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A Single-Amino-Acid Substitution in the C Terminus of PhoP Determines DNA-Binding Specificity of the Virulence-Associated Response Regulator from *Mycobacterium tuberculosis*

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The *Mycobacterium tuberculosis* PhoP–PhoR two-component system is essential for virulence in animal models of tuberculosis. Genetic and biochemical studies indicate that PhoP regulates the expression of more than 110 genes in *M. tuberculosis*. The C-terminal effector domain of PhoP exhibits a winged helix–turn–helix motif with the molecular surfaces around the recognition helix (α8) displaying strong positive electrostatic potential, suggesting its role in DNA binding and nucleotide sequence recognition. Here, the relative importance of interfacial α8–DNA contacts has been tested through rational mutagenesis coupled with *in vitro* binding-affinity studies. Most PhoP mutants, each with a potential DNA contacting residue replaced with Ala, had significantly reduced DNA binding affinity. However, substitution of nonconserved Glu215 had a major effect on the specificity of recognition. Although lack of specificity does not necessarily correlate with gross change in the overall DNA binding properties of PhoP, structural superposition of the PhoP C-domain on the *Escherichia coli* PhoB C-domain–DNA complex suggests a base-specific interaction between Glu215 of PhoP and the ninth base of the DR1 repeat motif. Biochemical experiments corroborate these results, showing that DNA recognition specificity can be altered by as little as a single residue change of the protein or a single base change of the DNA. The results have implications for the mechanism of sequence-specific DNA binding by PhoP.

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Much of the reason for the success of *Mycobacterium tuberculosis* as an intracellular pathogen lies in its ability to adapt to its host environments through signal transduction leading to switching on of complex transcriptional programs.[1] It is now known that the major response of the bacterium to environmental changes is through classical two-component regulatory systems via histidine–aspartate phosphorelay between the sensor kinase and the response regulator.[2] A number of recent studies revealed that PhoP of the PhoPR system controls a variety of functions including synthesis of complex pathogenic lipids, hypoxia response through DosR cross-talking, respiratory metabolism, secretion of the major T-cell antigen ESAT-6, and stress response[3–9] (for a review, see Ref. [10]). Further supporting the role of PhoP in regulation of *M. tuberculosis* virulence, two recent articles suggest that a point mutation in PhoP contributes to avirulence and also accounts for the absence of polyketide-derived acyltrehaloses in *M. tuberculosis* H37Ra.[11,12]

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Abbreviations used: EMSA, electrophoretic mobility shift assay; GdnHCL, guanidine hydrochloride.

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PhoP, a member of the OmpR/PhoB subfamily of response regulators, is composed of two functional domains, an N-terminal receiver domain (PhoPN) and a C-terminal effector domain (PhoPC), involved in DNA binding. The only reported interaction of PhoP from *M. tuberculosis* H37Rv involves binding of the regulator to its own promoter leading to autoregulation.[8] This observation was extended to show that the primary interaction believed to promote autoregulation involves sequence-specific recognition of two PhoP protomers to the target DNA comprising a 9-bp direct repeat motif (present within the *phoP* promoter region).[13] Moreover, biochemical evidences suggest head-to-head binding of two molecules of full-length PhoP monomers on a direct repeat motif projecting their N-termini toward one another.[13] Although global gene expression profiling shows that 44 genes are up-regulated and another 70 genes are down-regulated by PhoP in *M. tuberculosis*,[5] the origins of DNA binding affinity and sequence specificity of the regulator remain largely unknown.

The crystal structure of PhoPC clearly shows that the primary DNA binding of the protein involves a winged helix–turn–helix motif[14] [Protein Data Bank (PDB ID) 2PMU] and the surface around the PhoP residues that constitute the recognition helix (residues Asn212–Tyr224 of α8) display strong positive electrostatic potential, indicating that these residues are likely to be critical in DNA binding and nucleotide sequence recognition. To this end, we used structure-guided mutagenesis to obtain single alanine substitutions of 10 solvent-exposed residues spanning α8. Our results of rational mutagenesis coupled with a study of the DNA binding affinity of the α8–DNA interface in the complex formed by PhoP and its cognate DNA demonstrate that most PhoP mutants have significantly reduced DNA binding affinity while possessing near-wild-type stability. However, alanine substitution of Glu215 of α8 shows a major effect on the specificity of DNA recognition. Using structural insights coupled with biochemical analyses, we found that Glu215 of PhoP appears to establish a base-specific interaction with $(G/C)^9$ of the upstream repeat motif (DR1 of DR1,2) to contribute significantly to the recognition specificity of the regulator.

Amino acid mutagenesis of the PhoP–DNA interface

To decipher the origins of binding affinity and sequence specificity, we set out to identify amino acid residues of α8 that are important for sequence-specific DNA binding of PhoP. The three-dimensional structure of *M. tuberculosis* PhoPC (residues 144–247) and the modeled structure of the PhoPC–DNA complex[14] served as a guide for selection of amino acid residues to be mutated. Substitution sites of amino acid residues (as shown in Fig. 1a) with solvent-exposed side chains were selected, since these residues are expected to form base-specific hydrogen bonds, ionic interactions, or van der Waals contacts with the DNA duplex and are therefore likely to contribute to the level of DNA binding affinity and recognition specificity.

Wild-type and mutant PhoP proteins from *M. tuberculosis* H37Rv (Table 1) were purified as fusion proteins containing an N-terminal polyhistidine tag. To examine the effect of Ala substitution of PhoP on the overall structure and/or fold, mutant proteins were compared for their stability. To this effect, equilibrium denaturation of PhoP and PhoP mutants by guanidine hydrochloride (GdnHCl) was monitored by change in CD ellipticity at 220 nm. Expectedly, both wild-type and mutant proteins underwent loss of structure (native to denatured) with increasing GdnHCl concentration. The values for free energy of denaturation in the absence of GdnHCl ($\Delta G_u^{H_20}$) and the change in free energy with GdnHCl (m) obtained from multiple denaturation experiments are listed in Table 2. Eight of the 10 mutants have $\Delta G_u^{H_20}$ values within ± 0.5 kcal/mol of wild-type PhoP. Of the other two mutants, PhoPS219A and PhoPR222A showed a reduction in stability of 0.9 and 0.7 kcal/mol, respectively, compared to the wild-type protein.

We next investigated DNA binding of purified wild-type and mutant proteins by electrophoretic mobility shift assay (EMSA) using an oligonucleotide-based DR1,2 probe consisting of two direct repeat units (DR1 and DR2). To this effect, His tags were cleaved using Thrombin Clean Cleavage kit from Sigma. When incubated with end-labeled DR1,2 DNA, the majority of the point mutants (7 out of 10) under the conditions examined showed at least 10-fold reduced DNA binding affinity (based on the limits of detection in this assay and based on other gels; not shown) (Fig. 2). However, PhoPE215A (lanes 8–10, Fig. 2a), PhoPS216A (lanes 11–13, Fig. 2a), and PhoPY217A (lanes 2–4, Fig. 2b) formed a single retarded band stable to gel electrophoresis. As reference, wild-type PhoP at identical protein concentrations bound efficiently to the end-labeled DR1,2 probe (lanes 8–10, Fig. 2c). It should be noted that over a range of protein concentrations, the three mutant proteins (PhoPE215A, PhoPS216A, and PhoPY217A) showed wild-type PhoP-like DNA-binding properties with comparable affinity. From these results, we conclude that (i) most of the amino acid residues of the PhoP recognition helix with exposed side chains (residues Asn212, Val213, Ser219, Tyr220, Tyr222, Tyr223, and Lys224) are critical affinity determinants for PhoP–DNA interactions, and (ii) residues Glu215, Ser216, and Tyr217 do not appear to have any significant role in determining the DNA binding affinity of *M. tuberculosis* PhoP.

Specificity changes when PhoP residue Glu215 is substituted

To ascertain whether amino acids 215–217 contribute to the specificity of DNA recognition, the binding of PhoP and the three mutant proteins

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