Regulation of the SigH stress response regulon by an essential protein kinase in *Mycobacterium tuberculosis*

Sang Tae Park*, Choong-Min Kang†, and Robert N. Husson*‡

*Division of Infectious Diseases, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115; and †Department of Biological Science, Wayne State University, Detroit, MI 48202

Edited by John J. Mekalanos, Harvard Medical School, Boston, MA, and approved July 2, 2008 (received for review February 4, 2008)

SigH is a key regulator of an extensive transcriptional network that responds to oxidative, nitrosative, and heat stresses in *Mycobacterium tuberculosis*, and this sigma factor is required for virulence in animal models of infection. SigH is negatively regulated by RshA, its cognate anti-sigma factor, which functions as a stress sensor and redox switch. While RshA provides a direct mechanism for sensing stress and activating transcription, bacteria use several types of signal transduction systems to sense the external environment. *M. tuberculosis* encodes several serine-threonine protein kinase signaling molecules, 2 of which, PknA and PknB, are essential and have been shown to regulate cell morphology and cell wall synthesis. In this work, we demonstrate that SigH and RshA are phosphorylated *in vitro* and *in vivo* by PknB. We show that phosphorylation of RshA, but not SigH, interferes with the interaction of these 2 proteins *in vitro*. Consistent with this finding, negative regulation of SigH activity by RshA *in vivo* is partially relieved in strains in which *pknB* is over-expressed, resulting in increased resistance to oxidative stress. These findings demonstrate an interaction between the signaling pathways mediated by PknB and the stress response regulon controlled by SigH. The intersection of these apparently discrete regulatory systems provides a mechanism by which limited activation of the SigH-dependent stress response in *M. tuberculosis* can be achieved. Coordination of the PknB and SigH regulatory pathways through phosphorylation of RshA may lead to adaptive responses that are important in the pathogenesis of *M. tuberculosis* infection.

anti-sigma factor | sigma factor | transcription regulation | phosphorylation

SigH, an alternative sigma factor of *Mycobacterium tuberculosis* and other mycobacterial species, is a central regulator of the response to oxidative, nitrosative, and heat stresses. SigH directly regulates both effectors of the response to these stresses and additional transcription regulators that control expression of a broad range of stress response genes (1–4). The SigH-dependent activation of this extensive stress response regulon is critical for *M. tuberculosis* virulence, as a *sigH* mutant is highly attenuated in the mouse model of infection (5).

SigH activity is regulated at the transcriptional level via autoregulation of the *sigH* promoter, and posttranslationally via interaction with its cognate anti-sigma factor, RshA. This protein is a member of the Zinc-associated anti-sigma (ZAS) family, several members of which, including RshA, have been shown to function as redox switch proteins (3, 6–8). The interaction of RshA with SigH is disrupted under oxidizing conditions, allowing SigH to associate with core RNA polymerase and activate transcription of stress response genes and additional transcription regulators. The autoregulation of the *sigH* promoter results in rapid, strong induction of the SigH regulon following oxidative stress, which is maintained until redox homeostasis is reestablished and the positive transcriptional feedback loop is interrupted by binding of SigH by RshA (3).

While RshA functions as both a sensor of oxidative stress and a mechanism for transducing this stress to activate SigH-dependent transcription, *M. tuberculosis*, like other bacteria, also relies on a range of signaling systems to sense the external environment and transduce these signals into adaptive physiology. In addition to several typical bacterial 2-component signal transduction systems, the *M. tuberculosis* genome encodes 11 eukaryotic-like serine/threonine protein kinases (STPK's) (9, 10). Two of these, PknA and PknB, are coexpressed and function in the regulation of cell morphology and cell wall synthesis (11–13). The genes encoding PknA and PknB are highly transcribed during exponential growth and exhibit a marked decrease in expression upon entry into stationary phase (11).

In our ongoing work to identify substrates of *M. tuberculosis* PknB, we observed that SigH was phosphorylated *in vivo*. Independently, a recent report demonstrated that several putative *M. tuberculosis* anti- and anti-anti-sigma factor proteins, including RshA, can be phosphorylated by one or more *M. tuberculosis* STPKs (14). In that work PknB and PknE, but not PknA or PknK, targeted RshA *in vitro*. Whether RshA is phosphorylated *in vivo*, and if so what the functional effects of this phosphorylation might be, however, was not determined. Based on these observations we investigated the role of phosphorylation in the SigH-RshA stress response transcription regulatory system.

Here we demonstrate that PknB phosphorylates both SigH and RshA, *in vivo*. We further show that although phosphorylation of SigH does not affect the SigH-RshA interaction, phosphorylation of RshA results in decreased binding of SigH by RshA. In a *pknB* overexpression background, we demonstrate decreased inhibition of SigH function by RshA, consistent with the decreased binding of SigH by phospho-RshA seen *in vitro*. Phosphorylation of RshA by PknB provides a means to modulate the primary mechanism by which SigH activity is regulated through its interaction with RshA.

Results

***In Vivo* and *in Vitro* Phosphorylation of SigH and Identification of Phosphorylation Sites.** To search for proteins phosphorylated by PknB, we made an *M. bovis* bacillus Calmette–Guérin strain in which a copy of *M. tuberculosis pknB* is expressed under the control of the inducible acetamidase promoter (15, 16). A combination of 2 dimensional gel electrophoresis (2D GE), immunoblot analysis with a phospho-threonine (phospho-T)-specific antibody and mass spectrometry were used to identify

Author contributions: S.T.P., C.-M.K., and R.N.H. designed research; S.T.P. and C.-M.K. performed research; S.T.P. and C.-M.K. analyzed data; and S.T.P. and R.N.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

‡To whom correspondence should be addressed. E-mail: robert.husson@childrens.harvard.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0801143105/DCSupplemental.

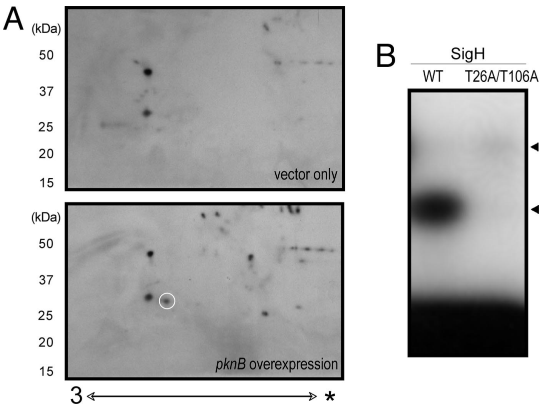

Fig. 1. *In vivo* and *in vitro* phosphorylation of SigH. (*A*) Total proteins of *M. bovis* bacillus Calmette-Guérin strains harboring pMH94 vector only or pMH94-*pknB* were prepared from mid-log phase cultures after 24 h of induction and separated by 2D GE. Protein was immunoblotted with a phospho-T-specific antibody. The circle indicates the spot identified as SigH protein. The asterisk at the right edge of the pI range indicates that a non-linear pH 3–10 IEF strip was used and the pI at the right side of the portion of the blot shown cannot be accurately estimated. (*B*) Recombinant SigH and SigH-T26A/T106A proteins were incubated with GST-PknB in kinase buffer containing 1 μCi of [γ-^{32}P]ATP at 25 °C for 30 min. The proteins were resolved by SDS/PAGE, followed by autoradiography. The upper arrowhead indicates the position of PknB and the lower arrowhead indicates the position of SigH.

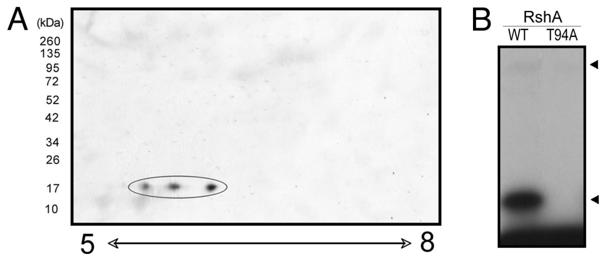

Fig. 2. *In vivo* and *in vitro* phosphorylation of RshA. (*A*) RshA-FLAG was expressed in *M. tuberculosis* H37Rv; total proteins were extracted and separated by 2D GE, followed by immunoblotting with an anti-FLAG antibody. The ovals indicate signals corresponding to RshA-FLAG proteins. (*B*) Recombinant RshA and RshA-T94A proteins were incubated with GST-PknB in kinase buffer containing 1 μCi of [γ-^{32}P]ATP at 25 °C for 30 min. The proteins were resolved by SDS/PAGE, followed by autoradiography. The upper arrowhead indicates the position of PknB and the lower arrowhead indicates the position of RshA.

proteins that showed increased phosphorylation in the *pknB* overexpression strain compared to *M. bovis* bacillus Calmette-Guérin containing the vector only. Following induction, several specific spots were found in immunoblots from *pknB*-overexpressing cells (Fig. 1*A*). When analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) one of these spots was identified as SigH. One peptide from this protein was identified as having 1 or 2 phosphorylated residues: threonine (Thr) at residue 106 and/or Thr at residue 110 [supporting information (SI) Fig. S1]. These residues fall between region 2 and region 4 of SigH in a region that is thought to function as an unstructured linker domain (17). They are not within the part of SigH that corresponds to the amino-terminal domain of the SigH orthologue SigR that has been shown to be involved in the interaction of SigR with its cognate anti-sigma factor RsrA (18).

To confirm that SigH is phosphorylated in *M. tuberculosis* containing native levels of PknB, we made a strain in which *M. tuberculosis sigH* fused to the FLAG epitope sequence at the carboxyl-terminus was expressed under the control of the inducible acetamidase promoter (15, 16). Total protein was extracted from this *sigH*-FLAG-expressing strain, separated by 2D GE, immunoblotted with anti-FLAG antibody, stripped, and then reprobed with phospho-T specific antibody. A cluster of spots of slightly different pI but identical migration in the SDS/PAGE dimension were found in the anti-FLAG blot image. A similar group of spots were detected in the same region in the phospho-T-specific antibody blot images, at positions consistent with the predicted mass and pI of SigH-FLAG (25.2 kDa, pI = 4.82) (Fig. S2). This pattern of adjacent spots differing slightly in pI is strongly suggestive of differences in the extent of phosphorylation (0, 1, or 2 phosphates) of SigH.

To further investigate the phosphorylated residue(s) in SigH, we performed *in vitro* phosphorylation with recombinant SigH protein and PknB-GST that were purified as previously described (2, 11). Phosphorylated SigH was separated by SDS/PAGE, digested with trypsin, and analyzed by LC-MS/MS. Two phosphorylated peptides corresponding to SigH were identified, and Thr at residue 26 and 106 were identified as the phosphorylated residues (data not shown). To determine whether additional sites might be phosphorylated, *in vitro* phosphorylation was performed using native recombinant SigH and SigH in which Ala was substituted for Thr at position 26 and 106 (T26A/T106A). In parallel experiments using equal amounts of native SigH and SigH-T26A/T106A, the native protein was robustly phosphorylated, whereas the T26A/T106A form of SigH showed no evidence of phosphorylation (Fig. 1*B*). Taken together, the *in vitro* and *in vivo* phosphorylation data, strongly support the identification of SigH is an *in vivo* target of PknB in *M. tuberculosis*, and indicate that it is phosphorylated on one and possibly 2 Thr residues.

***In Vivo* and *in Vitro* Phosphorylation of RshA and Identification of Phosphorylation Sites.** To determine whether RshA is phosphorylated *in vivo* in *M. tuberculosis*, we made an *M. tuberculosis* strain in which *M. tuberculosis rshA* fused to the FLAG epitope tag sequence was expressed under the control of the inducible acetamidase promoter (15, 16). Total protein was extracted from this *rshA*-FLAG-expressing strain, separated by 2D GE using a linear pI range 5–8 isoelectric focusing (IEF) strip, and immunoblotted with anti-FLAG antibody. As shown in Fig. 2*A*, 3 antibody-reactive spots of the same size but slightly different pI were detected. The location of these spots is consistent with the size and pI of RshA-FLAG (12.3 kDa, pI = 5.52). The region containing these spots was excised from 3 gels, digested with trypsin and analyzed by LC-MS/MS. Five peptides corresponding to RshA were identified, including one phosphorylated peptide in which Thr-94 was identified as the phosphorylated residue (Fig. S3).

To further investigate the phosphorylated residue(s) in RshA, we performed *in vitro* phosphorylation with recombinant native RshA-6X-His tagged protein and PknB-GST. Phosphorylated RshA was digested with trypsin and analyzed by LC-MS/MS. One phosphorylated residue of RshA was identified (data not shown), which was the same residue (Thr-94) that was found to be phosphorylated *in vivo*. To determine whether additional sites might be phosphorylated, *in vitro* phosphorylation was performed using native recombinant RshA and RshA in which Ala was substituted for Thr at position 94 (T94A). In parallel experiments using equal amounts of native RshA and RshA-T94A, the native protein was strongly phosphorylated, whereas the T94A form showed no evidence of phosphorylation (Fig. 2*B*). These data indicate that T94 is the only residue in RshA that is phosphorylated by PknB. This site is very near the carboxyl terminus of this 101-residue protein and is adjacent to helix 4 of the recently defined anti-sigma domain, which is critical for sigma-anti-sigma binding and is conserved across a broad range of anti-sigma factors (19). This phosphoacceptor residue is

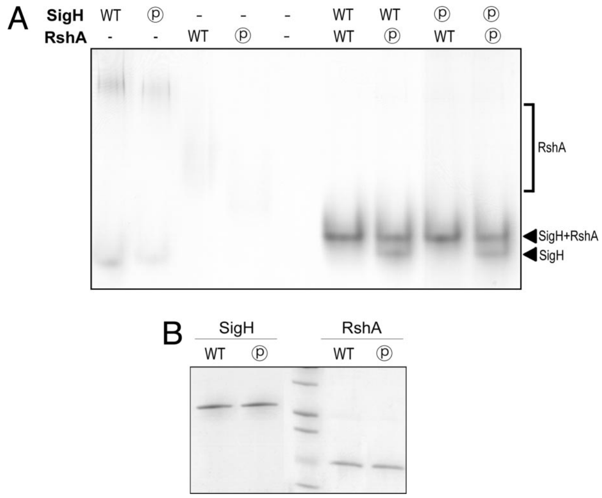

Fig. 3. Native gel binding analysis of RshA and SigH. (*A*) Phosphorylated or non-phosphorylated recombinant SigH and RshA were mixed together in a 1:1 molar ratio in the presence of 1 mM THP and incubated for 60 min at 30 °C. Protein mixtures were resolved by native-PAGE. RshA runs as a smear under these conditions. (*B*) SDS/PAGE gel showing that identical amounts of phosphorylated and non-phosphorylated SigH and RshA were used in these experiments.

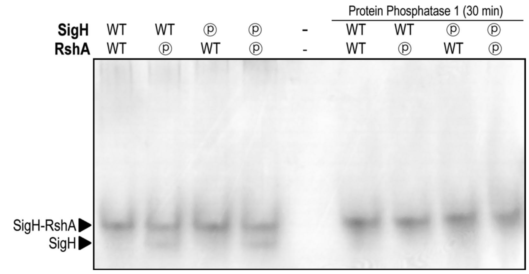

Fig. 4. Effect of phosphatase treatment on the interaction of SigH and phospho-RshA. SigH and RshA were purified, phosphorylated *in vitro* by GST-PknB, and mixed together in a 1:1 molar ratio in the presence of 1 mM THP. These mixtures were divided into 2 fractions, one of which was incubated with serine/threonine phosphatase for 30 min at 30 °C. All protein mixtures were resolved by native-PAGE.

broadly conserved among strains of *M. tuberculosis* and among other mycobacterial species. It is less widely conserved in other Actinomycetes that have SigH homologues, in contrast to PknB which is conserved throughout the group. These observations suggest that phosphorylation of RshA may be a conserved regulatory mechanism among the mycobacteria, but that it is not limited to the pathogenic species.

Effect of Phosphorylation on the Interaction between SigH and RshA. To examine the effect of phosphorylation on the SigH-RshA interaction *in vitro*, these proteins were purified, phosphorylated by PknB, and protein complex formation was analyzed by native gel electrophoresis. *M. tuberculosis* SigH and RshA proteins were prepared as previously described (3) and incubated with PknB-GST or kinase-inactive PknBK40M-GST in kinase reaction buffer (11). Phosphorylated and non-phosphorylated forms of SigH were purified from the pass-through fraction following incubation of the kinase reaction with glutathione-Sepharose (which retained PknB-GST or PknBK40M-GST). Phosphorylated and non-phosphorylated forms of 6X-His-RshA were purified by nickel affinity chromatography (Novagen). Phosphorylation by native PknB-GST and the absence of phosphorylation by PknBK40M-GST were confirmed in parallel reactions using [^{32}]ATP (data not shown).

Purified SigH and RshA were incubated together in a 1:1 molar ratio in the presence of the reducing agent THP (Tris(hydroxypropyl)phosphine) and subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE) as described in Materials and Methods. As shown in Fig. 3, in the absence of phosphorylation, SigH and RshA interact to form a complex, as previously described (3). Phosphorylation of SigH did not have a visible effect on this interaction, with a single band corresponding to the SigH-RshA complex visible in the gel. When phosphorylated RshA was incubated with either unmodified or phosphorylated SigH, however, a less intense band corresponding to the SigH-RshA complex was seen, and a lower band, corresponding to free SigH appeared. Free RshA runs as a faint smear under these conditions and is not visible as a discrete band.

To verify that the observed effect on the SigH-RshA interaction was the result of RshA phosphorylation, we examined the effect of treatment with a serine/threonine-specific phosphatase on the SigH-RshA interaction. Purified SigH and RshA (phosphorylated or non-phosphorylated) were incubated together in a 1:1 molar ratio for 30 min and divided into 2 samples. Serine/threonine phosphatase (New England Biolabs) was added to one of these samples, incubated for 30 min, and subjected to non-denaturing PAGE. The lower, free SigH bands seen in lanes that contain phosphorylated RshA disappeared following serine/threonine phosphatase treatment of the samples (Fig. 4). This result confirms the role of RshA phosphorylation in decreasing complex formation between SigH and RshA.

Reduced Inhibition by Phospho-RshA of the SigH-Mediated Disulfide Stress Response *in Vivo*. To investigate the functional effect of RshA phosphorylation on the SigH-RshA interaction in vivo, we performed disk diffusion assays in the rapid growing mycobacterium *M. smegmatis*. In these assays, the zone of inhibition of growth surrounding a disk containing diamide is measured on an agar plate onto which a uniform lawn of bacteria has been spread. Diamide is a highly specific disulfide stress reagent and resistance to this stress in mycobacteria is dependent on SigH activity, through its regulation of the thioredoxin reductase operon (2). Over-expression of RshA markedly increases susceptibility to diamide in this assay (2, 3). The *rshA* gene was cloned and placed downstream of the constitutive *hsp60* promoter in a replicating plasmid. This construct was then transformed into wild type *M. smegmatis* and into *M. smegmatis* strains expressing native or kinase-inactive forms of *M. tuberculosis pknB*.

The results of these experiments are shown in Fig. 5*A*. In wild type *M. smegmatis* the zone of inhibition is small and no measurable difference in zone diameter was observed between wild type and strains over-expressing either native or kinase-inactive *pknB*. As previously reported, in wild type *M. smegmatis* over-expressing *M. tuberculosis rshA*, a much larger zone of inhibition with a sharp border is seen, consistent with inhibition of SigH activity by RshA (3). The zone of inhibition in the strain over-expressing *rshA* and native *pknB* was significantly smaller than the zone in the strain over-expressing *rshA* in the wild type background, suggesting increased SigH activity resulting from decreased binding by phosphorylated RshA. In contrast the zone diameter was not significantly different in the strain over-expressing *rshA* and kinase-inactive *pknB*.

To confirm that the inhibition of SigH activity by RshA was decreased by phosphorylation of RshA, we performed additional disk diffusion assays with strains in which native RshA or RshA T94A were over-produced. As shown in Fig. 5*B*, after 48h incubation, the *M. smegmatis* strain over-expressing *pknB* and

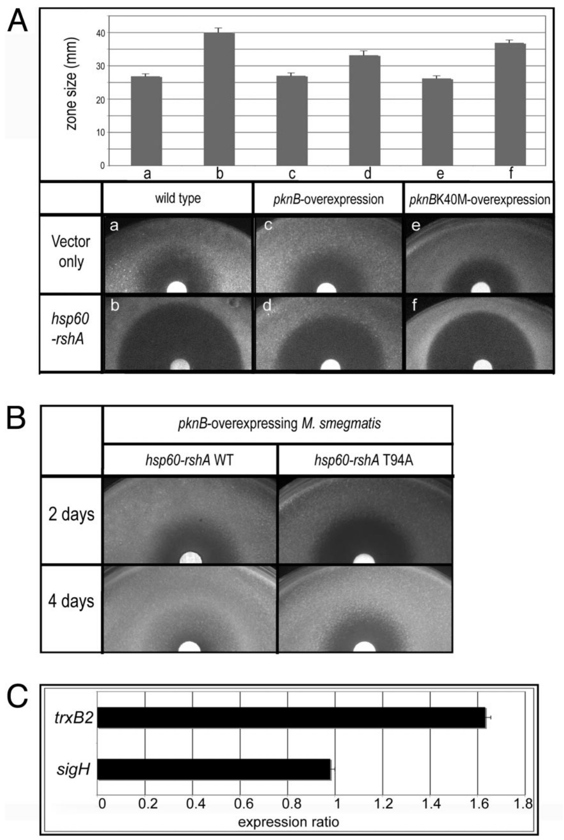

Fig. 5. Effect of *pknB* expression on inhibition of SigH activity by RshA. (*A*) Wild type (WT), *pknB* over-expressing, and *pknB*-K40M over-expressing strains of *M. smegmatis* containing *rshA* over-expressing constructs or vector only were grown to mid-log phase and plated to form a homogeneous lawn onto which a paper disk containing 5 μl of 1 M diamide was placed. The plates were photographed after 2 days of incubation at 37 °C. A larger zone of inhibition indicates inhibition of bacterial growth by diamide, consistent with decreased SigH activity. The graph represents measurements of 6 zone diameters for each strain. The error bars represent $\pm$ 1 standard deviation. (*B*) Strains over-expressing *pknB* and *rshA* or *rshA*^{T94A} were grown to mid-log phase and plated to form a homogeneous lawn onto which a paper disk containing 5 μl of 1 M diamide was placed. The plates were photographed after 2 days and 4 days of incubation at 37°C. (*C*) Effect of *pknB* expression on SigH-regulated genes in *M. tuberculosis*. Quantitative RT-PCR analysis of *sigH*, and *trxB2* transcript levels in active *pknB*-overexpressing and inactive *pknB*-K40M-overexpressing *M. tuberculosis*. Transcripts were normalized to *sigA* whose expression is constitutive (30). The graph presents relative fold expression of each gene after normalization, combining results from 3 independent RNA preparations with 2 technical replicates each. The error bars represent $\pm$ 1 standard error mean.

rshA showed a smaller zone size than strain over-expressing *pknB* and *rshA*^{T94A}, consistent with decreased inhibition of SigH activity by phosphorylated vs. non-phosphorylated RshA. To further assess the difference between these 2 strains, we incubated these plates for 2 additional days. As shown in the lower panels of Fig. 5*B*, the strain over-expressing *pknB* and native *rshA* showed a much smaller zone with prolonged incubation, whereas the strain over-expressing *pknB* and *rshA*^{T94A} showed little change in zone size. These results strongly suggest that decreased inhibition of SigH activity by RshA in the strain over-expressing *pknB* results from phosphorylation of Thr-94 of RshA, consistent with the *in vitro* data showing decreased binding of SigH by phospho-RshA compared to binding by unphosphorylated RshA.

To determine whether there is increased SigH-regulated transcription in the *pknB* over-expression strain, as predicted by the *in vitro* binding data and the *rshA* over-expression phenotypes, quantitative real time PCR was performed. As shown in Fig. 5*C*, the expression of *trxB2*, which encodes the SigH-regulated thioredoxin reductase gene that is required for resistance to diamide stress, was 1.6-fold higher in the strain expressing native *pknB* relative to the strain expressing the inactive form of *pknB*. In contrast to *trxB2*, expression of *sigH* itself was not significantly different in the 2 strains. Two other SigH-regulated genes were also examined; like *trxB2*, *dnaK* showed increased expression (1.4-fold) in the *pknB* native vs. *pknB*^{K42M} over-expression strain, while mRNA levels of Rv2466c, a gene of unknown function, was the same in the 2 strains (data not shown).

Discussion

The interaction of the essential PknB signal transduction pathway with the SigH-regulated stress response regulon provides insight into a mechanism by which sigma-anti-sigma factor interactions, and thus sigma factor activity, can be modulated by an apparently independent signaling pathway. Our finding that phosphorylation of RshA decreases its interaction with SigH *in vitro*, and that this modification leads to increased SigH activity *in vivo*, suggests that this mechanism for modulating the SigH stress response regulon is likely to be important in mycobacterial adaptation to stress. As SigH is essential for *M. tuberculosis* virulence (5), this kinase-mediated regulation of a SigH activity may be critical for tuberculosis pathogenesis.

We did not investigate whether additional STPKs might also interact with the SigH regulon, a possibility suggested by a report showing *in vitro* phosphorylation of RshA by PknE as well as PknB (14). Given the breadth and importance of this regulon in the *M. tuberculosis* stress response, additional regulatory inputs would not be surprising. Similarly, the possibility that other anti-sigma factors, or other transcriptional regulators, may be targeted by *M. tuberculosis* STPKs *in vivo* remains to be investigated.

Given the exquisite sensitivity of the SigH-RshA interaction to oxidative stress and the resulting ability to rapidly increase *sigH* transcription and SigH activity from very low basal levels to very high levels in response to stress, what role might phosphorylation of RshA play in regulating SigH activity? RshA functions as a redox switch protein, with disruption of the SigH-RshA interaction upon oxidation of RshA. Because *sigH* transcription is driven from a SigH-regulated promoter, disruption of the SigH-RshA complex creates a positive feedback loop that leads to rapid and strong induction of SigH-regulated gene expression (3). While this rapid onset, high-level expression may be ideal for coping with acute or severe stresses, a more limited response may be beneficial under other conditions. The partial inhibition of the SigH-RshA interaction that results when RshA is phosphorylated would allow such a graded activation of the SigH regulon.

Limited induction of transcription of the *trxB2* operon in the *pknB* over-expression strain supports this model and is consistent with the small but consistent decrease in oxidative stress susceptibility in this strain. We observed, however, that expression of only some SigH-regulated genes was increased in this strain. One possible explanation is that, in the absence of stress when *sigH* expression is very low, phosphorylation of RshA *in vivo* would release very limited amounts of SigH. Concentrations of RNA polymerase holoenzyme incorporating SigH would remain very low, so that increased transcription would occur only at strong SigH-dependent promoters; of those known, the SigH-regulated *trxB2* and *dnaK* are the strongest (2). An alternative explanation for these differences is that phosphorylation of SigH, which would also occur in the setting of increased *pknB* expression but which does not affect on the SigH-RshA interaction, might alter SigH binding or transcription activation at individual promoters. This possibility is consistent with the wide range of reported effects of phosphorylation on the function of eukaryotic transcription factors and, less commonly, prokaryotic regulators, such as effects on specific DNA binding, interaction with other transcription regulators and protein localization (20–22).

Why might PknB activity and SigH be linked? We previously demonstrated that *pknB* expression is highest during logarithmic aerobic growth and decreases in stationary phase (11). Early in the course of *in vivo* infection, *M. tuberculosis* replicates in the lung at rates similar to maximal *in vitro* aerobic growth rates (23). In this setting, limited activation of the SigH-regulated stress responses may be important for coping with the oxidative stress that accompanies aerobic growth, but which may not be sufficient to inactivate RshA. In addition, many host defense mechanisms use oxidative and nitrosative stress molecules for which SigH-regulated defenses are critical. Phosphorylation of RshA may provide a means for early or partial activation of the SigH regulon in response to oxidative or nitrosative stresses that are not potent enough to inactivate RshA.

How does this regulatory mechanism fit in the context of what is known regarding alternative sigma factor regulation? A recent publication demonstrated phosphorylation of a putative *M. tuberculosis* anti-anti-sigma factor by the *M. tuberculosis* STPK PknD (14). This work suggested a role for phosphorylation in regulating alternative sigma factor-dependent transcription, however, the sigma factor affected and the functional effects of phosphorylation on specific transcription were not defined. Our results provide direct evidence for a mechanism by which anti-sigma factor phosphorylation by a bacterial STPK regulates sigma factor activity.

In *Bacillus subtilis* well defined systems of reversible phosphorylation via kinase and phosphatase activities regulate the sporulation sigma factor SigF and the general stress response sigma factor SigB (24–26). In these systems, the phosphorylation state of sigma factor regulators results from the balance of dedicated kinases and phosphatases that are typically encoded in the same operon as the sigma factor. A similar system may operate in mycobacteria in regulating the general stress response sigma factor SigF, however the components of this system have not been fully defined (27, 28). In contrast to the *B. subtilis* paradigm, SigH and PknB are independently regulated, and PknB has an essential role in regulating cell shape and cell wall synthesis that is distinct from the stress response transcription regulation mediated by SigH (11, 12).

Our results demonstrate a mechanism for regulating alternative sigma factor activity via anti-sigma factor phosphorylation by a bacterial STPK. This mechanism provides a means for partial or graded activation of the SigH regulon, in contrast to the rapid high-level induction that results from inactivation of RshA by oxidative stress. This mechanism further serves to define a link between the regulation of growth and cell wall synthesis by PknB and stress response regulation by SigH. This linkage is consistent with a systems view of bacterial physiology in which multiple signal transduction and regulatory pathways work in concert to allow adaptation to changing environmental conditions.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions. *M. smegmatis*, *M. bovis* bacillus Calmette-Guérin and *M. tuberculosis* cultures were grown at 37 °C in Middlebrook 7H9 liquid medium (DIFCO) supplemented with 10% ADC (ADC = 5% [wt/vol] BSA fraction V, 2% [wt/vol] glucose and 0.85% [wt/vol] NaCl), and 0.05% (wt/vol) Tween-80 (7H9-ADC-Tw), or on Middlebrook 7H9-ADC agar plates. Hygromycin (50 μg/ml) and Kanamycin (50 μg/ml) were added to culture media as indicated. *M. bovis* bacillus Calmette-Guérin overexpressing *pknB* was grown in 7H9-ADC-Tw without inducer to late exponential phase and reinoculated into fresh medium with 0.2% (wt/vol) acetamide. *E. coli* DH5 (Invitrogen) was used as a host strain for cloning experiments and was grown in LB broth or solid medium with kanamycin (25 μg/ml) or carbenicillin (100 μg/ml) where appropriate. *E. coli* BL21(DE3) (Invitrogen) was used as a host strain for protein expression experiments and was grown in LB broth or solid medium.

Protein Purification and Site Directed Mutagenesis. *M. tuberculosis* SigH protein was purified as described (2) from *E. coli* BL21(DE3) transformed with pRH1458 which carries the *M. tuberculosis sigH* gene in pTYB1 (New England Biolabs). The *M. tuberculosis* 6X-His-RshA protein was purified as described (3) from *E. coli* BL21(DE3) transformed with pRH1504 which carries the *M. tuberculosis rshA* gene in pET-3c (Novagen). To produce SigH and RshA proteins in which the phosphoacceptor Thr was changed to Ala, site-directed mutagenesis was performed by overlap PCR using the Quickchange II XL kit (Stratagene). Active (GST-PknB) and inactive (GST-PknBK40M) forms of the kinase domain of PknB were expressed as amino-terminal GST fusion proteins and purified using glutathione-Sepharose 4B resin (Pierce) as previously described (11).

***In Vitro* Kinase Assays.** *In vitro* kinase assays were performed using 200 ng of GST-PknB or GST-PknBK40M protein mixed with recombinant SigH or RshA in 20 μl of kinase buffer (50 mM Tris·HCl at pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$). The reaction was started by adding 1 μCi of [γ-^{32}P]ATP, and incubated at 25 °C for 30 min. Reactions were terminated by adding SDS gel-loading buffer (50 mM Tris·HCl at pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), and the proteins were resolved by SDS/PAGE. Gels were stained with GelCode Blue (Pierce), dried, and exposed to film.

2D Gel, Immunoblot, and LC-MS/MS Analysis. For protein extraction, *M. bovis* bacillus Calmette-Guérin cells harboring pMH94 plasmid alone or pMH94-*pknB* were grown in liquid medium to early stationary phase and then acetamide was added to 0.2%. *M. tuberculosis* cells harboring pMV306AC-*sigH*-FLAG or pMV306AC-*rshA*-FLAG were grown in liquid medium to mid log phase and then acetamide was added to 0.2%. After 24 h of incubation, cells were pelleted, washed with 40 mM Tris buffer (pH 7.5), and resuspended in TRIzol (Invitrogen) in a Lysing Matrix B tube (QBiogene). *M. tuberculosis* cells harboring pMV261-*rshA*-FLAG were grown in liquid medium to late log phase and were pelleted, washed, and resuspended as above. The samples were lysed in a MagNAlyser device (Roche), and total mycobacterial protein was extracted according to the supplier's manual (Invitrogen). The protein pellet was resuspended in 1% (wt/vol) SDS and purified with the ReadyPrep 2D Cleanup Kit (Bio-Rad). 500 μg of total protein from each sample was rehydrated into isoelectric focusing strips with a pH range of 3–10, 3–6, or 5–8 (Bio-Rad). Isoelectric focusing was performed for 35,000 V-h in a PROTEAN IEF Cell (Bio-Rad). Second-dimension SDS/PAGE was performed using 10% Tris·HCl gels, and proteins were partially electrotransferred to a PVDF membrane. Immunoblot blot analysis was performed using a phospho-T polyclonal antibody (Cell Signaling Technology) as described above.

The protein spot from *M. bovis* bacillus Calmette-Guérin was excised from 2-D gels and subjected to a modified in-gel trypsin plus chymotrypsin digestion procedure (29). The resulting peptides were subjected to electrospray ionization and entered into an LCQ DECA ion-trap mass spectrometer (ThermoFinnigan). Eluting peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences (and protein identity) were determined by matching protein databases with the acquired fragmentation pattern by the software program Sequest (ThermoFinnigan).

The protein samples from *M. tuberculosis* and *in vitro* kinase assays were digested in-gel with trypsin (Promega) (29). The resulting peptide extract was collected, dried down, and resuspended in 5% acetonitrile and 2% formic acid before direct injection into an LC/MS system comprising a microautosampler, Suvery HPLC pump and an LTQ linear ion trap mass spectrometer (ThermoFinnigan). The LC-system featured a PepMap trapping column (Dionex) and an in-house packed reversed phase column using C18 Magic (3 mm, 200 Å; Michrom Bioresourcepacking material and PicoTip Emitters (New Objective). The peptides were eluted with a 15 min linear gradient and data acquired in a data dependent fashion, i.e., the 6 most abundant species were selected for automated fragmentation. The product ion spectra were searched against a custom *M. tuberculosis* database using Mascot (Matrix Science) and Protein Pilot (Thermo).

Protein Binding Assays. The non-denaturing PAGE protein binding assay was modified from the previously reported method (3). SigH (4 μM) was mixed with RshA (4 μM) in 20 μl of binding buffer [40 mM Tris·HCl pH 8.0, 10 mM $MgCl_2$, 0.01 mM EDTA, 20% (vol/vol) glycerol] in the presence of 1 mM THP

(Tris(hydroxypropyl)phosphine). After incubation for 60 min at 30 °C, proteins were separated on a non-denaturing 7.5% acrylamide gel at 700 mW. The gel and buffer were maintained at the desired temperature by running in the 4 °C room. For phosphatase treatment, purified SigH and RshA were phosphorylated at 30 °C for 30 min as described above and divided to 2 samples. Serine/threonine phosphatase (New England Biolabs) was added to one sample, both samples were incubated for 30 min and subjected to non-denaturing PAGE.

Disk Diffusion Assays. Approximately 10^6 *M. smegmatis* cells were uniformly spread onto 7H10-ADC-Tw agar medium, and a paper disk impregnated with 5 μl of 1 M diamide solution was placed onto the lawn. The susceptibility of *M. smegmatis* strains was determined by measuring the zone of inhibition after 2 days of growth at 37 °C. Photographs of the plates were taken using a Kodak Image Station 4000MM.

Quantitative Real Time PCR. *M. tuberculosis* cells harboring pMH94-*pknB* or $-$*pknB* DN were grown in liquid medium to mid log phase and then acetamide was added to 0.1%. After 3 h, cells were pelleted and resuspended in TRIzol (Invitrogen) in a Lysing Matrix B tube (QBiogene). The samples were lysed in a MagNAlyser (Roche), and RNA was isolated according to the supplier's manual (Invitrogen). Transcript quantities for *sigH*, Rv2466c, *dnaK*, and *trxB2* were determined relative to the amount of *sigA* transcript by quantitative real time PCR with the QuantiFast SYBR RT-PCR kit (Qiagen) and ABI Prism 7000 Sequence Detection System (Applied Biosystems). The ABI SDS (Sequence Detection Software) version 1.2 was used for data analysis. The primer sets used are shown in Table S1.

ACKNOWLEDGMENTS. The authors thank Simon Dove, Paula Watnick, and members of the Husson laboratory for discussion and critical review of the manuscript. This work was supported by National Institutes of Health Grants AI37901 and AI59702 to RNH.

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