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Phosphorylation Regulates Mycobacterial Proteasome[§]

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Mycobacterium tuberculosis **possesses a proteasome system that is required for the microbe to resist elimination by the host immune system. Despite the importance of the proteasome in the pathogenesis of tuberculosis, the molecular mechanisms by which proteasome activity is controlled remain largely unknown. Here, we demonstrate that the α-subunit (PrcA) of the *M. tuberculosis* proteasome is phosphorylated by the PknB kinase at three threonine residues (T84, T202, and T178) in a sequential manner. Furthermore, the proteasome with phosphorylated PrcA enhances the degradation of Ino1, a known proteasomal substrate, suggesting that PknB regulates the proteolytic activity of the proteasome. Previous studies showed that depletion of the proteasome and the proteasome-associated proteins decreases resistance to reactive nitrogen intermediates (RNIs) but increases resistance to hydrogen peroxide (H_2O_2). Here we show that PknA phosphorylation of unprocessed proteasome β-subunit (pre-PrcB) and α-subunit reduces the assembly of the proteasome complex and thereby enhances the mycobacterial resistance to H_2O_2 and that H_2O_2 stress diminishes the formation of the proteasome complex in a PknA-dependent manner. These findings indicate that phosphorylation of the *M. tuberculosis* proteasome not only modulates proteolytic activity of the proteasome, but also affects the proteasome complex formation contributing to the survival of *M. tuberculosis* under oxidative stress conditions.**

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Introduction

Approximately one-third of the world's population is infected with *Mycobacterium tuberculosis*, and in the majority of these individuals, the organism is in the latent state. Because *M. tuberculosis* inhabits the phagosomes of macrophages that produce reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs), it must avoid destruction by sensing and responding to these stress signals. *M. tuberculosis* possesses a wide variety of surface receptors and signaling molecules, including eleven eukaryotic-like Ser/Thr protein kinases (STPKs), which may transduce these environmental signals (Leonard *et al*., 1998).

Previously, we identified Wag31 and Rv1422 as *in vivo* substrates of PknA and PknB (Kang *et al*., 2005). We further found that the phosphorylation of Wag31 is important for optimal cell growth and morphology via controlling peptidoglycan biosynthesis at the cell poles (Kang *et al*., 2008; Jani *et al*., 2010). Other studies showed that PknB phosphorylated PbpA (a septal peptidoglycan-synthesizing enzyme), GarA (a regulator of central carbon metabolism), and EF-Tu (translation elongation factor Tu), and that PknA phosphorylated FtsZ (a protein essential for cell division) and MurD (a ligase involved in the peptidoglycan synthetic pathway) (Villarino *et al*., 2005; Dasgupta *et al*., 2006; Thakur and Chakraborti, 2006, 2008; Sajid *et al*., 2011a). More recently, InhA (an enzyme in mycolic acid biosynthesis) and PstP (a PP2C phosphatase) have also been identified as substrates of both PknA and PknB (Khan *et al*., 2010; Sajid *et al*., 2011b). These studies suggested that PknA and PknB are key signaling molecules that regulate many aspects of mycobacterial physiology. In this study, we have identified the proteasome to be a substrate of PknA and PknB.

In eukaryotes, the proteasome is responsible for protein turnover important for several cellular processes including the proteolysis of regulatory proteins, as well as irreversibly misfolded and potentially toxic proteins (Voges *et al*., 1999). While proteasomes are ubiquitous in archaea (Zwickl, 2002), bacterial proteasomes are apparently confined to Actinomycetes, such as *Mycobacterium*, *Streptomyces*, and *Nitrospora* (De Mot *et al*., 1999). In *M. tuberculosis,* strains in which the proteasome core genes (*prcBA*) or the proteasome-associated genes (*pafA* and *mpa*) are depleted have impaired growth in the presence of nitric oxide (NO) (Darwin *et al*., 2003; Gandotra *et al*., 2007). Interestingly, however, the same mutants are more resistant to H_2O_2 (Darwin *et al*., 2003; Gandotra *et al*., 2007). Similarly in *Streptomyces coelicolor* deletion of the proteasome increased resistance to organic peroxide,

coinciding with an increase in the level of haloperoxidase (De Mot *et al*., 2007).

While eukaryotic proteasomes are composed of several different α- and β-subunits to generate the 20S core complex (Groll *et al*., 1997), bacterial proteasomes are composed of only one or two types of α- and β-subunits. For example, the *M. tuberculosis* proteasome consists of 14 α-subunits encoded by *prcA* and 14 β-subunits encoded by *prcB*, which assemble into a four-ringed $\alpha_7\beta_7\beta_7\alpha_7$ complex (Hu *et al*., 2006; Lin *et al*., 2006). The *M. tuberculosis* proteasome is thought to assemble through a half-proteasome intermediate that contains one α-ring and one β-ring (Li *et al*., 2010). Upon apposition of two half-proteasomes, the propeptide of β-subunits is processed to yield a fully assembled and proteolytically active holo-proteasome complex (Zuhl *et al*., 1997). While the β-propeptide usually promotes the proteasome assembly in both prokaryotic and eukaryotic 20S proteasomes, the β-propeptide of the *M. tuberculosis* 20S appears to serve as a thermodynamic hurdle for 20S assembly (Zuhl *et al*., 1997; Kwon *et al*., 2004; Li *et al*., 2010).

Almost all proteins that are targeted for proteasomal degradation in eukaryotes are covalently tagged with ubiquitin, although some "natively disordered" proteins can be degraded by the proteasome without ubiquitin modification (Tofaris *et al*., 2001; Kerscher *et al*., 2006). Prokaryotic ubiquitin-like protein (Pup), encoded by the Rv2111c gene located upstream of *prcB* was found to target proteins for proteolysis by the *M. tuberculosis* proteasome (Pearce *et al*., 2008; Striebel *et al*., 2014). Several natural substrates of the *M. tuberculosis* and *M. smegmatis* proteasomes have been discovered (Pearce *et al*., 2006; Burns *et al*., 2009; Festa *et al*., 2010). The *M. tuberculosis* proteasome uses accessory proteins including Mpa (*Mycobacterium* proteasomal ATPase), PafA (proteasome accessory factor A), and Dop (deamidase of Pup) (Darwin *et al*., 2003; Lamichhane *et al*., 2006; Striebel *et al*., 2009). Mpa, which has sequence similarity to eukaryotic proteasomal ATPases, binds, unfolds, and delivers degradation substrates into the proteasome core (Burns *et al*., 2010; Striebel *et al*., 2010; Wang *et al*., 2010).

Although the structure and function of the proteasome in *M. tuberculosis* have been extensively investigated, the regulation of its function remains elusive. In eukaryotic and archaeal proteasomes, co- and post-translational modifications such as N-terminal acetylation (Kimura *et al*., 2000), O-linked glycosylation (Zachara and Hart, 2004), S-glutathionation (Demasi *et al*., 2003), and N-myristoylation (Lee and Shaw, 2007) appear to modulate proteasome function. Phosphorylation has also been recognized in both core and regulatory subunits of the eukaryotic and archaeal proteasome, which may regulate proteolytic activity or proteasome complex stability (Rivett *et al*., 2001; Bose *et al*., 2004; Horiguchi *et al*., 2005; Zhang *et al*., 2007; Humbard *et al*., 2010). In this work, we report the phosphorylation of the *M. tuberculosis* proteasomal subunits by the Ser/Thr protein kinases PknA and PknB. We determined that the α-subunits are phosphorylated by PknB at three threonine (Thr, T) residues in a sequential manner, which may regulate proteasomal activity. We also found that PknA, by phosphorylating unprocessed β-subunit (pre-PrcB) and α-subunit, inhibits proteasome assembly, which in turn renders mycobacterial cells more resistant to H_2O_2. Thus, this study suggests that phosphorylation of the proteasome by PknA/B not only modulates proteolytic activity of the proteasome, but also contributes to the survival of *M. tuberculosis* under oxidative stress conditions.

Materials and Methods

Bacterial strains, media, and growth conditions

M. tuberculosis H37Rv, *M. tuberculosis* $mc^2$6230, and *M. smegmatis* $mc^2$155 cultures were grown at 37°C in Middlebrook 7H9 liquid medium (Difco, USA) supplemented with 10% ADC [Albumin-dextrose-catalase consisting of 5% (w/v) BSA fraction V powder, 2% (w/v) glucose, 0.04% (w/v) catalase, and 0.85% (w/v) NaCl], and 0.05% (w/v) Tween 80, or on Middlebrook 7H9-ADC agar plates supplemented with 0.05% (w/v) glycerol. Kanamycin (50 μg/ml), hygromycin (50 μg/ml) or apramycin (50 μg/ml) was added to culture media as indicated. *Escherichia coli* TOP10 (Invitrogen, USA) was used as a cloning host, and *E. coli* BL21 (DE3) for expression of recombinant proteins. Cultures were grown at 37°C in LB broth or solid medium with apramycin (50 μg/ml), kanamycin (50 μg/ml), chloramphenicol (34 μg/ml) or ampicillin (50 μg/ml) where appropriate.

Plasmid construction

For *in vitro* kinase assay of the chimeric proteasome complex, *strep-prcA* was PCR amplified using the genomic DNA of *M. tuberculosis* H37Rv and primers N-Strep PrcA-5-Ndegtg and N-strep PrcA-3-xba (All the primers are listed in Supplementary data Table S2), and cloned into an integrating plasmid pCK202 carrying an acetamide-inducible promoter, and named as pCK206 (All the constructs are listed in Supplementary data Table S1). For *in vivo* phosphorylation analysis of PrcA in *ΔprcBA M. smegmatis*, *prcBA-strep* was PCR amplified by using primers TBprcBA pUAB100-F and TBprcBA-C-strep3′, and cloned behind a constitutive *hsp60* promoter in pCK315 (named as pCK322). For testing the phosphorylation of wild-type and mutant forms of PrcA in *E. coli*, *prcBA-strep* was PCR amplified by using primers C-StrepTBprcB-5 and TBprcBA-C-strep3′, and cloned under T7 promoter in pACYCDeut-1 (Novagen, USA) (named as pCK343). Plasmids expressing *prcAT84A*, *prcAT178A*, and *prcAT202A* were generated by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA) and primers indicated in Supplementary data Table S2 (named as pCK364, pCK348, and pCK349). Plasmids for phosphomimetic forms of PrcA (PrcAT84E, PrcAT(84,202)E, and PrcA(84,202,178)E) were constructed similarly (named as pCK382, pCK385, and pCK386). To construct a *ΔprcBA M. smegmatis* strain (KMS98), 5′- and 3′-flanking regions of the $prcBA_{Ms}$ genes was amplified by PCR by using primers Dn-PrcA-F, Dn-PrcA-R, Up-PrcB-F, and Up-PrcB-R, and the genomic DNA of wild-type *M. smegmatis* $MC^2$155. The hygromycin resistance gene was inserted between the two flanking regions in pRH1351 (Kang *et al*., 2008) and named as pCK260. To test *in vivo* proteolysis of Ino1, 6*His-pup* and 6*His-pup-prcBA* were amplified PCR by using primers Pup(tb)bglII-F,

Pup(tb)-R, and prcAtb-R, and the genomic DNA of *M. tuberculosis* H37Rv as a template, and cloned in pCK315 (names as pCK437 and pCK438).

Purification of recombinant proteins

To purify the chimeric proteasome complex, *M. smegmatis* KMS65 was grown to mid-log phase where expression of *strep-prcA* was induced by 0.2% acetamide for 4 h. Strep-PrcA was purified by using the Strep-Tactin Affinity Purification Kit (Novagen). The *M. tuberculosis* proteasome complex for the *in vivo* phosphorylation was purified from *ΔprcBA M. smegmatis* carrying pCK322 plus pCK5 (KMS109), pCK7 (KMS108), or pMH94 vector (KMS157) by overexpressing *pknB* at mid-log phase with 0.2% acetamide for 4 and 16 h. A parallel flask without acetamide was also cultured for 16 h. PrcA-strep was purified by using the Strep-Tactin Affinity Purification Kit (Novagen) as described above. To test the phosphorylation sites in PrcA, *E. coli* BL21 (DE3) cells were transformed with a pACYCDuet-1 plasmid containing wild-type (pCK343) or each mutant form of PrcA (pCK364, pCK348, and pCK349) and subsequently with pCK4 or pCK21. Each transformant was cultured in Overnight Express Autoinduction medium (Novagen) at 37°C for 16 h. Each strep-tagged PrcA form was purified as described above. PrcAT84E, PrcAT(84,202)E, and PrcAT(84,202,178)E were purified similarly by culturing *E. coli* BL21 (DE3) containing pACYCDuet-1, pCK382, pCK385, or pCK386 in Overnight Express Autoinduction medium. To test the effect of PknA on proteasome complex formation in *E. coli*, pCK343 was transformed into *E. coli* BL21 (DE3) containing pCK3 or pCK4, which were cultured in Overnight Express Autoinduction medium at 37°C for 10 h, and PrcA-strep was purified as described above.

2-D PAGE, immunoblot, and mass spectrometry

Total protein extraction from *M. tuberculosis* harboring pMH94 plasmid alone, pCK5, or pCK7 and subsequent 2-D SDS-PAGE were performed as described previously (Kang *et al*., 2005). The same amount (200 μg) of each total lysate protein was analyzed in 2-D SDS-PAGE (4–12% Bis-Tris Criterion XT Precast Gel, Bio-Rad, USA) with IPG strips with a pH range of 3–10 (Bio-Rad). Immunoblotting analysis was performed with a phospho-T polyclonal antibody and phospho-signals were compared by exposing the blots for the same 15 sec in the ChemiDoc XRS system (Bio-Rad). For the *in vivo* phosphorylation of PrcA, 30 μg of each purified PrcA-Strep was similarly analyzed in 2-D SDS-PAGE with IPG strips of pH 4–7 (Bio-Rad). Then, Western blots were performed by a phospho-T antibody, and subsequently with a strep-II antibody (Novagen) after stripping off the first antibody by using a stripping buffer [100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl, pH 6.7] at 50°C for 30 min. Mass spectrometry for the identification of the phosphorylation sites in PrcA was performed as previously described (Kang *et al*., 2005). To analyze the proteasome complex in *M. smegmatis* overexpressing *pknA*, 15 μg of PrcA-strep plus PrcB complex used in Fig. 4A was analyzed in 2-D SDS-PAGE with Flamingo Stain (Bio-Rad).

In vitro kinase assay

To test the phosphorylation of PrcA *in vitro*, 50 pmol of GST-PknA, GST-PknB, GST-PknAK42M, or GST-PknBK40M protein was incubated with 300 pmol of strep-PrcA and $PrcB_{Ms}$ complex in 20 μl of kinase buffer (50 mM Tris-HCl; 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 1 h. Reactions were terminated by adding SDS gel-loading buffer and the proteins were resolved by SDS-PAGE, followed by staining with GelCode Blue (Pierce, USA). Phosphorylation of the phosphomimetic forms of PrcA was examined by incubating 3 μg of the proteasome complex containing PrcAT84E, PrcAT(84,202)E or PrcAT(84,202,178)E with 1 μg of GST-PknB and 1 mM ATP. The relative phosphorylation levels of each protein were determined by immunoblot with a phospho-T antibody and GelCode staining of parallel reactions.

In vivo degradation of Ino1

M. smegmatis strains, KMS190, KMS191, KMS192, and KMS193 were cultured overnight in 7H9 medium and incubated with 0.2% acetamide for 6 and 12 h. 25 μg of the total lysate protein was subjected to immunoblotting with Ino1, PrcB, and PknB antibodies. To test the degradation of Pup~Ino1 in the presence of epoxomicin, KMS192 was cultured overnight with 50 μM epoxomicin (Sigma, USA) or the vehicle DMSO, and subsequently *pknB* was expressed with 0.2% acetamide for 6 h. Cells were lysed, and 25 μg of the lysate protein was used for Western blots as described above.

H_2O_2 susceptibility assays

To examine the effect of the proteasome on *M. smegmatis* survival in the presence of H_2O_2, wild-type (KMS197) and the *M. smegmatis DprcBA* strains (KMS128 and KMS116) were cultured overnight in 7H9 medium (50 mg/ml hygromycin and 50 mg/ml apramycin), diluted to OD_{600}=0.01 in 7H9 medium, treated with 4 mM H_2O_2, and incubated at 37°C for 45 min. The cultures were serially diluted into a buffer containing 0.8% NaCl and 0.01% Tween 80 and plated on 7H9 plates for CFU determination. The influence of *pknA*-expression on the survival in the presence of H_2O_2 was tested by using the *M. smegmatis DprcBA* strain constitutively expressing *prcBA* with induction of *pknA*, *pknB*, or vector (KMS108, KMS109, and KMS157) overnight in 7H9 containing hygromycin, apramycin, and kanamycin. As a control, *DprcBA M. smegmatis* carrying *pknA*-expression cassette alone (KMS201) was included. To induce the expression of kinases, overnight culture was diluted to initial OD_{600}=0.05 and incubated with 0.1% acetamide for 5 h. To test for sensitivity to H_2O_2 cultures were further treated with 4 mM H_2O_2 and incubated at 37°C for 45 min, followed by serial dilution to determine CFU.

Effect of H_2O_2 on proteasome assembly and PknA phosphorylation

DprcBA M. smegmatis that constitutively expresses *prcBA* and contains *pknA*- (KMS109) or *pknB*-expression cassette

(KMS108) was cultured with 0.1% acetamide to moderately express the kinase genes for 6 h. Cells were then treated with 2 mM H_2O_2 for 1.5 and 4 h. The *M. tuberculosis* $mc^2$6230 strain (attenuated *M. tuberculosis* strain, obtained from Dr. William Jacobs lab, Albert Einstein College of Medicine), was also cultured to log phase and treated with or without 2 mM H_2O_2 for 24 h. 20 µg total lysate protein purified with TRIzol was used for SDS-PAGE followed by Western blot with a PrcB polyclonal antibody. Autophosphorylation of PknA in response to H_2O_2 was tested in *E. coli* BL21 (DE3) containing a pGEX-4T-3-*pknA* plasmid (pCK3) cultured in the presence of 0.5 mM IPTG for 6 h, and then exposed to 2 mM H_2O_2 for 0, 60, and 120 min. The levels of the PknA phosphorylation and the GST-PknA were determined by Western blottings with phospho-T and GST antibodies.

Results

Phosphorylation of PrcA in *M. tuberculosis*

To study the functions of PknA and PknB in *M. tuberculosis*, we previously sought to identify their substrates by a proteomic approach using 2-D SDS-PAGE, immunoblotting with a phospho-(S/T)Q antibody, and mass spectrometry (Kang *et al.*, 2005). Using this approach, we identified two substrates, Wag31 and Rv1422. This initial search, however, was limited to proteins that had the phosphorylated TQ motif due to the phospho-(S/T)Q antibody being used. To further understand the role of these kinases, we attempted to identify additional substrates by a similar proteomic search, but with a phospho-T antibody. Throughout this report, all genes and proteins for kinases (PknA and PknB) and proteasome (PrcA and PrcB) are from *M. tuberculosis* unless otherwise indicated.

Approximately 15 protein spots were found to have stronger phospho-signal in *pknB*-overexpressing *M. tuberculosis* than the vector-only control or *pknA*-overexpressing cells (Fig. 1A). Mass spectrometry analysis of a spot from *pknB*-overexpression cells (3rd row, Fig. 1A) revealed PrcA as a potential substrate of PknB with two phosphorylated residues, T84 and T202 (Supplementary data Figs. S1 and S2). The presence of a weak but distinct immunoblot signal at the same location in cells with vector alone or *pknA*-overexpression (1st and 2nd rows, Fig. 1A) indicates that PrcA is phosphorylated in wild-type *M. tuberculosis* and is likely to be a physiologic target of PknB *in vivo*.

To examine the phosphorylation of PrcA *in vitro*, strep-tagged *M. tuberculosis* PrcA was expressed and purified from wild-type *Mycobacterium smegmatis*. Processed $PrcB_{Ms}$ of *M. smegmatis* (2nd row, Fig. 1B; Supplementary data Fig. S3) was co-eluted with strep-PrcA in approximately equimolar amounts. This "chimeric proteasome" showed proteolytic activity against a fluorogenic substrate Suc-LLVY-Amc (Lin *et al.*, 2006) (Supplementary data Fig. S5), indicating that *M. tuberculosis* PrcA forms an active proteasome complex with $PrcB_{Ms}$ in *M. smegmatis*. *In vitro*, Strep-PrcA was specifi-

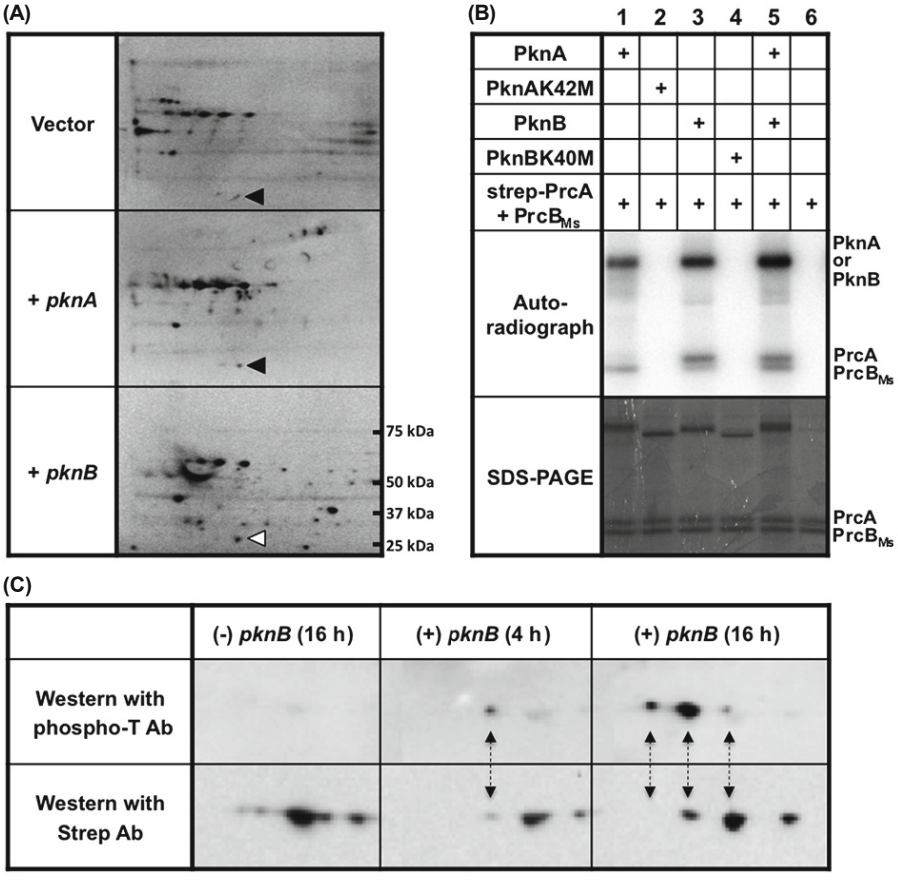

Fig. 1. Phosphorylation of PrcA by PknB. (A) PrcA is a potential substrate of PknB in *M. tuberculosis*. Whole-cell lysate (200 µg) of *M. tuberculosis* H37Rv containing pMH94 vector alone, pMH94-*pknA* (pCK5), or pMH94-*pknB* (pCK7) grown with 0.2% acetamide for 24 h was analyzed in a 2D-Western blot with a phospho-T antibody. Mass spectrometry analysis of a spot with stronger Western signal from cells with *pknB*-overexpression (◅) identified PrcA as a potential substrate of PknB that contains two phosphorylation sites (T84 and T202). A weak but distinct immunoblot signal at the same location in cells with vector alone or *pknA*-overexpression is also shown (◂). (B) *In vitro* phosphorylation of PrcA by PknB. Wild-type *M. smegmatis* was transformed with pMH94-*strep-prcA* (pCK206), and the expression of *strep-prcA* was induced by 0.2% acetamide for 4 h at mid-log phase. Affinity purification of strep-PrcA co-eluted $PrcB_{Ms}$, the b-subunit of the *M. smegmatis* proteasome. Chimeric complex of strep-PrcA and $PrcB_{Ms}$ was incubated with GST-PknA, GST-PknB, or kinase-inactive mutant protein (GST-PknAK42M or GST-PknBK40M) in the presence of [γ-^{32}P]ATP followed by SDS-PAGE, Coomassie blue staining (bottom row), and autoradiography (upper row). (C) *In vivo* phosphorylation of PrcA by PknB. Strep-PrcA was purified from the *M. smegmatis ΔprcBA* strain expressing *prcB-prcA-strep* (pCK322) and *pknB* (pCK7). *pknB* was overexpressed at mid-log phase by incubating with 0.2% acetamide for 0, 4, and 16 h. PrcA-strep was affinity purified and analyzed by 2-D SDS-PAGE followed by immunoblottings, first with a phospho-T antibody and subsequently with a strep antibody. Arrows indicate the corresponding spots on the two immunoblots.

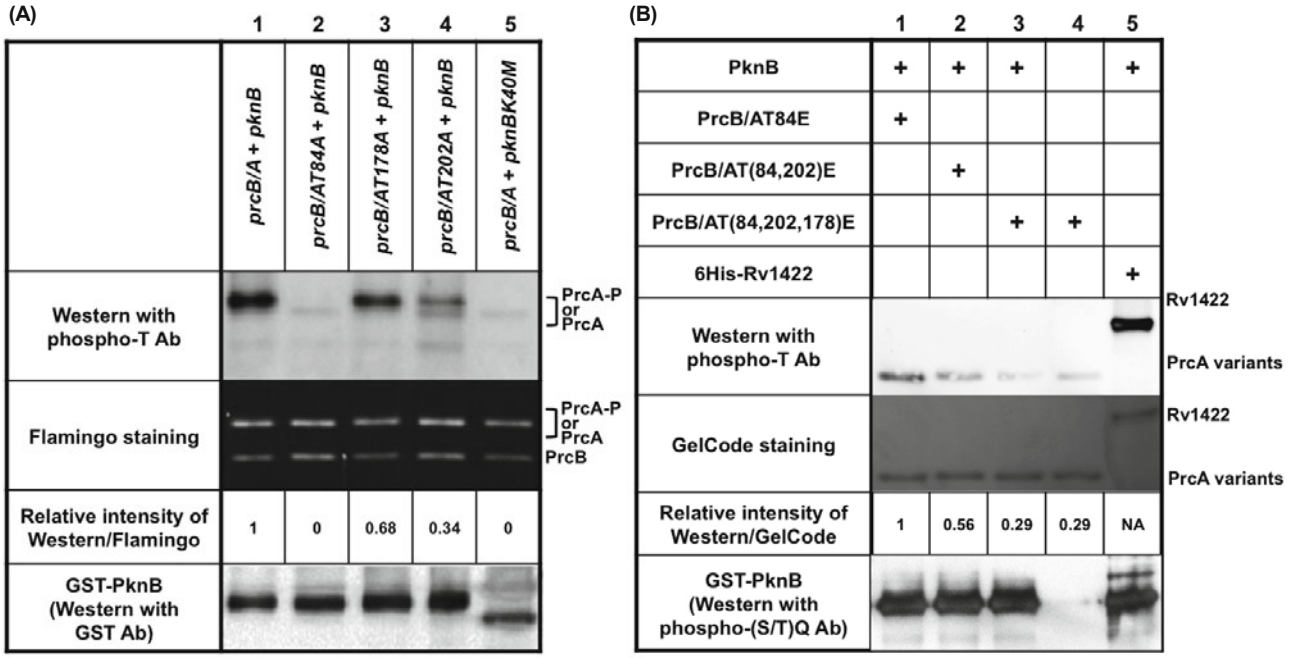

Fig. 2. Sequential phosphorylation of PrcA. (A) Verification of the phosphorylation sites in PrcA. Wild-type (pCK343) and substituted forms of PrcA (pCK348, pCK349, and pCK364) were co-expressed with PrcB and purified from *E. coli* BL21 (DE3) containing a pGEX-4T-3 plasmid expressing *pknB* (pCK4) or a kinase-inactive *pknBK40M* (pCK21). Intensity of phospho-signal in the immunoblot with a phospho-T antibody (row 1) relative to the amount of protein loaded (row 2) was determined by Quantity One Software (Bio-Rad) and shown in row 3. Levels of GST-PknB or GST-PknBK40M were determined by immunoblot with a GST antibody (row 4). (B) Phosphorylation of the phosphomimetic variants of PrcA-strep *in vitro*. PrcAT84E (lane 1), PrcAT(84,202)E (lane 2) or PrcAT(84,202,178)E (lane 3) was incubated with GST-PknB and ATP, and the relative phosphorylation levels of each protein (3rd row) were determined by immunoblot with a phospho-T antibody (2nd row) and GelCode staining of parallel reactions (3rd row). Note that PrcAT(84,202,178)E shows a weak background signal from immunoblot with a phospho-T antibody in the absence of PknB (lane 4). Rv1422, a known substrate of PknB, was included as a control (lane 5). Levels of GST-PknB included were determined by immunoblot with a phospho-(S/T)Q antibody (row 4).

cally phosphorylated by PknB but not by PknA (compare lanes 1 and 3, Fig. 1B), consistent with the *in vivo* phosphorylation shown in Fig. 1A. Interestingly, $PrcB_{Ms}$ from *M. smegmatis* was also phosphorylated by PknA and PknB (lanes 1 and 3), suggesting that PrcB of *M. tuberculosis* may be phosphorylated by one or both of these kinases.

To further validate the phosphorylation of PrcA *in vivo*, we examined the phosphorylation of strep-PrcA in the presence of *pknB*-overexpression in *M. smegmatis* (Fig. 1C). For this, we first constructed a *ΔprcBA* strain of *M. smegmatis* (Supplementary data Fig. S6). Unlike in *M. tuberculosis* where proteasome depletion impaired its growth on agar plates and, to a small degree, in liquid culture, the $prcA_{Ms}$ and $prcB_{Ms}$ genes have been deleted in *M. smegmatis* without affecting growth (Knipfer and Shrader, 1997). The *pknB*-overexpression construct was introduced into the chromosomal *attB* locus of *ΔprcBA M. smegmatis*, which was then further transformed with a replicating plasmid containing *prcB-prcA-strep* from *M. tuberculosis* under a constitutive *hsp60* promoter (Singh *et al.*, 2006). Strep-PrcA from cells with or without *pknB*-overexpression was purified and analyzed by 2-D SDS-PAGE and immunoblots, first with a phospho-T antibody and subsequently with a strep antibody after stripping off the phospho-T antibody. While the levels of strep-PrcA loaded in 2-D SDS-PAGE were similar (bottom row, Fig. 1C), the phosphorylation level of strep-PrcA increased as *pknB* was expressed over time (top row), indicating that PrcA was phosphorylated by PknB *in vivo*. After 4 h of *pknB* induction, a phospho-signal spot was clearly present, and at 16 h of *pknB* induction, a total of three phosphorylated PrcA spots were identified. Mass spectrometry of these spots revealed a third phosphorylation site (T178) in addition to the previously identified T84 and T202 (Supplementary data Fig. S4).

Sequential phosphorylation of PrcA

To further study the phosphorylation of the three Thr residues, wild-type and mutant alleles of *prcA* in which each relevant Thr codon was altered to Ala were co-expressed with *prcB* and purified from *E. coli* expressing *pknB*. When the phosphorylation level of each mutant PrcA was measured by immunoblotting with a phospho-T antibody and normalized by the amount of PrcA loaded, we found that PrcAT84A was not phosphorylated at all (lane 2, Fig. 2A), and that PrcAT178A and PrcAT202A showed 68% and 34% of the wild-type PrcA phosphorylation level, respectively (lanes 3 and 4, Fig. 2A). Phosphorylation signals were visible in the region slightly above the main band of the PrcA proteins, consistent with phosphorylation of this protein altering its mobility during SDS-PAGE (Feaver *et al.*, 1994). Phosphorylation of the PrcA proteins was specifically due to the kinase activity of PknB because no phospho-signal was detected from PrcA purified from cells with the kinase-defec-

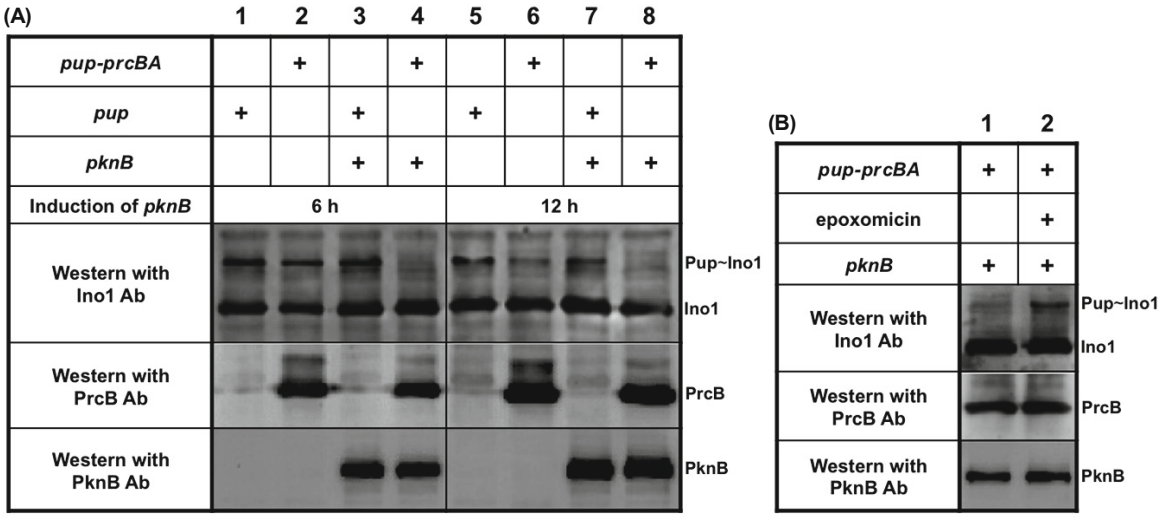

Fig. 3. Effect of PrcA phosphorylation on proteasomal degradation of Ino1 *in vivo*. (A) Degradation of Pup~Ino1 by the proteasome is enhanced by PknB. The *M. smegmatis ΔprcBA* strain carrying *6His-pup-prcBA* (pCK438) or *6His-pup* (pCK437) under the *hsp60* promoter was cultured until mid-log phase and *pknB* (pCK7) was induced by adding 0.2% acetamide for 6 and 12 h. 25 μg of total lysate protein from each cell was used for immunoblots with Ino1, PrcB, and PknB antibodies. Location of Pup~Ino1 was determined based on its size and recognition by the Ino1 antibody. (B) PknB enhances proteolytic activity of the proteasome, not the depupylation of Pup~Ino1. The *M. smegmatis ΔprcBA* strain carrying *6His-pup-prcBA* (pCK438) and acetamide-inducible *pknB* (pCK7) was grown until mid-log phase with 50 M epoxomicin (lane 2) or the vehicle DMSO (lane 1), and *pknB* was induced by adding 0.2% acetamide for 6 h. 25 μg of total lysate protein was subjected to immunoblotting as in (A).

tive PknBK40M (lane 5, Fig. 2A). In addition, PknB must directly phosphorylate PrcA because *E. coli* has neither a homolog of PknB nor the proteasome. The sum total of phosphorylation levels of the three PrcA mutants was approximately equal to that of wild-type PrcA, suggesting that the three Thr residues comprise the main phosphorylation sites in PrcA. Furthermore, these data suggest a sequential phosphorylation event of PrcA in which T84 phosphorylation is the prerequisite for that of T202, which is in turn required for T178 phosphorylation.

If the three Thr residues are sequentially phosphorylated and if Thr (T) to Glu (E) mutation mimics the phosphorylated form of PrcA as observed in our previous study on Wag31 (Jani *et al*., 2010), we predicted that the phosphomimetic PrcAT84E would be phosphorylated more than the doubly phosphomimetic PrcAT(84, 202)E, which in turn should be phosphorylated to a greater extent than the triply phosphomimetic PrcAT(84, 202, 178)E. To test this possibility, these phosphomimetic forms of PrcA were co-expressed with PrcB, purified from *E. coli*, and incubated with PknB *in vitro*. Rv1422, a known substrate of PknB (Kang *et al*., 2005), was included as a control. While the overall phosphorylation levels of these PrcA mutant forms were lower than what we observed *in vivo* (Fig. 2A) or that of Rv1422 (lane 5, Fig. 2B), the magnitude of the phosphorylation levels was PrcAT84E > PrcAT(84,202)E > PrcAT(84,202,178)E (Fig. 2B). This result further supports the sequential phosphorylation event of PrcA by PknB.

The role of PrcA phosphorylation in proteolytic activity of the proteasome

The phosphorylation of PrcA may be a mechanism by which the proteasome modulates its proteolytic activity in *M. tuberculosis*. To test this possibility, the proteolytic activity against a peptide substrate (Suc-LLVY-Amc) of the proteasome containing phosphorylated PrcA (16 h sample in Fig. 1C) was compared to the activity of the same proteasome complex in which PrcA had been dephosphorylated. Dephosphorylation of PrcA did not show a clear effect on the proteolytic activity against this peptide *in vitro* (Supplementary data Fig. S7).

We then examined whether PrcA phosphorylation affects the degradation of a *bona fide* substrate *in vivo*. For this experiment we used Ino1, a known substrate of the proteasome in both *M. tuberculosis* and *M. smegmatis* (Burns *et al*., 2009; Festa *et al*., 2010; Watrous *et al*., 2010). Quantitative immunoblot analysis on cell lysates was performed using an antibody raised against *M. tuberculosis* Ino1 that also detects *M. smegmatis* Ino1 (Burns *et al*., 2010). To examine effects of PrcA phosphorylation on Ino1 degradation, we introduced constructs constitutively expressing *M. tuberculosis pup* or *pup-prcBA* into an *M. smegmatis ΔprcAB* strain in which expression of *M. tuberculosis pknB* could be induced. The presence of the *M. tuberculosis* PrcBA complex decreased pupylated Ino1 (Pup~Ino1) levels in the *M. smegmatis ΔprcBA* strain (compare lanes 1 and 2 or lanes 5 and 6, Fig. 3A), indicating that the *M. smegmatis* Ino1 is also degraded by the *M. tuberculosis* proteasome. In the presence of the proteasome complex, the Pup~Ino1 level was decreased to a much greater extent by *pknB*-overexpression (compare lanes 3 and 4, or lanes 7 and 8).

Since these data cannot differentiate between Pup~Ino1 degradation and Pup~Ino1 depupylation, a similar experiment was performed in the presence of the proteasome inhibitor, epoxomicin. Inclusion of epoxomicin promoted an increase of Pup~Ino1 similar to levels observed when bacteria lacked PknB, suggesting that *pknB*-overexpression and

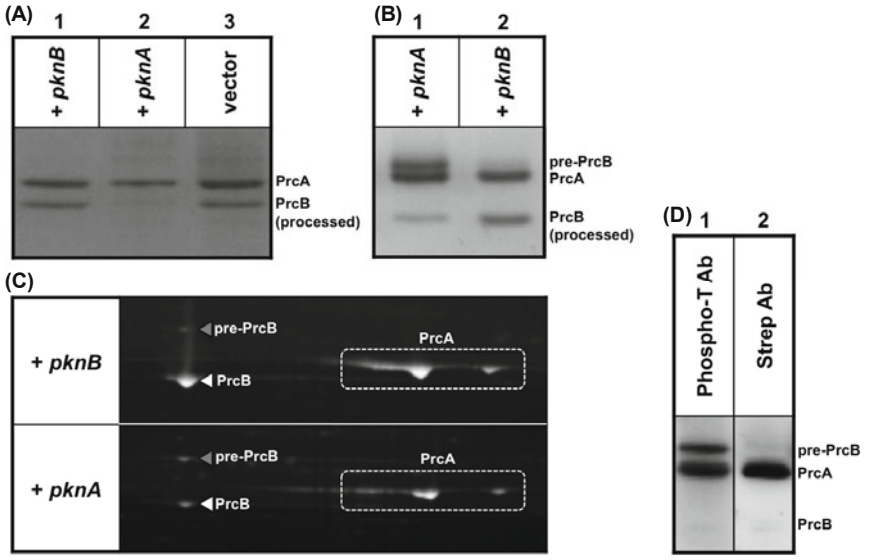

Fig. 4. PknA affects proteasome assembly via phosphorylating pre-PrcB and PrcA. (A) Effect of PknA on proteasome integrity in *M. smegmatis*. PrcA-strep was purified from *M. smegmatis ΔprcBA* expressing *pknA*, *pknB,* or vector following induction with 0.2% acetamide for 16 h. Approximately 3 μg of PrcA-trep plus co-purified PrcB was analyzed in 1-D PAGE and stained with Coomassie blue. Note that the total amount of PrcA-strep plus PrcB from *M. smegmatis* expressing *pknA* was 5–10 fold lower than *pknB*-expressing or vector alone cells and thus we had to adjust the loading to obtain this figure. (B) Effect of PknA on proteasome integrity in *E. coli*. 5 μg of PrcA-strep plus co-purified PrcB was analyzed in 1-D PAGE and stained with Coomassie blue. As in (A), we had to adjust the loading because *pknA*-xpression in *E. coli* also caused low level of PrcA-strep plus PrcB. (C) 5-fold more protein than was used in (A) was analyzed in 2-D PAGE and stained with Flamingo Stain. (D) PrcA and pre-PrcB are phosphorylated by PknA. 1 μg of PrcA-strep plus co-purified PrcB from *E. coli* expressing *pknA* (lane 1 in B) was analyzed by immunoblotting with phospho-T (lane 1) and strep (lane 2) antibodies.

subsequent phosphorylation enhance proteolytic activity of the proteasome (Fig. 3B). Taken together, these results suggest that the direct phosphorylation of PrcA by PknB is the most likely cause of enhanced proteolysis of proteasomal substrates, although an indirect effect via another substrate of PknB affecting proteasome activity cannot be excluded.

PknA affects the processing of PrcB during proteasome assembly

Because PrcB from *M. smegmatis* was phosphorylated by PknA *in vitro* (Fig. 1B), we wanted to test whether *M. tuberculosis* PrcB is also phosphorylated by PknA. For this, we expressed *prcB-prcA-strep* and attempted to purify the proteasome complex from the *M. smegmatis ΔprcBA* strain expressing *pknA*, similar to the experiment for PrcA phosphorylation in Fig. 1C. As controls, the *ΔprcBA M. smegmatis* strains overexpressing *pknB* or containing vector alone were included. While the formation of holo-proteasome or half-proteasome was not affected by *pknB*-overexpression (lanes 1 and 3, Fig. 4A; Supplementary data Fig. S8), *pknA*-overexpression markedly decreased recovery of mature PrcB (lane 2, Fig. 4A), suggesting that phosphorylation by PknA inhibited proteasome assembly or made the proteasome complex unstable.

To examine if this effect of PknA on the proteasome is direct, we examined the proteasome integrity in the presence of *pknA*- or *pknB*-expression in *E. coli* because *E. coli* possesses neither a PknA homolog nor the proteasomal system (Fig. 4B). While the formation of holo-proteasome appeared to be unaffected by *pknB*-expression, *pknA*-expression resulted in a mixture of half-proteasome and holo-proteasome, suggesting that PknA affects the processing of PrcB during the assembly of holo-proteasome. This result also

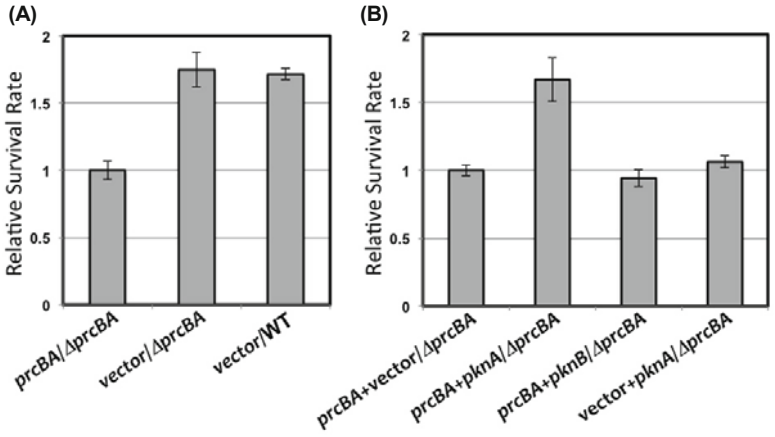

Fig. 5. Mycobacterial resistance to H_2O_2 is increased by *pknA*-expression. (A) *ΔprcBA M. smegmatis* with constitutive *prcBA* expression (1st bar) or vector alone (2nd bar), or wild-type *M. smegmatis* with vector alone (3rd bar) was treated with 4 mM H_2O_2 for 45 min and CFUs were determined. Survival rate was calculated by dividing the CFUs at 45 min with H_2O_2 by the CFUs at 45 min without H_2O_2. For relative survival rate, the survival rate of the *M. smegmatis ΔprcBA* cells with *prcBA* (1st bar) was set as one. p-value for the difference (two-tailed, unpaired t-tests): *prcBA*/*ΔprcBA* vs. vector/*ΔprcBA* = 0.038, and vector/*ΔprcBA* vs. vector/WT = 0.26. (B) The *M. smegmatis ΔprcBA* cells expressing *prcBA* in the presence of *pknA*-(2nd bar), *pknB*-overexpression (3rd bar) or vector alone (1st bar) was treated with 4 mM H_2O_2 for 45 min and the relative survival rate was determined as described in (A). As a control, *ΔprcBA M. smegmatis* that contains only *pknA*-overexpression cassette without *prcBA* was included (4th bar). p-value for the difference (two-tailed, unpaired t-tests): *prcBA*+vector/*ΔprcBA* vs. *prcBA*+*pknA*/*ΔprcBA* = 0.012, *prcBA*+vector/*ΔprcBA* vs. *prcBA*+*pknB*/*ΔprcBA* = 0.309, and *prcBA*+vector/*ΔprcBA* vs. vector+*pknA*/ *ΔprcBA* = 0.198. All the data shown are from a representative experiment of three biologic replicates each performed with triplicate plating. Data are represented as means +/- 1 SD.

suggests that PknA exerts this effect by directly phosphorylating PrcB or PrcA since *E. coli* possesses neither a PknA homolog nor the proteasomal system.

Further examination of the PrcA-strep purifications from *M. smegmatis* (lanes 1 and 2, Fig. 4A) was then performed by loading 5-fold more proteins in a 2-D SDS-PAGE experiment with detection of proteins using a fluorescent stain (Fig. 4C). This revealed weak but distinct spots of pre-PrcB and PrcB from *pknA*-overexpressing cells, compared to a near-complete processing of PrcB in *pknB*-overexpressing cells. Of note, the total amount of PrcA-strep plus PrcB purified from both *M. smegmatis* and *E. coli* expressing *pknA* was 5 to 10 fold lower than that of *pknB*-expressing or vector alone cells, suggesting that PknA may also affect the stability of the half- and/or holo-proteasome complex.

To test which proteasomal subunit is phosphorylated by PknA, the mixture of PrcA-strep and PrcB purified from *E. coli* expressing *pknA* (lane 1, Fig. 4B) was analyzed in immunoblots with phospho-T and strep antibodies (Fig. 4D). Strong phospho-signals were found from pre-PrcB and PrcA but not from processed PrcB, suggesting that PknA phosphorylates the half-proteasome and thereby regulates its assembly into the holo-proteasome.

Since the total proteasome level was decreased by *pknA*-expression (Figs. 4A and 4B), it was also possible that PknA destabilized the half-proteasome and/or the holo-proteasome complex after the assembly. To test this possibility, we incubated the holo-proteasome complex with PknA or PknB *in vitro* and examined its integrity by an in-gel proteasome assay in a native PAGE. Incubation with active PknA or PknB disrupted neither the holo-proteasome complex nor its proteolytic activity (Supplementary data Fig. S9). Taken together, these results suggest that PknA phosphorylation of PrcA and/or pre-PrcB inhibits the assembly of the holo-proteasome.

Impact of PknA on oxidative stress resistance via proteasome assembly

Previous studies demonstrated that depletion of the proteasome and the proteasome-associated proteins (Mpa and PafA) rendered *M. tuberculosis* more susceptible to nitric oxide but increased its resistance to H_2O_2 by about 2–3 fold (Darwin *et al.*, 2003; Gandotra *et al.*, 2007). Since we found that *pknA*-expression diminished the holo-proteasome level (Fig. 4), we sought to determine whether *pknA*-expression also enhances mycobacterial resistance to H_2O_2. To test this possibility, we first examined if depletion of the proteasome causes a similar phenotype in *M. smegmatis*. In contrast to the results in *M. tuberculosis*, the survival of *ΔprcBA M. smegmatis* following exposure to H_2O_2 (2nd bar, Fig. 5A) was almost identical to that of wild-type *M. smegmatis* (3rd bar). These results suggest that the proteasome in *M. smegmatis* may have different physiological functions than that

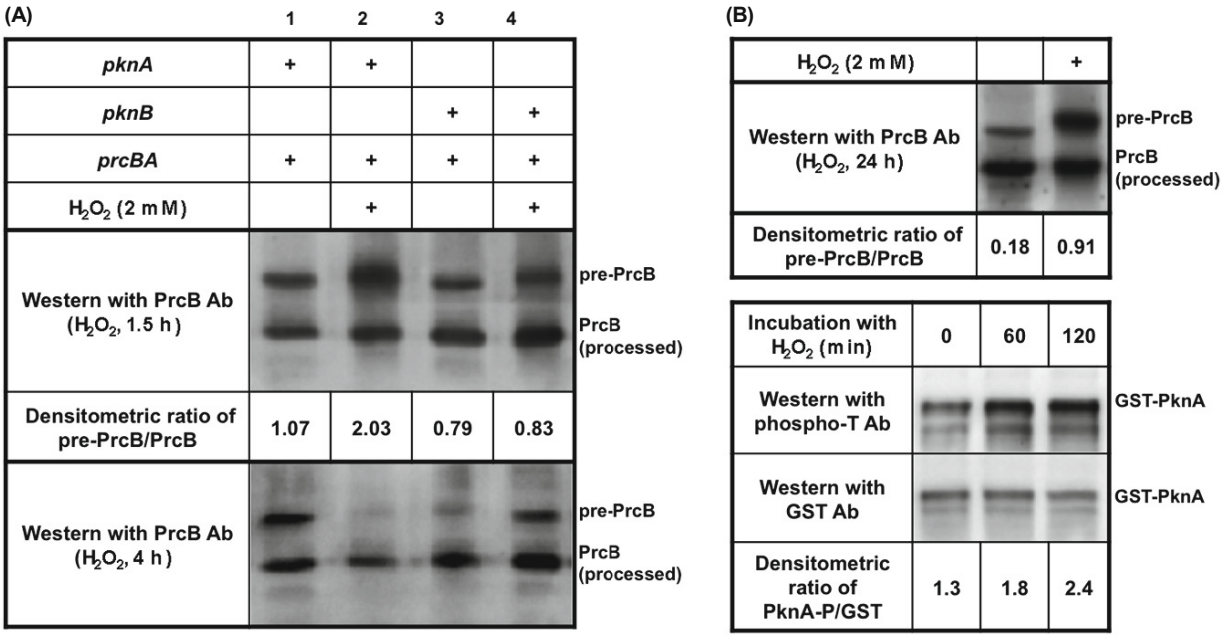

Fig. 6. PknA plays an important role in H_2O_2 stress response. (A) H_2O_2 impedes the formation of holo-proteasome in a PknA-dependent manner. (A) The *M. smegmatis ΔprcBA* strain that constitutively expresses *prcBA* (pCK322) and contains *pknA*- (pCK5) or *pknB*-expression (pCK7) cassette was cultured with 0.1% acetamide for 6 h to induce *pknA* or *pknB*. Cells were then treated with 2 mM H_2O_2 for additional 1.5 and 4 h. 20 μg total lysate protein from each culture was analyzed in immunoblot with a polyclonal PrcB antibody to test the processing of PrcB. Densitometric ratio of pre-PrcB and processed PrcB was determined by dividing the intensity of the Western signal from pre-PrcB by that of processed PrcB measured by Quantity One software (Bio-Rad). (B) Formation of the holo-proteasome is also affected by H_2O_2 in *M. tuberculosis*. *M. tuberculosis* mc^{2}6230 was cultured to log phase and treated with or without 2 mM H_2O_2 for 24 h. 20 μg total lysate protein from each culture was analyzed in immunoblot with a polyclonal PrcB antibody. The ratio of pre-PrcB to PrcB was determined as in (A). (C) Autophosphorylation of PknA increases in response to H_2O_2. *E. coli* BL21 (DE3) containing a pGEX-4T-3-*pknA* plasmid (pCK3) was cultured in the presence of 0.5 mM IPTG for 6 h, and then exposed to 2 mM H_2O_2 for 0, 60, and 120 min. The levels of the PknA phosphorylation and the GST-PknA were determined by immunoblottings with phospho-T (row 1) and GST (row 2) antibodies, respectively. Densitometric ratio of the phosphorylation level and the GST-PknA level was determined as in (A).

of *M. tuberculosis*, possibly reflecting their different habitats.

Interestingly, the introduction of *M. tuberculosis prcBA* into *ΔprcBA M. smegmatis* decreased the resistance to H_2O_2 as in *M. tuberculosis* (survival rate of *ΔprcBA M. smegmatis* (2nd bar, Fig. 5A) was 75% higher than that of the mutant expressing *M. tuberculosis prcBA-strep* (1st bar); $p<0.038$). This result provided us a tool to test whether *pknA*-expression, which influences proteasome integrity, can enhance resistance to H_2O_2 of *ΔprcBA M. smegmatis* carrying *prcBA*. Figure 5B shows that *pknA*-expression in the presence of *prcBA* (2nd bar) confers 67% higher resistance to H_2O_2 than cells containing vector only (1st bar) ($p<0.012$). This increased resistance to H_2O_2 by *pknA*-expression was statistically significant and was obtained consistently over three independent experiments. In contrast, co-expression of *pknB* and *prcBA* did not change the resistance to H_2O_2 (3rd bar, Fig. 5B), consistent with the intact proteasome complex formation under *pknB*-overexpressing condition shown in Fig. 4A. In addition, *pknA*-overexpression alone did not change the bacterial survival rate in the presence of H_2O_2 (4th bar, Fig. 5B), indicating that PknA contributes to H_2O_2 resistance mainly through the proteasome.

If PknA increases the resistance to H_2O_2 via the proteasome, we predicted that H_2O_2 stress would inhibit the formation of holo-proteasome in a PknA-dependent manner. To test this prediction, we performed immunoblotting with a PrcB antibody and examined the formation of the holo-proteasome under H_2O_2 treatment in an *M. smegmatis ΔprcBA* strain that constitutively expresses *prcBA* in the presence of *pknA*- or *pknB*-expression (Fig. 6A). Two-fold more pre-PrcB was accumulated in cells expressing *pknA* after 1.5 h H_2O_2 treatment (compare lanes 1 and 2, upper panel, Fig. 6A), but not in cells with *pknB*-expression (lanes 3 and 4). Longer treatment of H_2O_2 dramatically diminished the levels of both pre-PrcB and processed PrcB in *pknA*-expressing cells while processing of PrcB was near complete in *pknB*-expressing cells (lower panel, Fig. 6A). The decrease in PrcB levels in H_2O_2-treated and *pknA*-expressing cells (lane 2, lower panel, Fig. 6A) was not due to the potential effect of prolonged *pknA*-expression on *prcBA* transcription because comparable levels of the *prcB* transcript were found in those cells with or without H_2O_2 treatment (Supplementary data Fig. S10). More importantly, processing of PrcB in *M. tuberculosis* was also greatly retarded by H_2O_2 treatment (Fig. 6B). Finally, when *E. coli* cells expressing *pknA* were cultured in the presence of H_2O_2, we found that PknA increased its autophosphorylation level (Fig. 6C), suggesting that PknA can be activated by H_2O_2. Taken together, these data suggest that PknA plays an important role in the oxidative stress response by impeding the formation of holo-proteasome in *M. tuberculosis*.

Discussion

Many studies in eukaryotic and archeal proteasomes have demonstrated phosphorylation of both 20S proteasomal and regulatory subunits. However, the role of phosphorylation in various proteasome systems is somewhat confusing because both positive and negative effects on proteasomal activity and stability/assembly have been observed. For example, phosphorylation in α3 and α7 subunits of the proteasome from rat liver appeared to increase the proteolytic activity and the formation of the 26S complex, but hyper-phosphorylation of the same subunits induced by ethanol stress decreased the proteasome activity through affecting its stability (Mason *et al*., 1996; Bardag-Gorce *et al*., 2004). Interferon-γ has been shown to decrease the phosphorylation of α3 and α7 subunits, which in turn destabilized the regular 26S complex but increased the assembly of immunoproteasome during the immune response (Rivett *et al*., 2001). Similarly, phosphorylations in the regulatory subunits of proteasomes have also shown diverse effects on proteasome activity or assembly. For instance, phosphorylation of Rpt6 increased the activity and assembly of the 26S proteasome but phosphorylation on Rpn2 decreased proteasomal activity (Satoh *et al*., 2001; Zhang *et al*., 2007; Lee *et al*., 2010; Jarome *et al*., 2013). Phosphorylation of PA28 also appears to enhance the activity of the immunoproteasome (PA28-20S proteasome complex) (Li *et al*., 1996). Though somewhat complex, these findings suggest that phosphorylation is an important regulatory mechanism of proteasome function in eukaryotes.

In this work, we identified a sequential phosphorylation of the three Thr residues in PrcA by the PknB Ser/Thr kinase, which enhanced the degradation of the proteasomal substrate, Ino1, suggesting that PknB may regulate proteolytic activity of the *M. tuberculosis* proteasome. We also determined that the PknA kinase phosphorylated both pre-PrcB and PrcA resulting in inhibition of processing of pre-PrcB and assembly of the holo-proteasome. We also demonstrate that holo-proteasome complex formation can be inhibited through activation of PknA by H_2O_2, which may then contribute to enhanced resistance to H_2O_2. This mechanism may be important *in vivo*, where *M. tuberculosis* is subjected to oxidative stress within the host macrophage.

We identified three phosphorylation sites in PrcA. Two of these, T178 and T202, are located on the surface of the proteasome molecule. In particular, T202 is located within a surface loop that is disordered and is not resolved in the *M. tuberculosis* proteasome crystal structure (Hu *et al*., 2006). These data suggest that PknB can readily bind and phosphorylate these two Thr residues. The addition of two negatively charged substituents within this region could potentially alter the structure of PrcA and therefore its interactions with accessory proteins such as Mpa and ultimately affect the proteasomal activity. This interpretation is supported by our data indicating the enhanced degradation of Pup~Ino1 by *pknB*-overexpression *in vivo*, although we do not have evidence for direct interaction between Mpa and the proteasome containing the phosphorylated PrcA. Interestingly, the third phosphorylated residue, T84, is located within the internal chamber, near the junction between the PrcA and PrcB subunits, suggesting that T84 must be phosphorylated before the assembly into the half-proteasome. This prediction is consistent with the sequential phosphorylation of PrcA where T84 is the first residue to be phosphorylated.

Although it is unclear how exactly the sequential phosphorylation in PrcA is controlled, examination of the relative position of the three Thr residues and the chemical bonds

of neighboring residues may provide a clue. There exists a hydrogen-bonded network connecting T84 and T202, and phosphorylation at T84 may influence the local structure surrounding T202, which may then position T202 appropriately for phosphorylation by PknB (Supplementary data Fig. S11). In the native proteasome structure, the side chain of T178 is hydrogen-bonded to the backbone carbonyl of L233, and so is unavailable for phosphorylation by PknB prior to phosphorylation of T202. However, following T202 phosphorylation, it could form an ionic interaction with R189 resulting in the displacement of the C-terminus of PrcA, which would subsequently makes T178 available for phosphorylation (Supplementary data Fig. S11).

The *M. tuberculosis* proteasome is known to have a low intrinsic proteolytic activity with an apparent closed gate. Deletion of the α-subunit N-terminal octapeptide increases the activity by opening the gate (Lin *et al*., 2006). While the pupylated Ino1 level was decreased by PknB-expression in *M. smegmatis in vivo*, PrcA phosphorylation did not affect the proteasomal degradation of the peptide substrate *in vitro*. This finding suggests that PrcA phosphorylation may not modulate the proteolytic activity of the proteasome by causing a conformational change to open the gate. Rather, it may do so by contributing to other processes such as interaction with Mpa carrying a substrate or direct interaction with a substrate.

Darwin *et al*. (2003) and Gandotra *et al*. (2007) have independently shown that depletion of the proteasomal system resulted in 2- to 3-fold higher resistance of *M. tuberculosis* against H_2O_2 stress. Consistently, we have found that PknA hindered the proteasome assembly, which increased mycobacterial resistance to H_2O_2. We also demonstrated that the H_2O_2 stress activates PknA. This activation, by inhibiting proteasome assembly could provide a mechanism to enhance *M. tuberculosis* resistance to H_2O_2 *in vivo*. It has been shown that the H_2O_2 level remains high even after 1 h of infection while the NO level increases only after 72 h in *M. tuberculosis*-infected macrophages (Vishwanath *et al*., 1997). Because the *pknA* transcript is only seen at the early stage of *M. tuberculosis* infection in human macrophages (Malhotra *et al*., 2010), it is tempting to speculate that PknA may contribute to the resistance of *M. tuberculosis* to H_2O_2 during the early stages of infection. PknA may sense the H_2O_2 stress signal at this stage and affect the assembly of the proteasome, which may then lead to survival of oxidative stress proteins such as catalase-peroxidase (KatG), whose activity has been shown to correlate with resistance to H_2O_2-mediated killing of *M. tuberculosis* (Manca *et al*., 1999).

Many aspects of proteasome phosphorylation remain to be defined. There are likely to be additional substrates of the PrcA-phosphorylated proteasome. Further study will also be required to determine how the phosphorylation of pre-PrcB and PrcA regulates the assembly of the proteasome complex. Nonetheless, our findings demonstrate novel mechanisms for regulating proteasome activity via phosphorylation by bacterial STPKs. These mechanisms provide means not only for regulation of proteolytic activity of the proteasome, but also for at least partial resistance to oxidative stress of *M. tuberculosis*, in contrast to the rapid high-level resistance via SigH-dependent stress response (Song *et al*., 2003). These mechanisms further provide a link between the regulation of growth and cell wall synthesis by the PknA/B kinases and stress response regulation by the proteasome. This linkage is consistent with a systems view of bacterial physiology in which multiple signaling and regulatory pathways work in concert to allow adaptation to ever-changing environmental conditions.

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