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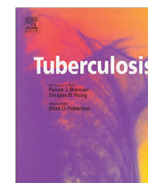

Mycobacterium tuberculosis $MtrA_{Y102C}$ is a gain-of-function mutant that potentially acts as a constitutively active protein

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ABSTRACT

The MtrAB histidine-aspartate signal transduction of mycobacteria includes the response regulator MtrA and sensor kinase MtrB. We recently showed that *Mycobacterium smegmatis ΔmtrB* is filamentous, defective for cell division, cell shape maintenance and shows compromised MtrA target gene expression. Interestingly, overproduction of phosphorylation competent *M. tuberculosis* $MtrA_{Y102C}$ reverses the Δ*mtr*B mutant phenotype, although the genetic basis of phenotype reversal is unknown. Here we show that introduction of D56N mutation in $MtrA_{Y102C}$ completely abolished its phosphorylation potential yet the double mutant protein retained a partial ability to reverse the *mtr*B mutant phenotype indicating that phosphorylation activity is not necessary for the function of $MtrA_{Y102C}$. The phosphorylation-defective $MtrA_{D56N-Y102C}$ protein bound its target promoters *rip*A and *fbp*B efficiently. Together, these results support a hypothesis that the gain-of-function phenotype of $MtrA_{Y102C}$ is in part due to its ability to function as a constitutively active protein in the absence of phosphorylation.

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1. Introduction

The histidine-aspartate two-component signal transduction system (2CRS) consisting of membrane bound sensor kinase and cytosolic response regulator (RR) is one of the several regulatory networks that bacteria often use to modulate their gene expression in response to changes in environmental conditions.[1,2] The genome of *Mycobacterium tuberculosis* contains 11-paired 2CRS, several orphan sensor kinases and response regulators.[3] The organization and sequences of the components of the 2CRS are well conserved across all mycobacterial members indicating that their functions are likely conserved. While the published data suggest that the activities of all characterized 2CRS contribute to *M. tuberculosis* survival under stressful growth conditions and promote pathogen proliferation upon infection [reviewed in],[4] majority of their targets and the pathways affected are largely unknown. MtrAB 2CRS includes MtrA RR and MtrB sensor kinase. MtrA RR contains a N-terminal signal receiving regulatory domain and a C-terminal DNA-binding effector domain.

The MtrAB system is one of the two essential 2CRS in *M. tuberculosis*[5-7] and it likely plays important role(s) in DNA replication, cell division and the maintenance of cell-wall homeostasis.[8-12] Overproduction of MtrA compromises *M. tuberculosis* ability to proliferate upon infection in macrophages and mice lungs whereas simultaneous overproduction of MtrB along with MtrA reverses the growth defect.[9] These studies indicate that optimal proliferation of *M. tuberculosis* upon infection depends in part on the ratio of phosphorylated MtrA (MtrA~P) to MtrA and that one role of the MtrB kinase is to regulate MtrA phosphorylation state.[9] These studies also revealed that D56 residue is important for MtrA phosphorylation. MtrB sensor kinase exhibits autophosphorylation activity, interacts with and transphosphorylates MtrA.[13]

MtrA is poorly phosphorylated in vitro; the published crystal structure data indicate that the orientation of MtrA signal-receiving and DNA-binding domains promote inactive conformation, thereby inhibition of MtrA phosphorylation activity.[14] These studies also showed that Y102 residue is located at the interface of MtrA signal-receiving and DNA-binding domains.[14] $MtrA_{Y102C}$ protein is phosphorylation competent[11] and its overproduction restored *mtr*B mutant phenotype including the expression of the MtrA-regulon indicating that $MtrA_{Y102C}$ functions independent of MtrB sensor kinase activity. While the genetic basis of *mtr*B mutant phenotype reversal by $MtrA_{Y102C}$ is unknown, one possibility is that replacement of tyrosine at position 102 with cysteine produced a gain-of-function MtrA that is also phosphorylation competent. This possibility implies that phosphorylation activity is not relevant for its function and that $MtrA_{Y102C}$ acts as a constitutively active protein. Another possibility is that MtrA is phosphorylated by one of the remaining sensor kinases when MtrB function is lost and that Y102C mutation facilitates such phosphorylation activity, hence restoration of the *mtr*B mutant phenotype. Experiments presented in this study are designed to address the first possibility. We show by creating and characterizing the $MtrA_{Y102C}$

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protein defective for phosphorylation that $MtrA_{Y102C}$ functions as constitutively active for phosphorylation.

2. Materials and methods

2. 1 Growth and Culture conditions

Escherichia coli strain Top10 F' was used for cloning purposes and was propagated in Luria Bertani (LB) broth or LB agar containing various antibiotics as needed (ampicillin (Amp) at 100 μg/ml, kanamycin (Km) at 50 μg/ml and hygromycin (Hyg) at 200 μg/ml). *M. smegmatis* mC^2155 (wild type, WT) and its derivatives were cultured in Middlebrook 7H9 broth or 7H10 agar supplemented with albumin-dextrose-catalase-sodium chloride and appropriate antibiotics as needed (Km at 25 μg/ml or Hyg at 10 μg/ml). Growth of broth cultures was monitored by absorbance measurements at A600.

2. 2 Molecular techniques

Construction of plasmid pDS4 expressing $mtrA_{Y102C}$ from the amidase promoter was previously described.[11] $MtrA_{Y102C}$ mutation, created by QuikChange site-directed mutagenesis kit (Stratagene Corp., La Jolla, CA), was described previously. Using a similar strategy, oligonucleotide primer-pair D56N_F (5'-CTGGTGTTATTGAACTTGATG-3') and D56N_R (5'-CATCAAGTTCAATAACACCAG-3') and the same kit, the $MtrA_{D56N-Y102C}$ mutation was created and the corresponding plasmid was named pNM29. Transformation of *M. smegmatis* mC^2155 and confirmation of recombinant strains was as described.[15] Coding regions from pDS4 and pNM29 were PCR amplified and cloned in pMALC4e and the corresponding plasmids, pDS3[13] and pKS47 (this study) were electroporated into *E. coli* ArcticExpress (DE3) RIL (Stratagene Corp., La Jolla, CA). Recombinant $MtrA_{Y102C}$ and $MtrA_{D56N-Y102C}$ proteins were purified essentially as described.[13]

2.3 Phosphorylation assay

E. coli EnvZ kinase was phosphorylated for 5 min in a phosphorylation buffer (PB) containing 50 mM Tris–HCl, pH 7.5, 50 mM KCl and 1 mM DTT supplemented with 20 mM $MgCl_2$ (Mg^{2+}), 10 mM $CaCl_2$ (Ca^{2+}) or both (Mg^{2+} and Ca^{2+}). Reactions were initiated by adding ^{32}P-ATP and incubated for 2, 5, 10, 20 and 30 min at 37°C. An aliquot of the phosphorylated EnvZ was mixed with either MtrA, $MtrA_{Y102C}$ or $MtrA_{D56N-Y102C}$, incubated for 15 min, resolved by SDS–PAGE, dried gel exposed to a phosphoimager screen and scanned in a Bio-Rad Molecular Imager.

2.4 Gel mobility shift assay

Electrophoretic mobility shift assay (EMSA) to detect MtrA binding to MtrA target promoters, *PfbpB* and *PripA*, were carried out using FITC-labeled promoter DNA in a buffer containing 50 mM Tris–HCl pH 7.0, 50 mM sodium chloride, 10 mM magnesium chloride, 10 mM calcium chloride, 1 mM DTT, 0.1 mM EDTA, 5% glycerol, 0.01% NP-40, 0.05 ng/μl sheared salmon sperm DNA, 250 nM poly dI/poly dC, 100 nM FITC-labeled promoter fragments and indicated concentrations of phosphorylated or non-phosphorylated MtrA as described.[12] The samples were incubated for 15 min at 37°C and resolved by 5% TAE native gel electrophoresis (120 V, 4°C, 40 min). MtrA protein was phosphorylated by EnvZ in the presence of 20 mM cold ATP for 15 min at 37°C. FITC-labeled *PfbpB* or *PripA* were prepared by amplification of respective cloned promoter regions using FAM-labeled T7 universal and Sp6 primers.[12]

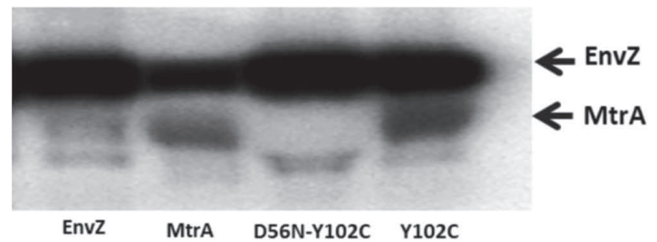

Figure 1. Phosphorylation of MtrA mutants: EnvZ protein was autophosphorylated by incubating with γ-^{32}P-ATP in 1X PB buffer for 60 min. Phosphorylated EnvZ was then incubated with MBP-MtrA, MBP-$MtrA_{Y102C}$ or MBP-$MtrA_{D56N-Y102C}$ for 5 min, proteins were resolved by SDS-PAGE and phosphorylated proteins visualized in a BioRad molecular imager.

2.5 Microscopy

M. smegmatis WT and Δ *mtrB Pami::*$mtrA_{D56N-Y102C}$ cells were grown with 0.02% acetamide and imaged by brightfield microscopy as described.[11] A Nikon Eclipse 600 microscope fitted with a 100X Nikon Plan Fluor oil-immersion objective with a numerical aperture of 1.4 was used for bright-field microscopy. Images were acquired using a Photometrics Coolsnap ES camera and Metamorph (version 6.2) imaging software (Universal Imaging Corporation). All images were acquired at identical exposure times and optimized to similar extents with respect to brightness and contrast using Adobe Photoshop CS4.

3. Results and Discussion

3. 1 $MtrA_{D56N-Y102C}$ is not proficient for phosphorylation

We showed earlier that $MtrA_{D56N}$ is defective for phosphorylation[9] whereas $MtrA_{Y102C}$ is phosphorylation competent and complements the defects due to loss of MtrB.[11] To separate the phosphorylation activity from its function, we introduced D56N mutation in the $MtrA_{Y102C}$ background and tested whether the double mutant protein is functional in reversing the *mtr*B mutant phenotype including binding to MtrA targets as proficiently as $MtrA_{Y102C}$. To this end, the mutant gene was cloned downstream of T7 promoter and amidase promoters for expression in *E. coli* and mycobacteria, respectively (see methodology for details). To test the phosphorylation ability, the recombinant $MtrA_{D56N-Y102C}$ was incubated with EnvZ kinase and ^{32}P-γ-ATP, and autoradiography was performed. Both MtrA and $MtrA_{Y102C}$ proteins were used as controls. As can be seen, the $MtrA_{D56N-Y102C}$ protein was not phosphorylated under these conditions whereas both MtrA and $MtrA_{Y102C}$ showed distinct phosphorylation indicating that introduction of D56N mutation in $MtrA_{Y102C}$ abolished the latter's phosphorylation ability (Fig. 1).

3.2 $MtrA_{D56N-Y102C}$ complements M. smegmatis mtrB mutant

To test whether $MtrA_{D56N-Y102C}$ functions like $MtrA_{Y102C}$ in reversing *mtr*B mutant phenotype, we electroporated pNM29, an integration proficient vector expressing *mtr*A $_{D56N-Y102C}$ from acetamide-inducible amidase promoter, into *M. smegmatis mtr*B mutant. The recombinant strain was grown with 0.2% acetamide for 24 h prior to visualizing the cells by brightfield microscopy. Consistent with the published data, the *mtr*B mutant was clumpy, elongated and often contained bulbous heads when compared to parent *M. smegmatis* [see Fig. 2, panels i and ii], whereas the *mtr*B mutant overproducing $MtrA_{Y102C}$ following growth in the presence of acetamide showed near WT phenotype (Fig. 2, panel iii, see also[11]). Interestingly, the

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