Protein Kinase G Is Required for Intrinsic Antibiotic Resistance in Mycobacteria

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Antibiotic resistance and virulence of pathogenic mycobacteria are phenotypically associated, but the underlying genetic linkage has not been known. Here we show that PknG, a eukaryotic-type protein kinase previously found to support survival of mycobacteria in host cells, is required for the intrinsic resistance of mycobacterial species to multiple antibiotics.

Although intrinsic antibiotic resistance and host persistence have been viewed as two isolated characteristics of mycobacterial species, in vitro and in vivo observations have demonstrated phenotypic correlations between these two phenomena (10, 14, 21). Mycobacterium tuberculosis surviving in a persistent state during latent infection usually displays a reduced antibiotic susceptibility compared to that of the bacterium during active growth (12, 27–29). Conversely, exposure to antibiotics may also induce a transformation of M. tuberculosis from an active growth to a persistent state (11).

As an intracellular pathogen, the key factor deciding the successful persistence of M. tuberculosis in the host is its ability to survive within infected cells (15). Beside several host factors, mycobacterial lipids, and the RD1 specialized secretion system (4, 23, 25), mycobacterial protein phos- phatases and kinases are also emerging as key players in this process (1, 25, 26). The eukaryotic-type protein kinase G (PknG) is involved in mycobacterial survival within macrophages, presumably by blocking lysosomal delivery (26). It has been generally thought that factors required for pathogenicity such as PknG are not encoded in nonpathogenic mycobacteria, for example, Mycobacterium smegmatis (8, 26). However, recent studies showed that pknG is ubiquitously present in the Mycobacterium genus (7, 17). The M. tuberculosis PknG (PknG_Mtb) and M. smegmatis PknG (PknG_Msm) share almost 80% identity and appear at syntenic loci of the genomes (7) (Fig. 1A). These observations suggested that PknG might provide physiological functions to both nonpathogenic and pathogenic mycobacteria besides its role in pathogenicity of the latter species.

Another feature that makes eradication of M. tuberculosis difficult is its profound intrinsic antibiotic resistance (14). In addition to a repertoire of dedicated antibiotic resistance mechanisms, the thick and hydrophobic cell envelope constitutes an effective barrier decelerating the influx of antibiotics (13, 14). In a screen to identify genome-wide drug resistance determinants of mycobacteria, a library of more than 5,000 transposon mutants derived from wild-type M. smegmatis mc²155 (22) was generated by Himar1-mediated mutagenesis (13). The library was subjected to an exhaustive screen to identify mutants sensitive to single or multiple antibiotics. One of the identified mutants, MAR4, displayed increased sensitivity to many clinically important antibiotics of diverse chemical structures and mechanisms of action. These include antibiotics targeting cell wall synthesis (imipenem, vancomycin, and ethambutol), protein synthesis (erythromycin), folate synthesis (sulfachloropyridazine), and transcription (rifampin [rifampicin]) (Table 1). Notably, rifampin and ethambutol are frontline tuberculosis drugs. MICs for five representative antibiotics quantified by using the Etest assay (AB Biodisk, Sweden) as previously described (13) indicated that MAR4 was 8-, 12-, 15-, 4-, and >8-fold more susceptible than the wild-type strain to erythromycin, vancomycin, imipenem, ethambutol, and rifampin, respectively (Table 1).

Arbitrary PCR was done as previously described (13, 18) to map the transposon insertion in MAR4 to msmeq_0786, the gene encoding PknG_Msm (Fig. 1A). The pknG_Msm disruption in MAR4 was confirmed by PCR using primers flanking the gene, MS-PknG1 (GAATTCCATATGACTTCACCCGAGAACC) and MS-PknG2 (AAGCTTCCATGAAAGCACGGTCGACGTG) (Fig. 1A). The PCR product generated from MAR4 template was larger than that of the wild type, consistent with Himar1 transposon insertion (Fig. 1B). Sequencing of the PCR product identified the insertion site at the dinucleotide TA₁₀¹₅₋₁₀₁₆, which introduced a stop codon after the triplet encoding the Ile³⁷⁸ residue and created a gene truncation (Fig. 1A, bottom). To confirm loss of the pknG gene product in MAR4, Western blot assays were done using a polyclonal antibody raised against the M. tuberculosis PknG (26). The antibody recognized a protein band of ~78 kDa corresponding to the molecular mass of PknG in cell extract of wild-type M. smegmatis which was undetectable in the extract of MAR4 (Fig. 1C).

To confirm that the multidrug-sensitive phenotype of MAR4 was due to pknG deletion but not secondary mutations or polar effects on downstream genes, a targeted pknG mutant (the ΔpknG_Msm strain) was constructed by the recombineering method with modifications (24). Plasmid pVN701B was first constructed by cloning the SpeI-XbaI fragment encoding the
mycobacteriophage Che9c recombination proteins gp60 and gp61 from pJV53 (24) into the XbaI site of pPR27 (19) (Fig. 2A). pVN701B was thus replicated from a temperature-sensitive origin of replication [Ori(Ts)] and expressed the counterselectable marker gene sacB, which is toxic for mycobacteria in the presence of sucrose (19, 20). These genetic elements facilitated homologous recombination and removal of pVN701B after gene deletion was completed. The pknGMsm deletion cassette (Fig. 2B) was constructed by cloning the chromosomal DNA sequences flanking pknGMsm (arrows in panel A) from genomic DNA of wild-type M. smegmatis mc2155 and MAR4. (C) Western blot assay using Pkn GMtb antibody that recognizes PknG in cell extracts of M. bovis BCG and wild-type M. smegmatis mc2155 but not in MAR4 extract.

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### TABLE 1. Susceptibility of M. smegmatis strains to antibiotics

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (mg/liter)</th>
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<tbody>
<tr>
<td></td>
<td>EM</td>
</tr>
<tr>
<td>mc2155</td>
<td>32</td>
</tr>
<tr>
<td>MAR4</td>
<td>4</td>
</tr>
<tr>
<td>mc2155/pknGK181M</td>
<td>12</td>
</tr>
<tr>
<td>ΔpknG_Msm strain</td>
<td>3</td>
</tr>
<tr>
<td>ΔpknG_Msm/pknGK181M strain</td>
<td>3</td>
</tr>
<tr>
<td>ΔpknG_Msm/pknG_Msm strain</td>
<td>32</td>
</tr>
<tr>
<td>ΔpknG_Msm/pknG_Msm strain</td>
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*Abbreviations: EM, erythromycin; VA, vancomycin; IP, imipenem; EB, ethambutol; RI, rifampin; ND, not determined.*
quences (Fig. 2B). Primers P1 (ATCGGCAGCAACCTGTTCTCGTT) and P2 (CTGCACGACTTCGAGGTGTTCGA) recovered the \( \Delta \text{pknG}_{\text{Adm}} \) deletion cassette and \( \text{pknG} \) locus in wild-type \( M. \text{smegmatis} \) mc\(^{2}\)155 and its derived \( \Delta \text{pknG}_{\text{Adm}} \) strain. (C) PCR confirmation of \( \text{pknG} \) deletion in eight random mutant candidates. (D) Removal of pVN701B by a temperature shift resulted in kanamycin sensitivity and sucrose resistance of the \( \Delta \text{pknG}_{\text{Adm}} \) strain. (E) Reverse transcription (RT)-PCR detection of \( \text{pknG} \) transcription in \( M. \text{smegmatis} \) strains. (F) Western blot detection of \( \text{PknG} \) in \( M. \text{smegmatis} \) strains. Samples of 35 µg of proteins (except for 10 µg of \( \Delta \text{pknG}_{\text{Adm}}/\text{pknG}_{\text{Adm}} \) sample) were loaded onto sodium dodecyl sulfate-polyacrylamide gels.

FIG. 2. Construction and complementation of the \( \Delta \text{pknG}_{\text{Adm}} \) strain. (A) Construction of pVN701B. (B) The \( \text{pknG}_{\text{Adm}} \) deletion cassette and \( \text{pknG} \) locus in wild-type \( M. \text{smegmatis} \) mc\(^{2}\)155 and its derived \( \Delta \text{pknG}_{\text{Adm}} \) strain. (C) PCR confirmation of \( \text{pknG} \) deletion in eight random mutant candidates. (D) Removal of pVN701B by a temperature shift resulted in kanamycin sensitivity and sucrose resistance of the \( \Delta \text{pknG}_{\text{Adm}} \) strain. (E) Reverse transcription (RT)-PCR detection of \( \text{pknG} \) transcription in \( M. \text{smegmatis} \) strains. (F) Western blot detection of \( \text{PknG} \) in \( M. \text{smegmatis} \) strains. Samples of 35 µg of proteins (except for 10 µg of \( \Delta \text{pknG}_{\text{Adm}}/\text{pknG}_{\text{Adm}} \) sample) were loaded onto sodium dodecyl sulfate-polyacrylamide gels.

Panel, indicating the presence of pVN701B. To remove pVN701B, cultures were shifted to 39°C on medium supplemented with only hygromycin. All of the recovered \( \Delta \text{pknG}_{\text{Adm}} \) clones became kanamycin sensitive and sucrose resistant, indicating the successful removal of the plasmid (Fig. 2D, right panel). Two complementation plasmids expressing \( \text{pknG}_{\text{Adm}} \) (pVN759) or \( \text{pknG}_{\text{Adm}} \) (pVN758) from the heat shock promoter were constructed as previously described (26) and used to transform into the \( \Delta \text{pknG}_{\text{Adm}} \) strain. Transformants were selected on 7H10 medium containing kanamycin (50 mg/liter).
Reverse transcription-PCR and Western blotting were used to analyze the transcription and translation of pknG, respectively. RNA samples were isolated from M. smegmatis strains using the RNeasy minikit (Qiaagen) and reverse transcribed by a standard method (7). The pknG cDNA was amplified using primers RTpknG1 (GCCACCGCACCTACACCGT) and RTpknG2 (GGTGTGGCGACCAGCAG), which recognized both Smegmatis and M. tuberculosis pknG sequences. Both reverse transcription-PCR and Western blotting (described above) readily detected transcription and translation of PknG in the wild-type M. smegmatis strain but not in the ΔpknG strain (Fig. 2E and F). Transformation of pVN578 or pVN579 restored both transcription and translation of PknG in the ΔpknG strain (Fig. 2E and F). Compared to M. bovis BCG (lane 1, Fig. 2F), a dramatic reduction of PknG signals was observed in wild-type M. smegmatis (lane 2, Fig. 2F). The reduced signal intensities likely reflected lower PknG production in M. smegmatis rather than a preferred specificity of the antibody for Mycobacterium bovis PknG (100% identical to PknG) because in trans expression of PknGΔdom or PknGΔmid from the same vector yielded comparable signals on Western blots (lanes 4 and 5, Fig. 2F). Interestingly, a recent study suggested that the differential expression of PknG may relate to its new function as a virulence factor in pathogenic mycobacteria (7). Antibiotic susceptibility was tested using the Etest assay as described above. Similarly to MAR4, the ΔpknGΔdom mutant became more sensitive to multiple antibiotics with diverse chemical structures and mechanisms of action (Table 1). In trans expression of pknGΔdom or pknGΔmid completely restored the multiple antibiotic resistance of the ΔpknGΔdom strain to wild-type levels (Table 1). Similar results were also obtained for wild-type M. tuberculosis, M. bovis BCG, their derived ΔpknG mutants (26), and the complemented strains expressing pknGΔmid from pVN578 (examples in Fig. 3), demonstrating that PknG provides a drug resistance function not only to the nonpathogenic M. smegmatis but also to pathogenic mycobacteria such as M. tuberculosis.

Furthermore, to assess whether the function of PknG in intrinsic antibiotic resistance is provided through its kinase activity, plasmid pPknGK181M, previously shown to create a dominant-negative effect by overexpressing a kinase-inactive form of PknG (26) (kindly provided by Jean Pieters, University of Basel), was introduced into wild-type M. smegmatis and the ΔpknGΔdom strain. Whereas overexpression of this dominant-negative PknGK181M form did not alter the antibiotic susceptibility of the ΔpknGΔdom strain, it resulted in an increased antibiotic susceptibility in the wild-type M. smegmatis strain, similar to the effect caused by pknG deletion (Table 1).

The nonspecific sensitivity of the pknG mutants to antibiotics with diverse chemical structures and mechanisms of action suggested two possibilities: (i) PknG controls activities of dedicated multiple antibiotic resistance systems such as efflux pumps or (ii) PknG controls essential cellular functions (for example, metabolism or cell envelope structure) whose disruption resulted in pleiotropic defects leading to the reduced drug resistance. Properties of the mycobacterial cell surface were characterized using previously described methods (3, 6). Compared to wild-type M. smegmatis, the cell wall of the ΔpknGΔdom strain displayed a significantly reduced hydrophobicity (Table 2), which correlates with a reduced association of Congo red, a lipid-bound hydrophobic dye that has been used as an indicator of phenotypic drug susceptibility (3). Reduced cell wall hydrophobicity has previously been reported to be important for multiple antibiotic resistance in mycobacteria (9, 13). Recent findings that PknG is involved in metabolic pathways such as the tricarboxylic acid cycle and glutamine utilization in Corynebacterium and Mycobacterium species (5, 16, 17) also favor the latter hypothesis. The fact that increased expression of PknG in the ΔpknGΔdom strain (lanes 4 and 5 in Fig. 2F) did not lead to a reduced drug susceptibility compared to wild-type...
**M. smegmatis** (Table 1) further suggested that PknG does not directly control dedicated antibiotic resistance systems.

In summary, we present comprehensive evidence supporting a role of PknG in the natural resistance of mycobacteria to multiple antibiotics. Both transposon-mediated and targeted disruption of pknG resulted in increased multiple antibiotic susceptibility. Together with previous reports (5, 26), our data suggest that signaling circuitries surrounding PknG might serve as a phenogenetic network that controls host persistence and intrinsic antibiotic resistance in mycobacteria (26). Pharmacological inactivation of PknG may thus not only inhibit the survival of pathogenic mycobacteria in macrophages (26) but also render the bacilli susceptible to a variety of readily available, clinically approved antibiotics. Further studies will need to address the underlying mechanisms that mediate PknG function in intrinsic antibiotic resistance of pathogenic mycobacteria.

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