**The Mycobacterium tuberculosis Ser/Thr Kinase Substrate Rv2175c Is a DNA-binding Protein Regulated by Phosphorylation**

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Recent efforts have underlined the role of serine/threonine protein kinases in growth, pathogenesis, and cell wall metabolism in *Mycobacterium tuberculosis*. Although most kinases have been investigated for their physiological roles, little information is available regarding how serine/threonine protein kinase-dependent phosphorylation regulates the activity of kinase substrates. Herein, we focused on *M. tuberculosis* Rv2175c, a protein of unknown function, conserved in actinomyctes, and recently identified as a substrate of the PknL kinase. We solved the solution structure of Rv2175c by multidimensional NMR and demonstrated that it possesses an original winged helix-turn-helix motif, indicative of a DNA-binding protein. The DNA-binding activity of Rv2175c was subsequently confirmed by fluorescence anisotropy, as well as in electrophoretic mobility shift assays. Mass spectrometry analyses using a combination of MALDI-TOF and LC-ESI/MS/MS identified Thr9 as the unique phosphoacceptor. This was further supported by complete loss of PknL-dependent phosphorylation of an Rv2175c_T9A mutant. Importantly, the DNA-binding activity was completely abrogated in a Rv2175c_T9D mutant, designed to mimic constitutive phosphorylation, but not in a mutant lacking the first 13 residues. This implies that the function of the N-terminal extension is to provide a phosphoacceptor (Thr9), which, following phosphorylation, negatively regulates the Rv2175c DNA-binding activity. Interestingly, the N-terminal disordered extension, which bears the phosphoacceptor, was found to be restricted to members of the *M. tuberculosis* complex, thus suggesting the existence of an original mechanism that appears to be unique to the *M. tuberculosis* complex.

In response to its environment, *Mycobacterium tuberculosis* (*M. tb*) activates or represses the expression of a number of genes to promptly adjust to new conditions. More precisely, during the infection process, cross-talk of signals between the host and the bacterium take place, resulting in reprogramming the host signaling network. Many of these stimuli are transduced in the bacteria via sensor kinases, enabling the pathogen to adapt its cellular response to survive in hostile environments. Although the two-component systems represent the classic prokaryotic mechanism for detection and response to environmental changes, the serine/threonine and tyrosine protein kinases (STPKs) associated with their phosphatases have emerged as important regulatory systems in prokaryotic cells (1–3). *M. tb* contains eleven STPKs (4, 5), and most are being investigated for their physiological roles and potential application for future development to combat tuberculosis (6). Through phosphorylation these STPKs are also thought to play important functions in cell signaling responses as well as in essential metabolic pathways. The cell wall of *M. tb* plays a critical role in the defense of this pathogen in the host, and changes in cell wall composition in response to various environmental stimuli are critical to *M. tb* adaptation during infection. Although little is known regarding the cell wall regulatory mechanisms in *M. tb*, there is now an increasing body of evidence indicating that these processes largely rely on STPK-dependent mechanisms (7–9).

Moreover, little information on the range of functions regulated by the STPKs is available, and the complicated mycobacterial phosphoproteome is still far from being deciphered. Understanding mycobacterial kinase biology has been severely impeded by the difficulty to identify direct kinase substrates and the subsequent characterization of the phosphorylation site(s). However, several recent studies have reported the iden-
tification and characterization of the phosphorylation sites in substrates related to various metabolic pathways in mycobacteria. These include the Fork Head-associated-containing protein GarA, a key regulator of the tricarboxylic cycle (10, 11); PbpA, a penicillin-binding protein required for cell division (12); Wag31, a homologue of the cell division protein DivIVA that regulates growth, morphology, and polar cell wall biosynthesis in mycobacteria (13); the β-ketoacyl carrier protein synthase mtFabH, which participates in mycolic acid biosynthesis (9); the anti-anti-sigma factor Rv0516c (14); the alternate synthase mtFabH, which participates in mycolic acid biosynthesis (13); the forkhead-associated-containing protein GarA, a key regulator of the tricarboxylic cycle (10, 11); and the division cell wall gene cluster, which encompasses several genes involved in cell wall synthesis and cell division (17, 18), raising the possibility that PknL might participate in the regulation of this gene cluster. Moreover, pknL (Rv2176) is adjacent to the Rv2175c gene, encoding a 16-kDa protein of unknown function. We further demonstrated that phosphorylation of the activation loop Thr-173 residue was required for optimal PknL-mediated phosphorylation of Rv2175c. Moreover, Rv2175c belongs to a mycobacterial “core” of 219 genes, identified by macroarray and bioinformatic analysis, common to M. tb- and Mycobacterium leprae-encoding proteins showing no similarity with proteins from other organisms. The presence of Rv2175c as a member of this set of genes emphasizes the importance of Rv2175c in the physiology of M. tb. In this context, we reasoned that the structural determination of Rv2175c would provide a valuable basis for a better understanding of the function of this protein.

Therefore, we have undertaken the structural determination of Rv2175c using multidimensional NMR techniques. Herein, we provide strong evidence that Rv2175c is a DNA-binding protein and investigated how phosphorylation of a unique Thr residue in the N-terminal domain of the protein affects its DNA-binding activity.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Strains used for cloning and expression of recombinant proteins were *Escherichia coli* DH5α (Invitrogen) and *E. coli* BL21(DE3)Star (Novagen). Strains were grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin.

**Cloning, Expression, and Purification of Rv2175c and Mutant Proteins**—The Rv2175c gene was amplified by PCR using *M. tuberculosis* H37Rv genomic DNA as a template and the following primers: #403, 5'-TAT ATA TCG TT CAT atg CCT GGC CGC GCA CCA GGC TCT-3' (containing an Ndel restriction site underlined) and, #404, 5'-TAT GGA TCC TCA ATA CGC CAT AGC CTG GGC CCG-3' (containing a BamHI restriction site underlined). This 441-bp amplified product was digested by Ndel and BamHI and ligated into pET15bTev, the variant of pPET15b (Novagen) that includes the replacement of the thrombin site coding sequence with a tobacco etch virus (TEV) protease site (19), and in pETPhos, the variant of pPET15bTev that is devoid of putative phosphoacceptors in the His tag fusion (20), thus generating pET15bTev_Rv2175c and pETPhos_Rv2175c, respectively. Site-directed mutagenesis was directly performed on pETPhos_Rv2175c using inverse-PCR amplification with the following self-complementary primers: 5'-GGCGGCCAATCGTCTGCGCGCGGTG-3' and 5'-GGCGGCCGACCCGCAAGTAGTGCGCTTGGTGCAGGCC-3' for Rv2175_T9A, 5'-GGCGGCCGACCCGCAAGTAGTGCGCTTGGTGCAGGCC-3' and 5'-GGCGGCCGACCCGCAAGTAGTGCGCTTGGTGCAGGCC-3' for Rv2175_T9D, 5'-GGCGGCCGACCCGCAAGTAGTGCGCTTGGTGCAGGCC-3' and 5'-GGCGGCCGACCCGCAAGTAGTGCGCTTGGTGCAGGCC-3' for Rv2175_T9E (the corresponding substitutions are shown in bold). The Rv2175c N-terminal truncated fragment lacking the first 13 residues was amplified by PCR using *M. tuberculosis* H37Rv genomic DNA as a template and the following primers: #615, 5'-TAA TAG CTC ATA TGG GCC GCA CAT TTC CCG CTG GGC AGC-3' and pETPhos_Rv2175c_T9A, pETPhos_Rv2175c_T9D, and pETPhos_Rv2175c_T9E (the corresponding substi-
tutions are shown in bold). All constructs were verified by DNA sequencing. Recombinant *E. coli* BL21(DE3)Star strains harboring the Rv2175c-expressing constructs pET15bTev_Rv2175c, pETPhos_Rv2175c, and pETPhos_Rv2175c_T9A, pETPhos_Rv2175c_T9D, and pETPhos_Rv2175c_T9E were used to inoculate 750 ml of LB medium supplemented with ampicillin and incubated at 37 °C with shaking. When A600 reached 0.6, isopropyl 1-thio-β-D-galactopyranoside was added at a final concentration of 0.2 mM, and growth was continued for an additional 3 h at 37 °C with shaking. Purifications of the Histagged proteins were performed as reported previously (8, 21). When required, the protein was treated with TEV protease. Finally, the purified protein was concentrated and applied to a Superdex 75 26/60 (Amersham Biosciences) size exclusion column, equilibrated in buffer 20 mM sodium acetate, pH 4.6, 300 mM NaCl to remove any remaining impurities and the cleaved tag when TEV protease cleavage was performed. The purified Rv2175c protein was identified by SDS-PAGE and stored at −20 °C until required. This protocol was carried out for all the non-labeled constructs and 15N labeled of wild-type and mutant Rv2175c constructs, excepted that the cultures were grown in a minimum media containing 15NH4Cl and 13C6 glucose as the sole nitrogen and carbon sources.

**Cloning, Expression, and Purification of the Recombinant PknL1-281 Protein**—The pknL PCR fragment encoding the kinase core domain corresponding to the kinase domain without the juxtamembrane linker of PknL (residues 1-281 out of 399) was amplified by PCR using *M. tuberculosis* H37Rv genomic DNA as a template and the following primers: #399, 5'-TAT ATG CCG TTG GAG AGC GCG CTT CTC-3' and #400, 5'-TAT TAC TGC TGA ATG CTC-3'.
Solution Structure of M. tuberculosis Rv2175c

(containing an NdeI restriction site underlined) and, #400, 5’-TAT GGG ACC TCA TTA CTC CTC GGC GAT CAG CTC CAG-3’ (containing a BamHI restriction site underlined). This 843-bp amplified product was digested by NdeI and BamHI and ligated into pET15bTev. E. coli BL21(DE3)Star cells were transformed with the pET15bTev_pknL1–281 vector expressing the PknL1–281 core domain. Purifications of the His-tagged protein was performed as above (8, 21). When required, the protein was treated with TEV protease according to the manufacturer’s instructions (Invitrogen).

Solution Structure of Rv2175c—NMR experiments were carried out at 14.1 Tesla on a Bruker Avance 600 spectrometer equipped with a 5-mm z-gradient 1H-13C-15N triple resonance cryoprobe. 1H, 13C, and 15N resonances were assigned using standard triple resonance three-dimensional experiments (22) recorded at 30 °C on 0.3 mM 15N- or 15N,13C-labeled Rv2175c protein samples dissolved in 10 mM sodium acetate, pH 4.6, 150 mM NaCl with 5% D2O for the lock. 1H chemical shifts were directly referenced to the methyl resonance of di-ethylsilapentane sulfonate, whereas 13C and 15N chemical shifts were referenced indirectly to the absolute frequency ratios 15N/1H = 0.101329118 and 13C/1H = 0.25144952. All NMR spectra were processed with GIFA (23). Nuclear Overhauser effect peaks identified on three-dimensional [1H,15N],[1H,13C][ and [1H,15N] three-dimensional nuclear Overhauser effect spectroscopy-HSQC were assigned through automated NMR structure calculations with CYANA 2.1 (24). Backbone φ and ψ torsion angle constraints were obtained from a data base search procedure on the basis of backbone (15N, HN, 13C\(^\text{N}\)), H\(^\text{a}\), and 13C\(^\text{b}\) chemical shifts using the program TALOS (25). Hydrogen bond restraints were derived using standard criteria on the basis of the amide 1H/2H exchange experiments and nuclear Overhauser effect data. When identified, the hydrogen bond was enforced using the following restraints: ranges of 1.8–2.3 Å for \(d(N-H, O)\), and 2.7–3.3 Å for \(d(N,O)\). The final list of restraints (from which values redundant with the covalent geometry have been eliminated) consisted of 477 intra-residue, 576 sequential, 278 medium-range (1 < i–j < 4), and 345 long range upper bound distance restraints, 190 backbone dihedral angle restraints (φ and ψ), and 66 hydrogen bond restraints. The 30 best structures (based on the final target penalty function values) were minimized with CNS 1.2 according the RECOORD procedure (26) and analyzed with PROCHECK (27). The root mean square deviations were calculated with MOLMOL (28). Structural statistics are given in (Table 1).

Electrophoretic Mobility Shift Assays—The DNA fragment used in this assay corresponds to 200 bp of non-relevant double stranded M. tuberculosis DNA. The 5’ ends of DNA were labeled using [γ-32P]ATP and T4 polynucleotide kinase. A typical assay mixture contained in 20 µl: 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% (v/v) glycerol, 0.5 mg of bovine serum albumin, radioactive DNA probe (2000 cpn·ml\(^{-1}\)), and various amounts of the purified Rv2175c proteins. After 30 min of incubation at room temperature, 20 µl of this mixture was loaded onto a native 4% (w/v) polyacrylamide TBE Ready Gel (Bio-Rad) and electrophoresed in 1% TBE (Tris-Borate-EDTA) buffer for 1 h at 100 V·cm\(^{-1}\). Radioactive species were detected by autoradiography after exposure using direct exposure to films.

Fluorescence Anisotropy—The 32-bp oligonucleotide with a 3’ reactive amine group (sequence 5’-CTC CAG GTC ACT GTG ACC TCC TC-3’) was purchased from Sigma Genosys. It was covalently labeled with Alexa Fluor 488 succinimidyl ester dye (Invitrogen). A 10-fold molar excess of the dye was added to a solution of DNA in 0.1 M sodium borate, pH 9.0, buffer, and the reaction was allowed to proceed at room temperature for 3 h with continuous agitation. The reaction was stopped by adding 10% Tris-HCl 1. M. After ethanol precipitation, the labeled oligonucleotide was further purified using reversed-phase chromatography on a C\(_2\)-C\(_18\) column. Finally, it was hybridized with a 1.1/1 molar excess of complementary strand. Steady-state fluorescence anisotropy binding titrations were carried out on a Tecan Saphire II microplate reader, using a 470 nm light emitting diode for excitation, and a monochromator set at 530 nm (bandwidth 20 nm) for emission.

In Vitro Kinase Assays—In vitro phosphorylation of PknL and Rv2175c proteins was carried out for 30 min at 37 °C in a reaction mixture (20 µl) containing buffer P (25 mM Tris-HCl, pH 7.0; 1 mM dithiothreitol; 5 mM MgCl\(_2\); 1 mM EDTA) with 200 µCi/ml [γ-32P]ATP. Phosphorylation of Rv2175c by PknL1–281 was performed with 3 µg of Rv2175c in 20 µl of buffer P with 200 µCi/ml [γ-32P]ATP and 500 ng of PknL1–281 for 30 min at 37 °C. The reaction was stopped by addition of an equal volume of 2× sample buffer, and the mixture was heated at 100 °C for 5 min. After electrophoresis, gels were soaked in 16% trichloroacetic acid for 10 min at 90 °C, and dried. Radioactive proteins were visualized by autoradiography using direct exposure to films. In vitro phosphorylation for NMR, EMSAs, and mass

### Table 1

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<tr>
<th>Protein</th>
<th>NMR and refinement statistics for Rv2175c protein structures</th>
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### Structure statistics

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### Ramachandran plot (%)

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### Average pairwise root mean square deviation (Å)

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<th>Backbone</th>
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<tr>
<td>Heavy</td>
<td>1.54 ± 0.18</td>
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* The pairwise root mean square deviation was calculated among 30 refined structures for residues 18–104 and 126–146.
spectrometry analysis was performed as described above except that [γ-32P]ATP was replaced by 5 mM non-radioactive ATP and incubated overnight.

**Mass Spectrometry Analysis**—Purified Rv2175c was subjected to in vitro phosphorylation by PknL_{1-28}, as described above with 5 mM cold ATP. Subsequent mass spectrometry analyses were performed as previously described (29).

**RESULTS**

Rv2175c **Solution Structure**—Rv2175c, a 146-amino acid protein with a calculated molecular mass of 16 kDa exhibits very weak similarity to transcriptional regulatory proteins harboring a DNA-binding domain (residues 32–52) with a helix-turn-helix (HTH) motif, as well as homologies to α-helix proteins like cyclines and cytochromes (17). Because bioinformatic analyses using several different web-based meta-servers failed to clearly identify Rv2175c as a typical DNA-binding protein, we decided to determine its structure, expecting that it would provide new insights with respect to its function. Rv2175c was isotopically labeled with 15N, and 15N-13C, expressed, and purified from *E. coli* carrying pET15bTev_Rv2175c. Following removal of its N-terminal His tag using TEV protease, purified Rv2175c was used for the structural determination by multidimensional NMR spectroscopy. By combining the information from the double and triple resonance heteronuclear experiments, we were able to assign all the amide group resonances (except Thr-29) for the non-proline residues (12 prolines), 93.8% of the other backbone resonances (Ca, C', and Hα), 76.7% of the Cβ resonances, and ~96% of the side-chain protons.

NMR experiments revealed that the N-terminal part (Met1–Ile49) and a large loop (Thr110–Asn122) of Rv2175c were fully disordered. The corresponding residues gave rise to the intense cross-peaks centered at 8.5 ppm in the HSQC spectrum (Fig. 1). Intriguingly, these two regions correspond to the less conserved parts of the protein found in different actinomycetes homologues. Noteworthy, the loop Thr110–Asn122 is present in mycobacteria but missing constantly in *Corynebacterium* or *Streptomyces* despite a sequence identity close to 60% for the core residues (Fig. 2). In addition, multiple sequence alignments of Rv2175c homologues revealed that, except for *M. tuberculosis* and *Mycobacterium bovis*, most homologues, including *Mycobacterium smegmatis*, lack the first twelve residues (Fig. 2). One notable exception is *Mycobacterium avium para tuberculosis*, which carries a 20-amino acid extension with respect to the *M. smegmatis* sequence.

We solved the structure of Rv2175c, which corresponds to a monomer constituted of two domains with six α-helices and two β-strands in total (Fig. 3, A and B) with flexibility existing between the N-terminal (residues Pro18 to Val77) and the C-terminal domains (residues Val78 to Tyr146). The 30 best structures were calculated and superimposed for the whole protein heavy atoms (except the first 16 residues) with a root mean square deviation of 1.1 Å, whereas the root mean square deviation calculated for the N-terminal or the C-terminal domain were of 0.7 Å and 0.85 Å, respectively (Fig. 4). Our data indicate that the N-terminal domain, with the topological α1, α2, β1, and β2 order, harbors an unusual prokaryotic winged helix-turn-helix (wHTH) DNA-binding motif missing the typical third helix (30) (Fig. 3, A and B). To our knowledge, the only characterized HTH DNA-binding proteins missing the third helix are the bacteriophage protein from the Xis nucleoprotein filament (31). In the Rv2175c N-terminal domain, the turn following the second strand allows the next six residues (Ile66 and Phe71) to cap the hydrophobic core formed by the two first helices of the wHTH, a role usually devoted to the third helix of the motif (Fig. 3C). Moreover, the Rv2175c C-terminal domain is composed of four anti-parallel helices (α3–α6) and their connecting turns turns together with a mobile 20-amino acid loop (residues Thr110–Asn122). If most of the hydrophobic residues are buried in the helix bundle, some remain partially solvent-exposed (Leu87, Val138, and Tyr146). Interestingly, in transcriptional regulators harboring a wHTH domain, the C-terminal segment usually possesses a dual role, a functional role as regulatory domain, and a structural role as an actor of protein dimerization when bound to DNA. The relative positioning of these two domains is dictated by contacts between the hydrophobic surface encompassing the residues from the C-terminal α-helices α4 (Ile46, Met48, and Phe103) and α3 residues (Val126 and Leu128) and the N-terminal residues Val22 and Phe79. Overall, the NMR solution structure determination of Rv2175c clearly identified structural features typically found in DNA-binding proteins with an original N-terminal wHTH domain as well as a C-terminal regulatory domain of unknown function.

Rv2175c **Is a DNA-binding Protein**—To confirm that Rv2175c is a DNA-binding protein two biochemical strategies were used. First, we confirmed by EMSA the ability of Rv2175c to bind non-relevant double-stranded DNA at low salt concentration. Because Rv2175c DNA targets remain unknown, the binding experiments were carried out using a typical assay mixture containing 50 mM NaCl with increasing amounts of purified Rv2175c. The results indicated that the Rv2175c protein interacted measurably with double-stranded DNA (Fig. 5A). An excess of cold DNA was also mixed along with the labeled probe, and the absence of a shifted probe indicated specificity of the binding activity of Rv2175c toward the probe.

DNA-binding activity of Rv2175c was further analyzed by fluorescence anisotropy determination, a method of choice to measure interaction between different molecules like protein/DNA. A 30-bp DNA segment was labeled with Alexa Fluor 488 dye and mixed with unlabeled Rv2175c. As expected, in the absence of salt, Rv2175c exhibited a substantial DNA-binding activity (Fig. 5C). However, increasing NaCl concentrations from 0 to 150 mM resulted in a dramatic decrease in the affinity for DNA, with a complete loss of activity culminating at 100 mM NaCl (Fig. 5C). The addition of salt raises the DNA-protein specificity and demonstrates that the anisotropy increase observed at low salt with the highest protein concentration is due to proper DNA-protein interactions, corresponding to a regain of specificity, rather than to a nonspecific aggregation. Taken together, our structural and biochemical data clearly establish the function of Rv2175c as a DNA-binding protein.

**Rv2175c Is Phosphorylated on a Unique Threonine Residue**—We recently showed that Rv2175c was exclusively phosphorylated on Thr residues, *in vitro* as well as *in vivo*, but the phosphorylation sites could not be identified (17). Therefore, to
decipher the role of the phosphorylation event it appeared necessary to identify which of the eight threonines of Rv2175c corresponded to the phosphorylated site(s). Thus, recombinant Rv2175c was incubated with cold ATP in the presence of PknL, and subjected to mass spectrometry analysis after tryptic digestion. ProteinPilot® data base searching software (version 2.0, Applied Biosystems), using the Paragon method with phosphorylation emphasis, was used to detect and identify the phosphorylated peptides. The sequence coverage of the protein was 91%, and phosphorylation occurred only on a peptide (5–12) with an 80-Da mass increment from 771.42 to 851.40 Da (monoisotopic mass). The MS/MS spectrum of the corresponding doubly charged ion at m/z 426.7 unambiguously confirmed the presence of the phosphate group on the threonine residue T9 (Fig. 6A), in agreement with our previous phosphoamino analysis, which indicated that phosphorylation of Rv2175c occurred exclusively on Thr residues (17).

Definitive identification of Thr9 as being the unique phosphorylation site in Rv2175c was achieved by site-directed mutagenesis to introduce a mutation that prevents specific phosphorylation (Thr9 to Ala9 replacement). This mutant was expressed, purified as an His-tagged protein in E. coli...
BL21(DE3) Star harboring pETPhos_Rv2175c_T9A, and used in an in vitro kinase assay. The recombinant Rv2175c_T9A was incubated along with [γ-32P]ATP and PknL1–281. The mixture was separated by SDS-PAGE and analyzed by autoradiography. As shown in Fig. 6B (upper panel), equal amounts of Rv2175c or mutant Rv2175c_T9A were used. Phosphorylation of Rv2175c_T9A was completely abrogated, compared with phosphorylation of Rv2175c, as evidenced by the absence of a specific radioactive band (Fig. 6B, lower panel). These results unambiguously demonstrate that Rv2175c_T9A has lost its ability to be phosphorylated by PknL. An additional round of mass spectrometry analysis was also performed directly on Rv2175c_T9A pre-treated with ATP and PknL, which failed to identify any additional phosphate group that could eventually have arisen as a compensatory mechanism to the loss of the Thr9 phosphorylation (data not shown).

Phosphorylation on the Rv2175c N-terminal Extension Modulates Its DNA-binding Activity—The structural elucidation of Rv2175c as a DNA-binding protein and the identification of its phosphorylation site led to the hypothesis that phosphorylation...
Solution Structure of M. tuberculosis Rv2175c

may influence the DNA-binding activity of Rv2175c. Following a strategy that has been successfully used to demonstrate that regulation of a substrate protein via phosphorylation is important during active cell growth in mycobacteria (Wag31), or for mycolic acid metabolism (mtFabH) (9, 13), we expressed and purified the phosphorylation mimics Rv2175c_T9D or Rv2175c_T9E. Indeed, previous studies have shown that acidic residues such as Asp or Glu qualitatively recapitulate the effect of phosphorylation with regard to functional activity (32). The option was taken to analyze and compare the DNA-binding activity of Rv2175c, Rv2175c_T9D, or Rv2175c_T9E using non-relevant DNA by EMSA and by fluorescence anisotropy at various NaCl concentrations.

FIGURE 4. Conformational flexibility in Rv2175c. Representation of the final ensemble comprising 30 NMR structures of Rv2175c. The N-terminal wHTH domain is represented in red while the putative C-terminal effector domain is represented in gray. The residues Met1 to Pro17 and Asp106 to Ala123 are not represented. The superimposition made on the sole wHTH domain (A) and the C-terminal domain (B) shows that some flexibility exists between the two domains.

FIGURE 5. DNA-binding activity of Rv2175c. A and B, gel electrophoretic mobility shift assays of the binding of Rv2175c to a non-relevant 200-bp DNA fragment. The radioactive 200-bp DNA probe was incubated with 0.4, 0.6, 0.8, and 1 μg of the purified Rv2175c, Rv2175c_T9D, or phosphorylated Rv2175c by PknL (pT9-Rv2175c) and analyzed by non-denaturing PAGE. As a control, non-radiolabeled DNA (cold probe) was added to the Rv2175 mix. C and D, anisotropy binding profile for binding of Rv2175c (B) and Rv2175c_T9D (C) to a 23-bp oligonucleotide labeled with Alexa Fluor 488 succinimidyl ester dye. The protein concentration was 4 nM. The buffer was 10 mM Tris-HCl, pH 7.5, with 0 mM NaCl (□), 50 mM NaCl (○), 100 mM (×), or 150 mM NaCl (▲). The anisotropy measurements were performed four different times and showed consistent order of magnitude.
As mentioned above, the EMSAs were carried out using a radioactive 200-bp non-relevant DNA fragment, except that Rv2175 was substituted by Rv2175c_T9D or Rv2175c_T9E (data not shown). Although Rv2175c protein interacted with this double-stranded DNA, the Rv2175c_T9D and Rv2175c_T9E phosphorylation mimics failed to shift the probe, indicating that they had lost their ability to bind to DNA (Fig. 5A). On the contrary, EMSA carried out using a Rv2175c_T9A mutant, a mutation that prevents the phosphorylation of Rv2175c, showed that this mutant retained its DNA-binding activity (data not shown), thus confirming the critical role of phosphorylation in Rv2175c to inhibit DNA binding. Moreover, EMSA assays were performed using Rv2175c phosphorylated by PknL in the presence of cold ATP, thus generating pT9-Rv2175c. As shown in Fig. 5B, the phosphorylated isoform of Rv2175c failed to bind DNA, thus confirming the critical role of phosphorylation to prevent DNA binding, as previously observed with the mimics mutants, T9D or T9E (Fig. 5A).

In addition, the fluorescence anisotropy method using the labeled 30-bp DNA fragment, which was mixed with unlabeled Rv2175c, Rv2175c_T9D, or Rv2175c_T9E, was performed to confirm the phosphorylation role in Rv2175c DNA-binding activity. As shown in Fig. 5C, Rv2175c exhibited a strong DNA-binding activity in the absence of salt, as expected whereas the affinity of Rv2175c_T9D for DNA in the absence of salt was comparable to that of the Rv2175c at 100 mM NaCl (Fig. 5D). Similar results were observed when Rv2175c_T9D was replaced by the other mimic mutant Rv2175c_T9E (data not shown). Together, these results indicate that mimicking phosphorylation by an acid residue at position 9 abrogates the DNA-binding activity of Rv2175c. This highlights the critical role of Thr9, which, following phosphorylation, negatively controls the DNA-binding activity of Rv2175c. This prompted us to investigate whether phosphorylation alters the structure of Rv2175c using two-dimensional and three-dimensional NMR experiments on the mimic mutants. However, our different analyses did not reveal major differences on a structural basis compared with the non-phosphorylated isoform of Rv2175c protein.

To investigate whether the N-terminal extension characterizing the M. tb protein was contributing to the DNA-binding activity of Rv2175c, fluorescence anisotropy of the Rv2175c mutant lacking the N-terminal extension (Rv2175c13–146) was recorded using the same conditions as mentioned above. This truncated protein exhibited a DNA-binding activity comparable to the one of full-length protein (data not shown), indicating that the first 13 N-terminal residues are not involved in the DNA-binding activity of the protein in the absence of phosphorylation, but act as a phosphoacceptor site that regulates Rv2175c DNA-binding activity.

Effect of Thr9 Phosphorylation on Rv2175c Structural Organization—In a second set of experiments, PknL was purified and used to phosphorylate a 15N-labeled sample of Rv2175c. The first series of experiment was performed in the

FIGURE 6. Identification of Rv2175c phosphorylation site. A, MS/MS spectrum of the doubly charged ion [M+2H]2+ at m/z 426.7 of a peptide (5-12) (monoisotopic mass: 851.40 Da). Unambiguous location of the phosphate group on Thr9 was showed by observation of the "y" C-terminal daughter ion series. Starting from the C-terminal residue, all y ions loose phosphoric acid (~98 Da) after the Thr9-phosphorylated residue. B, in vitro phosphorylation of the Rv2175c_T9A mutant. Purified Rv2175c and Rv2175c_T9A were incubated with PknL1-281 and [γ-32P]ATP. Samples were separated by SDS-PAGE (upper panel) and visualized by autoradiography (lower panel).
same conditions at pH 4.6, and a few chemical shift variations could be observed on the ^1H,^15N HSQC spectrum recorded after treatment with the kinases (Fig. 7, A and B). Only the Thr^9 residue and its close neighbors could be clearly detected, whereas no significant modifications of the Rv2175c core domain could be resolved by NMR for the phosphorylated isoform of Rv2175c in these conditions (Fig. 7B). Importantly, frequency resonance sequential attribution revealed a unique phosphorylation site on Thr^9, clearly identified as the most important chemical shift change, thus confirming our mass spectrometry analysis (Fig. 6A), and that PknL was clearly able to phosphorylate Rv2175c despite an obvious structural shift that could explain the role of Thr^9 phosphorylation. The downfield chemical shift change from Thr to pThr observed at pH 4.6 was around 0.5 ppm, compared to the 2 ppm observed in recent studies made at a higher pH (6.4) (21). Therefore, to determine whether the pH could be responsible for the failure to observe a structural shift, five samples of the protein were prepared at pH 4.6, 5.3, 6.1, 6.4, or 6.8. However, due to instability as the pH increased, around 90% of the protein precipitated. Consequently, only limited NMR data were obtained, particularly the ^[^1H, ^15N] HSQC experiment using different acquisition time. Although we could not record any other experiment to solve and compare the solution structures of the phosphorylated and non-phosphorylated isoforms at different pH, it is noteworthy that only the residue Thr (with a 1.5-ppm chemical shift) and its close neighbors were affected by phosphorylation without modifications of the core domain residues, indicating that pH did not affect any structural rearrangement (Fig. 7, C and D). It appears therefore tempting to hypothesize that PknL-dependent phosphorylation on Thr^9 does not engender major structural modifications on the Rv2175c monomer. However, it remains possible that the inhibitory mechanism triggered by phosphorylation may disturb protein dimerization and/or induce a structural rearrangement between the N-terminal and the C-terminal domains, or an interaction with a third partner beside DNA.

DISCUSSION
Recent years have been characterized by a tremendous increase in structural information on protein-DNA complexes and uncovered a remarkable structural diversity in DNA binding folds (33). A subclass of the HTH family known as the wHTH proteins are so named due to the presence of an additional wing immediately C-terminal to the HTH unit that mediates additional contacts with the DNA (30). Here, we have
Importantly, Rv2175c mutants mimicking the phosphorylation site in a flexible region of the protein is likely to promote optimal presentation/interaction between the phosphorylation site in the N-terminal disordered extension, is the unique protein from the Xis nucleoprotein filament (31), while the first two helices and the winged β-strand hairpin corresponding to the domains of the protein that directly interacts with the DNA are conserved.

Biochemical studies, including EMSA and fluorescence anisotropy, further confirmed that Rv2175c is capable to bind DNA at low salt concentrations. Taken, collectively, our data clearly establish the function of Rv2175c as a DNA-binding protein. Our biochemical studies uncovered that Thr9, laying within the N-terminal disordered extension, is the unique phosphoacceptor in Rv2175c. The presence of the phosphorylation site in a flexible region of the protein is likely to promote optimal presentation/interaction between the phosphorylation site and the kinase.

This work presents compelling evidence that Rv2175c encodes a DNA-binding protein and that this activity is inherent to the main core domain of the protein as a truncated version of Rv2175c protein lacking the first 13 residues presented the same DNA-binding properties than the wild-type Rv2175c. Importantly, Rv2175c mutants mimicking the phosphorylation isoform failed to express DNA-binding activity, supporting the view that phosphorylation of Thr9 negatively regulates Rv2175c activity. Surprisingly, structural comparison of the non-phosphorylated and PknL-phosphorylated isoforms of Rv2175c did not reveal major conformational changes. Our structural study revealed that Rv2175c behaves as a monomer in solution, a feature that may explain the failure to detect major structural changes. Because DNA-binding proteins, and particularly prokaryotic transcription factors, usually act as homodimers, one can speculate that phosphorylation of Thr9 interferes with cooperativity and/or oligomerization mechanisms linked to the DNA binding of a dimeric form of Rv2175c. Whether phosphorylation exerts its inhibitory effect on DNA binding by disrupting homotypic interactions between two monomers represents an attractive hypothesis. However, as a prerequisite to test this hypothesis, specific DNA target(s) of Rv2175c will have to be identified. Work is currently in progress to identify the putative regulon(s) controlled by Rv2175c using a chromatin immunoprecipitation (chip-on-chip) approach, which should lead to the Rv2175c-specific DNA targets and to determining their locations on a whole genome basis.

Multiple sequence alignments indicated that the disordered N-terminal sequence bearing the phosphoacceptor is only restricted to a few pathogenic mycobacterial species. In addition to M. tb and M. bovis a full conservation of this domain was found to be present in M. africanum, M. microti, and M. canetti (data not shown), but not in other pathogenic species such as M. marinum or M. leprae or in non-pathogenic species such as M. smegmatis. Overall, these observations support the occurrence of a regulation mechanism by phosphorylation of Rv2175c, which is unique to the M. tuberculosis complex. During the infection process, M. tb encounters numerous environmental conditions and induces or represses a number of genes for a quick adjustment to new conditions allowing the bacteria to adapt and survive within its host. Whether the phosphorylation-dependent mechanism controlling the activity of Rv2175c contributes to the adaptation of M. tb in its host requires further study.

Because these organisms display a complex life style comprising different environments and developmental stages, it is believed that their success results from their remarkable capacity to survive within the infected host, which is also linked to the presence of an unusual cell wall. It is therefore tempting to speculate that the PknL/Rv2175c couple participates in the modulation and adaptation of cell wall components in response to environmental changes. In this context it is important to mention that this kinase/substrate pair is present in the close vicinity of the dcv (division and cell wall synthesis) gene cluster, encompassing several genes involved in cell wall synthesis and cell division (17, 18). In addition, Kang et al. (34) demonstrated that the activity of Wag31, a homologue of the cell division protein DivIVA, is controlled by phosphorylation by mycobacterial STPKs and that Wag31 causes a dramatic morphological change in which one end of the cell becomes round rather than rod-shaped. Interestingly, wag31 (Rv2145c) belongs to the 30-kb dcv cluster. Whether Rv2175c acts as a transcriptional regulator involved in controlling the dcv gene cluster has not been elucidated yet. Access to the DNA target(s) of Rv2175c may help to understand how regulation via phosphorylation of Rv2175c in response to environmental changes contributes to the persistence of M. tb within the infected host. A detailed characterization of the regulon(s) will be a major step in defining the role(s) of Rv2175c in the M. tb physiological adaptation to its environment.

In prokaryotes, protein kinases can be classified into two superfamilies based on their sequence similarities and enzymatic specifications: (i) the histidine kinase superfamily, which belongs to the two-component systems (1). In this system, the stimulus causes the histidine kinase to autophosphorylate a conserved histidine residue, forming a highly reactive phosphodiester bond. The cognate response regulator catalyzes the phosphorly transfer to a conserved aspartate within its own receiver domain. The phosphorylated response regulator interacts with transcription factors, which in turn, up- or down-regulate the number of genes; and (ii) the superfamily of serine, threonine, and tyrosine kinases (2, 3). M. tb has only twelve paired two-component systems homologues compared with more than 30 present in E. coli or Bacillus subtilis, which may be a consequence of the presence of a relatively much higher number of STPKs in M. tb (4). In addition to the proposed roles of STPKs in pathogenesis and development, it is likely that some STPKs simply fulfill the role of the classic bacterial two-component system regulatory proteins. In this context, the PknL/Rv2175c pair presents functional analogy with two-component systems, in that PknL can be compared with the sensor kinase, and Rv2175c as the transcriptional factor controlled by phosphorylation. In support of this view, PknH has been shown
to phosphorylates EmbR (encoded by its adjacent gene), a putative transcriptional regulator of arabinosyl transferases EmbCAB (35, 36). Activation of EmbR upon phosphorylation by PknH induces transcription from the embCAB operon, leading to a higher lipoarabinomannan/lipomannan ratio (37). Moreover, in a recent report, it was demonstrated that PknK (Rv3080c) phosphorylates the AraC/XylS transcriptional regulator VirS (Rv3082c), which regulates expression of the mycobacterial monooxygenase (mymA) operon, and that PknK-mediated phosphorylation of VirS increases its affinity for mym promoter DNA (38). These few examples highlight the functional analogy of M. tb STPKs in regulating the activity of DNA-binding proteins in a way similar to the classic two-component couples.

In conclusion, our study clearly established the function of Rv2175c as a DNA-binding protein, whose activity is controlled by phosphorylation of a unique residue in the N-terminal domain of the protein. However, future work is required to identify and characterize the DNA target(s) of Rv2175c and its possible link with the dcw gene cluster. In combination with structural analysis of the PknL/Rv2175c complex, these studies may also provide the key to designing molecules for selective disruption of signal transduction that are specific to M. tb.

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