Rv3168 Phosphotransferase Activity Mediates Kanamycin Resistance in Mycobacterium tuberculosis

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Introduction

Tuberculosis is a worldwide epidemic disease caused by Mycobacterium tuberculosis, with an estimated one-third of the human population currently affected. Treatment of this disease with aminoglycoside antibiotics has become less effective owing to antibiotic resistance. Recent determination of the crystal structure of the M. tuberculosis Rv3168 protein suggests a structure similar to that of Enterococcus faecalis APH(3')-IIIa, and that this protein may be an aminoglycoside phosphotransferase. To determine whether Rv3168 confers antibiotic resistance against kanamycin, we performed dose-response antibiotic resistance experiments using kanamycin. Expression of the Rv3168 protein in Escherichia coli conferred antibiotic resistance against 100 µM kanamycin, a concentration that effected cell growth arrest in the parental E. coli strain and an E. coli strain expressing the Rv3168 D249A mutant, in which the catalytic Asp249 residue was mutated to alanine. Furthermore, we detected phosphotransferase activity of Rv3168 against kanamycin as a substrate. Moreover, docking simulation of kanamycin into the Rv3168 structure suggests that kanamycin fits well into the substrate binding pocket of the protein, and that the phosphorylation-hydroxyl-group of kanamycin was located at a position similar to that in E. faecalis APH(3')-IIIa. On the basis of these results, we suggest that the Rv3168 mediates kanamycin resistance in M. tuberculosis, likely through phosphotransferase targeting of kanamycin.

Keywords: Mycobacterium tuberculosis, Rv3168, aminoglycoside phosphotransferase, kanamycin resistance, antibiotics

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Recent studies on the genome sequence of *M. tuberculosis* have suggested candidate genes responsible for the acquisition of aminoglycoside resistance [12, 16], and we have previously reported the crystal structure of Rv3168, a putative aminoglycoside phosphotransferase [7]. Although lacking significant amino acid sequence similarity, the overall structure of Rv3168 was similar to that of *E. faecalis* APH(3’)-IIIa, which is a characterized aminoglycoside phosphotransferase. Moreover, the structure of the ATP-bound form of Rv3168 implied an ATP-binding mode similar to that of *E. faecalis* APH(3’)-IIIa. Together with the existence of a large negatively charged substrate-binding pocket located near the ATP-binding pocket of Rv3168, these data collectively suggest that Rv3168 is a candidate phosphotransferase that confers aminoglycoside antibiotic resistance in *M. tuberculosis* [7].

Here, we report that an *E. coli* strain in which the Rv3168 expression is induced exhibits resistance to a concentration of kanamycin that is lethal to strains in which the Rv3168 is not induced. Moreover, Rv3168 protein has phosphotransferase activity against kanamycin as a substrate. Finally, docking simulation of kanamycin into the Rv3168 structure suggests a possible binding of the kanamycin substrate and the enzyme.

### Materials and Methods

#### Antibiotic Resistance Test

The Rv3168 coding gene (Gene ID: 888778) was cloned into pPROEX HTa (Life Technology), and the resulting plasmid pPROEX HTa:Rv3168 was transformed into the *E. coli* BL21(DE3) strain. The strain was grown overnight and the culture, to the final concentration of 1%, was inoculated into 1 L of LB broth containing 10 mM MgCl₂, 5 mM ATP, 25 mM kanamycin, and 50 µM Rv3168 protein, was incubated overnight at room temperature, and 1 µl aliquot of the reaction mixture was spotted onto a cellulose-F TLC plate (Merck). For the controls, the ATP and ADP molecules and the reaction mixture without the protein were spotted as well. Ascending TLC was performed with a buffer containing saturated ammonium sulfate, 3 M sodium acetate, and isopropanol (80/6/2) in the closed chamber for 3 h. The hydrolysis of ATP was monitored by visualizing nucleotides under UV light.

#### Docking Simulation

The docking simulations of a kanamycin molecule to the Rv3168 structure was performed using the Autodock Vina program [15]. As a template, the crystal structure of Rv3168 in complex with Mg²⁺ and ATP was used (PDB code 3ATT). The water, acetate, calcium, and glyceral molecules were removed from the crystal structure of Rv3168 and hydrogen atoms were added in accordance with only polar atoms. The structure of kanamycin was obtained from PDBChem [2] and was prepared as pdbqt files. For the precise docking simulation, the substrate binding site of the Rv3168 structure was defined using Autodocking tools software. The simulation results were checked using the PyMOL software.

#### Results and Discussion

#### Kanamycin Resistance of Rv3168-Expressing *E. coli*

We previously reported the crystal structure of Rv3168 protein, a putative aminoglycoside phosphotransferase in *M. tuberculosis*. Based on the structural comparison of *M. tuberculosis* Rv3168 with *Enterococcus faecalis* APH(3’)-IIIa, a characterized aminoglycoside phosphotransferase that confers aminoglycoside resistance in this strain, we speculated that Rv3168 was a candidate phosphotransferase family enzyme.
Kanamycin Antibiotic Resistance by Rv3168

November 2013 | Vol. 23 | No. 11

conferring aminoglycoside resistance to *M. tuberculosis*. We first determined whether Rv3168 confers the antibiotic resistance effect when expressed in *E. coli*. A pPROEX HTa vector harboring an inducible Rv3168 gene was transformed into an *E. coli* strain, which was then treated using various concentrations of kanamycin. The Rv3168-expressing *E. coli* strain expressing the Rv3168 protein.

For the measurement of the antibiotic resistance activity of Rv3168 against kanamycin, the Rv3168 coding gene was cloned into the pPROEX HTa vector, and the resulting plasmid was transformed into *E. coli* strain BL21(DE3). The cell growth under various concentrations of kanamycin was measured spectrophotometrically at A₆₀₀. Each experiment was performed with and without IPTG induction, and presented with open and closed circles, respectively. pEX is an abbreviation of a pPROEX HTa vector. (A) The cell growth of the *E. coli* strain harboring pEX:Rv3168 without kanamycin. (B) The cell growth of the *E. coli* strain harboring an empty pEX vector under 100 µM kanamycin. (C–E) The cell growth of the *E. coli* strain harboring pEX:Rv3168 under 50, 100, and 200 µM kanamycin, respectively. (F) The cell growth of the *E. coli* strain harboring pEX:Rv3168D249A under 100 µM kanamycin.

Fig. 1. Kanamycin resistance of the *E. coli* strain expressing the Rv3168 protein.
exhibited resistance to 100 µM of kanamycin (Figs. 1A–1D), a concentration which effected growth arrest in the E. coli strains in which Rv3168 was not expressed and in strains harboring an empty pEX vector under 100 µM kanamycin. (C–E) The cell growth of the E. coli strain harboring pEX:Rv3168 under 50, 100, and 200 µM kanamycin, respectively. (F) The cell growth of the E. coli strain harboring pEX:Rv3168D249A under 100 µM kanamycin.

To confirm the kanamycin resistance activity of Rv3168, we collected aliquots of each of the above cultures, and spread them on LB solid medium with ampicillin. The viable cell growth showed marked results with the same tendency as those observed using spectrophotometric cell growth measurement (Fig. 2). The E. coli strain expressing Rv3168 showed high cell growth in the presence of 100 µM kanamycin, whereas no significant cell growth was observed for strains in which Rv3168 expression was not induced (Figs. 2A–2D). Moreover, the viable cell count of the E. coli strain expressing Rv3168 was markedly decreased by the addition of 200 µM kanamycin (Fig. 2E). As anticipated, the E. coli strain expressing the Rv3168D249A mutant had low viability in 100 µM kanamycin (Fig. 2F). Based on these
spectrophotometric and viable cell growth measurements, we concluded that Rv3168 confers antibiotic resistance at low concentrations of kanamycin.

**Phosphotransferase Activity of Rv3168**

To determine whether the kanamycin resistance was mediated by possible phosphotransferase activity of the Rv3168 protein, we performed a phosphotransferase activity assay by using the recombinant Rv3168 protein. When the reaction mixture containing 5 mM ATP, 50 mM kanamycin, and 50 µM of Rv3168 was incubated for 20 min, and spotted on a TLC plate, no significant phosphotransferase activity was detected (Fig. 3). Increasing the time to overnight, however, resulted in detectable ATP hydrolysis, indicating that the recombinant Rv3168 protein has very low phosphotransferase activity (Fig. 3). Moreover, ATP hydrolysis was undetectable in a reaction mixture containing the Rv3168D249A mutant protein instead of the wild-type Rv3168 protein (Fig. 3). These results indicate that the Rv3168 protein has kanamycin phosphotransferase activity, which may confer kanamycin resistance to *M. tuberculosis*. The low kanamycin phosphotransferase activity of the Rv3168 protein is consistent with the results showing that the Rv3168 protein conferred antibiotic resistance only in low concentrations of kanamycin. The low phosphotransferase activity and mild antibiotic resistance effect of Rv3168 in *E. coli* are conceivably attributable to the fact that the protein originates from the wild-type *M. tuberculosis* H37Rv strain, which does not show a strong antibiotic resistance to aminoglycosides. In general, mutations in the target proteins of antibiotics confer antibiotic resistance to the organism, as previously shown in the wild-type *M. tuberculosis* strain H37Rv, which acquired aminoglycoside resistance through mutations in the 30S ribosomal subunit. We speculated that mutations in the Rv3168 protein might increase its aminoglycoside phosphotransferase activity, thereby conferring greater aminoglycoside resistance to *M. tuberculosis*. With this in mind, the examination of Rv3168-coding sequences in aminoglycoside-resistant *M. tuberculosis* strains may shed light on whether specific mutations, if any, play a role in conferring resistance.

**Kanamycin Binding Simulation**

The previously reported crystal structure of Rv3168 showed that the highly charged large substrate binding pocket can accommodate a charged aminoglycoside substrate, and that Gly248, Asp249, Asn254, and Asp267 form an xxDxxxxNx kinase motif that is located in the tunnel connecting the ATP and substrate-binding pockets. We next modeled the complex structure of Rv3168 bound to kanamycin to identify the substrate binding mode of the protein. Unfortunately, we were unable to obtain the complex structure owing to the blocking of the substrate-binding entrance by a neighboring molecule of the *P2_12_2_1* crystal packing, thereby preventing the entry of a kanamycin molecule. We then performed autodocking simulation of a kanamycin molecule by using the ATP-bound form of the Rv3168 structure (PDB code 3ATT). In the simulated Rv3168-kanamycin complex structure, a kanamycin molecule was observed to fit well in the substrate-binding pocket (Fig. 4A). The charged kanamycin substrate appeared to be stabilized by the charged residues, including Asp50, Thr52, Glu57, Asp249, Arg251, Glu269, Thr344, Arg347, Arg348, and Glu353. The overall orientation of bound kanamycin in Rv3168 was distinct from that in *E. faecalis* APH(3')-IIIa, whereas the orientation and the binding mode of ATP were almost identical (Fig. 4A). Interestingly, when the simulated structure of the Rv3168-kanamycin complex was superimposed onto that of the kanamycin-bound *E. faecalis* APH(3')-IIIa, the phosphorylation-hydroxyl groups of kanamycin of the two proteins were observed to be located at the same positions (Fig. 4B). Moreover, the phosphorylation-hydroxyl group of kanamycin in Rv3168 is proximal to the catalytic Asp249 residue ~3.2 Å, which is comparable to that observed in the kanamycin-bound form of *E. faecalis* APH(3')-IIIa (Fig. 4B). These observations suggest that kanamycin is a
natural substrate for Rv3168, which functions as a kanamycin phosphotransferase with a reaction mechanism similar to that of \textit{E. faecalis} APH(3')-IIIa.

We also performed the antibiotic resistance test of Rv3168 against other aminoglycoside antibiotics such as neomycin and streptomycin, resulting in no significant resistance effect, and the protein exhibited no detectable phosphotransferase activity with these antibiotics as well (data not shown). Taken together, we suggest that Rv3168 does not have broad aminoglycoside substrate specificity, but rather the resistance is limited to kanamycin. In future studies, we recommend the detailed examination of this protein.

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References


\textbf{Fig. 4.} Docking simulation of a kanamycin molecule to the Rv3168 structure.

Docking simulation of a kanamycin molecule into the ATP-bound form of the Rv3168 structure was performed using the Autodock Vina program. (A) Comparison of kanamycin binding to Rv3168 (top) and \textit{E. faecalis} APH(3')-IIIa (bottom). The proteins are presented as electrostatic potential models. The bound kanamycin and ATP molecules are shown as stick models with cyan and yellow colors, respectively, and labeled. (B) Positions of bound kanamycin molecules. The simulated kanamycin-bound form of Rv3168 was superposed with the kanamycin-bound form of \textit{E. faecalis} APH(3')-IIIa. The catalytic Asp residues, the bound magnesium atoms, and kanamycin molecules of \textit{M. tuberculosis} Rv3168 are shown with cyan color and indicated as “Rv”. Those of \textit{E. faecalis} APH(3')-IIIa are shown with green color and indicated as “Ef”. The phosphorylation-hydroxyl atoms of Rv3168 and \textit{E. faecalis} APH(3')-IIIa are indicated by dotted circles with cyan and green colors, respectively. The distances between the catalytic Asp residue and the phosphorylation-hydroxyl atom of Rv3168 and \textit{E. faecalis} APH(3')-IIIa are shown with cyan and green colors, respectively.