KD\(^{Mtb}\) and RegX3\(^{Mtb}\) from pMH201-derived plasmids was induced by addition of acetamide to the growth medium to a final concentration of 0.2% (w/v) unless specific concentrations of acetamide are stated. Construction of the plasmids and a \(\Delta regX3\) conditional mutant of \(M.\) smegmatis is described in the supporting information.

Determination of colony-forming units

Samples (1 ml) were collected from \(M.\) smegmatis cultures at the indicated time points. The collected samples were homoge-
neously suspended by passing them ten times through a 25-gauge needle to break up cell clumps. 200 µl of the samples appropriately diluted with MOPS minimal medium were plated on glucose–MOPS agar plates supplemented with either 10 mM or 50 µM K2HPO4 and 0.2% (w/v) acetamide. The bacterial colonies growing on agar plates were counted after 72-h incubation at 37 °C.

DNA manipulation and electroporation

Recombinant DNA manipulations were conducted in accordance with standard protocols and the manufacturer’s instructions (70). Transformation of M. smegmatis with plasmids was carried out by electroporation as described previously (71).

Site-directed mutagenesis

To introduce point mutations into the genes encoding RegX3MTb and PknB-KDMTb, PCR-based mutagenesis was conducted using the QuikChange site-directed mutagenesis procedure (Stratagene, La Jolla, CA). Synthetic oligonucleotides 33 bases long and containing a mutated codon in the middle of their sequences were used to mutagenize the original codons (Table S2). Mutations were verified by DNA sequencing.

β-Galactosidase activity assay and determination of protein concentration

β-Galactosidase activity was measured spectrophotometrically as described elsewhere (72). A Bio-Rad protein assay kit was used to determine the protein concentration.

RT-PCR and real-time qPCR

RNA isolation from M. smegmatis strains, preparation of complementary DNA, RT-PCR, and real-time qPCR were conducted as described previously (73). The primers used for complementary DNA synthesis, RT-PCR, and real-time qPCR are listed in Table S2.

Protein purification

E. coli strains overexpressing RegX3MTb or PknB-KDMTb were grown aerobically at 37 °C in Luria–Bertani medium containing 100 µg/ml ampicillin (for E. coli strains containing the pIT7–7 derivatives) or 50 µg/ml kanamycin (for the E. coli strain carrying pETpknBhis) to an A600 of 0.4–0.6. Expression of the regX3MTb and pknBMTb genes was induced by addition of IPTG to a final concentration of 0.5 mM, and then cells were grown further for 4 h at 30 °C. The harvested cells from 200 ml of culture were resuspended in 5 ml of buffer A (20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 10 mM MgCl2, and 10 mM MnCl2). Cell-free crude extracts were obtained by centrifugation twice at 23,708 × g for 15 min. 500 µl of the 50% (w/v) slurry (bed volume, 250 µl) of Ni-Septarose high-performance resin (GE Healthcare) was packed into a column. After equilibration of the resin with 10 bed volumes of buffer A, cell-free crude extracts were loaded into the column. The resin was washed with 60 bed volumes of buffer A containing 5 mM imidazole and 60 bed volumes of buffer A containing 10 mM imidazole, and then His6-tagged RegX3MTb and His6-tagged PknB-KDMTb were finally eluted with 10 bed volumes of buffer A containing 250 mM imidazole. The eluted proteins were desalted using a PD-10 desalting column (GE Healthcare) equilibrated with the appropriate buffer.

Western blot analysis

To determine the amount of expressed His6-tagged RegX3MTb and PknB-KDMTb in cells, Western blot analysis was performed as described previously (74). To detect His6-tagged proteins, mouse monoclonal IgG against His-3 (Santa Cruz Biotechnology, Santa Cruz, CA; sc8036) was used at a 1:2,000 dilution. Alkaline phosphatase–conjugated anti-mouse IgG produced in rabbits (Sigma, A4312) was used at a 1:10,000 dilution for detection of the primary antibody.

Analysis of in vivo protein–protein interactions

Saccharomyces cerevisiae AH109 strains cotransformed with both the pGADT7linker and pGBKT7 derivatives were grown in synthetic defined dropout (SD) medium (Clontech, Palo Alto, CA) lacking leucine and tryptophan (SD/−Leu/−Trp). The overnight cultures were diluted with distilled water to an A600 of 0.6 and spotted onto both solid SD/−Leu/−Trp plates and histidine-deficient SD/−Leu/−Trp/−His plates for a spotting assay. These plates were incubated at 30 °C for 3–5 days.

In vitro kinase assay

Purified PknB-KDMTb was mixed with purified WT or mutant forms of RegX3MTb in 20 µl of reaction buffer (20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 10 mM MgCl2, and 10 mM MnCl2). Reactions were started by adding 100 µM cold ATP and 1,000 Ci/mmol [γ-32P]ATP and incubated at 30 °C. The reactions were terminated at the indicated time points by adding 5 µl of gel-loading buffer (250 mM Tris-Cl (pH 6.8), 50% (w/v) glycerol, 500 mM DTT, 10% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 0.5% (w/v) bromphenol blue) containing 100 mM EDTA. Proteins were resolved by SDS-PAGE. The gels were dried and exposed to films at room temperature overnight.

LC-MS/MS analysis for phosphorylated RegX3MTb

Phosphorylation reactions of 0.4 nmol of WT RegX3MTb were conducted in the presence of 0.4 nmol of PknB-KDMTb in 84 µl of reaction buffer (20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 10 mM MgCl2, 10 mM MnCl2, and 100 µM ATP) for 1 h at 30 °C. The reactions were terminated by adding 30 µl of gel-loading buffer containing 100 mM EDTA. The proteins were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (CBB), and the RegX3MTb bands were excised. The excised protein bands from SDS-PAGE gels were cut into small pieces, washed three times with 200 µl of HPLC-grade water, and destained with 200 µl of 1:1 (v:v) mixture of acetonitrile and ammonium bicarbonate (100 mM (pH 8.0)). The gel pieces were dehydrated for 5 min with 500 µl of 100% acetonitrile and incubated in a solution of 10 mM DTT in 100 mM ammonium bicarbonate (50 µl) for 30 min at 56 °C, followed by 55 mM iodoacetamide in 100 mM ammonium bicarbonate (50 µl) for 20 min at room temperature in the dark. Thereafter, the gel pieces were dehydrated again with 100% acetonitrile and rehydrated in a solution of 13 ng/µl of sequencing-grade modified trypsin (Promega, Madi-
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...son, WI) in 10 mM ammonium bicarbonate. The digestion was completed overnight at 37 °C. Peptides were extracted by incubating the gel pieces in a 1:2 (v:v) mixture of 5% formic acid and acetonitrile, and the solution was dried by vacuum centrifugation.

Peptide samples were reconstituted in 7 μl of 0.1% formic acid and injected from an auto sampler into a reverse-phase C18 column (20 cm × 75 μm inner diameter, 3 μm, 300 Å, packed in-house; Dr. Maisch GmbH) on an Eksigent multidimensional liquid chromatography system at a flow rate of 300 nl/min. Before use, the column was equilibrated with 95% mobile phase A (0.1% formic acid in H2O) and 5% mobile phase B (0.1% formic acid in acetonitrile). The peptides were eluted with a linear gradient from 10%–35% B over 100 min, followed by washing with 70% B and re-equilibration with 5% B at a flow rate of 300 nl/min with a total run time of 130 min. The HPLC system was connected to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA) operated in data-dependent acquisition mode. Survey full-scan MS spectra (m/z 400–2,000) were acquired in the Orbitrap with a resolution of 60,000. Source ionization parameters were as follows: spray voltage, 1.9 kV; capillary temperature, 275 °C. The MS/MS spectra of the 10 most intense ions from the MS1 scan with a charge state of 1 or more were acquired in the ion trap with the following options: isolation width, 2.0 m/z; normalized collision energy, 45%; dynamic exclusion, 60 s.

The acquired MS/MS spectra were subjected to a search against the in-house database (containing RegX3MtB and PknB-KDMtB sequences with a common contaminant database) using the SEQUEST HT software in Proteome Discoverer 2.2 (Thermo Fisher Scientific). Two missed trypsin cleavages were allowed, and the peptide mass tolerances for MS/MS and MS were set to ±0.6 Da and ±10 ppm, respectively. Other parameters used for the SEQUEST HT searches included fixed modification of carbamidomethylation at cysteine (57.02 Da), variable modification of oxidation at methionines (+15.99 Da), and phosphorylation at serine, threonine, or tyrosine (+79.97 Da). ptmRS was run in PhosphoRS mode to localize the phosphorylation site. A probability of 75% or higher was considered to confidently indicate a phosphorylation site.

**EMSA**

123-bp DNA fragments including a RegX3-binding site upstream of the phoA gene were used in the assay (specific DNA). The DNA fragments were generated by PCR using the primer set F/phoA_ESMA_123 (5'-AGTCAACGTGTCCGTCTCGAGCGGCTCTGTG-3') and R/phoA_ESMA_123 (5'-AGTCCGAATTCTGGAGCTCAGTAAAGC-3'). pNCphoA as a template. 60-bp control DNA fragments without the RegX3-binding site were amplified by PCR using pUC19 as a template (control DNA) and the primers pUC19_ESMA_F (5'-CCTCTAGAGTGCGACAGCG-3') and pUC19_ESMA_R (5'-AGGAAACAGCTATGACCATG-3'). Purified RegX3MtB proteins were incubated with 100 fmol each of specific and control DNA in buffer (20 mM MOPS (pH 8.0) containing 150 mM KCl) in a final volume of 10 μl for 20 min at 25 °C. To examine the effect of RegX3MtB phosphorylation by PknB-KDMtB on DNA binding, phosphorylation reactions were performed in 10 μl of kinase buffer (20 mM Tris-Cl (pH 7.5), 25 mM NaCl, 10 mM MgCl2, and 20 mM MnCl2) and 100 μM ATP containing 60 pmol of RegX3MtB and 30 pmol of the WT or mutant form (K40M) of PknB-KDMtB for 30 min at 30 °C, and then 100 fmol each of the specific and control DNA were added to the phosphorylation reaction mixtures. The binding reaction mixtures were incubated for 20 min at 25 °C. After addition of 2 μl of 6× loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, and 40% (w/v) sucrose), the mixtures were subjected to non-denaturing PAGE (6% (w/v) acrylamide) using 0.5× TBE buffer (41.5 mM Tris borate and 0.5 mM EDTA (pH 8.3)) at 50 V/cm for 1 h 50 min at 4 °C. The gels were stained with SYBR Green staining solution (Invitrogen) for 30 min. Bands were visualized using an UV illuminator.

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