The Non-catalytic “Cap Domain” of a Mycobacterial Metallophosphoesterase Regulates Its Expression and Localization in the Cell*

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Significance:
Metallophosphoesterases possess unique, poorly characterized cap domains located at the entrance to the active site. Despite highly conserved core catalytic domains, members of the metallophosphoesterase (MPE) superfamily perform diverse and crucial functions ranging from nucleotide and nucleic acid metabolism to phospholipid hydrolysis. Unique structural elements outside of the catalytic core called “cap domains” are thought to provide specialization to these enzymes; however, no directed study has been performed to substantiate this. The cap domain of Rv0805, an MPE from Mycobacterium tuberculosis, interacts with the cell wall of mycobacteria, possibly with the mycolyl-arabinogalactan-peptidoglycan complex, by virtue of its C terminus, a hitherto unknown property of this MPE. Using a panel of mutant proteins, we identify interactions between active site residues of Rv0805 and the CTE that determine its association with the cell wall. Finally, we show that Rv0805 and a truncated mutant devoid of the CTE produce different phenotypic effects when expressed in mycobacteria. Our study thus provides a detailed dissection of the functions of the cap domain of an MPE and suggests that the repertoire of cellular functions of MPEs cannot be understood without exploring the modulatory effects of these subdomains.

Conclusion: The functions of the Rv0805 metallophosphoesterase are regulated by the cap domain.

Significance: Cap domains may provide catalysis-independent features to metallophosphoesterases that shape their cellular functions.

Background: Metallophosphoesterases possess unique, poorly characterized cap domains located at the entrance to the active site. These enzymes are characterized by metal-dependent phosphoesterase activity against a variety of substrates that includes nucleic acids (3–5), cyclic nucleotides (5–8), and organophosphates (9). Members of this class of enzymes play crucial roles in diverse cellular processes such as DNA repair, cyclic nucleotide metabolism, and RNA processing.

The structures of several bacterial and eukaryotic MPEs have been described and reveal a conserved core α/β-sandwich-fold (5, 10–15). Metal ions, typically two cations such as Fe3+/Fe2+ or Mn2+, are coordinated in the active site of these enzymes by conserved His, Asp, and Asn residues, mutation of which results in markedly reduced catalytic activity (5, 10–15). Despite the availability of structural details, it has not been possible to successfully predict the substrates of several MPEs. Moreover, members of this enzyme class may use multiple substrates in vitro, best exemplified by GpdQ from Enterococcus faecalis, that possesses phosphomonoesterase and phosphodiesterase as well as phosphatetriesterase activities (16). We have previously characterized, both biochemically and structurally, the Rv0805 phosphodiesterase from the human pathogen Mycobacterium tuberculosis (8, 17, 18). It was initially identified as a homolog of CpdA from Escherichia coli and hence predicted to regulate mycobacterial 3',5'-cAMP levels. However, the ability of Rv0805 to hydrolyze a number of substrates in vitro, including 3',5'-cAMP, 2',3'-cAMP, bis-p-nitropheno- phosphonate (bispNPP), and p-nitropheno- phosphonate suggested promiscuity in its targets (7, 17). Higher turnover of these additional substrates than 3',5'-cAMP (7, 17) and cAMP-independent phenotypic and the transcriptional effects of Rv0805 overexpression in mycobacteria (17, 19) indicated that cAMP may not be the only physiologically relevant substrate of Rv0805.

The structure of Rv0805 shows a canonical MPE-like-fold, with a surface-exposed active site with bound Fe3+ and Mn2+ ions (17, 18). Docking studies with 3',5'-cAMP, 2',3'-cAMP, and bispNPP as well as co-crystallization with 5'-AMP identified interactions between active site metals and phosphate moieties as contributing to the primary recognition between

Metallophosphoesterases (MPEs) are a protein family with a wide phyletic distribution ranging from Eubacteria to Eukarya (1, 2). These enzymes are characterized by metal-dependent phosphoesterase activity against a variety of substrates that includes nucleic acids (3–5), cyclic nucleotides (5–8), and organophosphates (9). Members of this class of enzymes play crucial roles in diverse cellular processes such as DNA repair, cyclic nucleotide metabolism, and RNA processing.

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‡ To whom correspondence should be addressed. Tel: 91-80-22932542; Fax: 91-80-2360999; E-mail: sandhya@rmdg.iisc.ernet.in.
§ The abbreviations used are: MPE, metallophosphoesterase; bispNPP, bis-(p-nitropheno)phosphate; CTE, C-terminus extension; mAGP, mycolyl-arabinogalactan-peptidoglycan complex; OBG, n-octyl-β-D-glucoside; CRP, cAMP receptor protein.
Functions for the Cap Domain of the Rv0805 MPE

ExPERimental Procedures

Sequence Analysis—The sequence of Rv0805 was used as the seed sequence, and close and distant homologs were identified using PSI-BLAST analysis (www.ncbi.nlm.nih.gov). The sequences of homologs from *Mycobacterium leprae* (gi 15828187) and *Mycobacterium marinum* (gi 183984854) were used as representative closely related proteins, whereas *Nocardia farcinica* (*gi* 54026843), *Arthrobacter aurescens* (*gi* 119962173), *Rhodococcus rhodochrous* (*gi* 515042053), and *E. coli* (*gi* 85675835) were used as distant homologs. Multiple sequence alignment was built using ClustalW using default parameters, and their phylogenetic relationships were analyzed by neighbor-joining using MEGA 6 (21).

Mycobacterial Strains and Culture Conditions—*Mycobacterium smegmatis* secA2 (NR116) strain (22) was obtained from Dr. Miriam Braunstein (Dept. of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC). *M. smegmatis* mc²155 cultures were grown at 37 °C with shaking at 200 rpm in Middlebrook 7H9 medium (BD Biosciences) supplemented with 0.2% glycerol and 0.05% Tween 80. For *Mycobacterium bovis* bacillus Calmette-Guérin cultures OADC (oleic acid-albumin-dextrose-catalase) supplement (BD Biosciences) was included at a final concentration of 10% v/v. Hygromycin B (AMRESCO) was added to the culture medium at a concentration of 50 μg/ml wherever needed.

Cloning, Expression, and Purification of Recombinant Rv0805 and Its Mutants—Cloning of wild type Rv0805 or mutants D21A, H23A, D66A, N97A, H169A, H98A, H209A, H207A, and Rv0805Δ40 for expression as N-terminal hexahistidine-tagged proteins in *E. coli* has been described previously (2, 8, 17). To generate Rv0805Δ10 and Rv0805Δ20, PCR using Rv0805-fwd (8) and Rv0805_308_rev_NotI (5–8) primers, respectively, was performed on plasmid encoding wild type Rv0805. Amplicons were digested with XhoI and NotI and cloned into Sall-NotI-digested pPROExHT-C plasmid. Both clones were confirmed by sequencing (Macrogen).

Tagged Rv0805 and its mutants were purified using a protocol described earlier (17, 18) with minor modifications. Briefly, all proteins were overexpressed in *E. coli* BL21 strain grown in Terrific broth supplemented with FeCl₃ and MnCl₂ (10 μM each) using isopropyl-1-thio-β-D-galactopyranoside (500 mM) at 16 °C. After 16–20 h, cells were harvested and lysed in cold Rv0805 lysis buffer (50 mM Tris-HCl, pH 8.5, 100 mM NaCl, 5 mM β-mercaptoethanol, 10% glycerol, 10 μM FeCl₃, and 100 μM MnCl₂) by sonication. Soluble protein was loaded onto nickel-nitrilotriacetic acid resin and washed using lysis buffer supplemented with NaCl (500 mM) and imidazole (20 mM). Pure protein was eluted using lysis buffer containing imidazole (300 mM), desalted into lysis buffer, and stored at −70 °C until further use.

Rv0805 and its substrates, partially explaining the observed promiscuous substrate utilization (17). The presence of phosphate or phosphate mimics in the active site of Rv0805 and other MPEs (as seen from crystal structures) suggests that this may be a conserved feature of the MPE family of proteins (5, 11–14, 20). However, the structure of Rv0805 revealed features unique to this enzyme. Most striking among these was the formation of a swapped dimer consisting of two symmetric protomers of Rv0805. Swapped structural elements were constituted largely by the C terminus of the protein (Leu²⁶⁶–Ile²⁹⁰), placed at the entrance of the active site. Electron density for amino acids of the C terminus beyond Asp²⁹⁸ was not observed in the crystal structure of Rv0805, indicating that this region was flexible (17). Interestingly, residues Pro²⁷₈–Asp³¹⁸ of the C terminus of Rv0805 (henceforth referred to as the C-terminal extension (CTE)) do not bear sequence similarity to other MPEs, suggesting regulatory roles relevant in vivo rather than being involved in catalysis. In agreement with this, we showed earlier that Rv0805¹–²⁷₈ is catalytically competent, and the presence of the CTE only marginally improves utilization of linear substrates (17).

Unique structural elements outside of the core catalytic domain of MPEs have been referred to as cap domains as they may often form a cap on the active site of these proteins (Fig. 1). They may be present at the C terminus as in Rv0805 (17) (Fig. 1a), GpdQ (8) (Fig. 1b), and Lmo2642 (13) (Fig. 1c) or at the N terminus as in MPPED2 (11) (Fig. 1d). Cap domains are speculated to contribute to substrate selectivity (12) or in providing a surface for interacting proteins (11). However, a systematic study on their functions has not been performed so far, primarily due to poor information on the in vivo roles of most MPEs. In the present study we demonstrate that the C terminus of the cap domain of Rv0805 modulates levels of its expression and is important in interaction of the protein with the mycobacterial cell envelope. We also identify a link between the active site and the CTE of Rv0805, opening up the possibility of similar interactions in other members of this enigmatic enzyme family.

FIGURE 1. Cap domains of MPEs. Cap domain of Rv0805 (a, PDB ID 3IB8; residues 266–318), GpdQ (b, PDB ID 2ZOA), Lmo2642 (c, PDB ID 2XMO), and MPPED2 (d, PDB ID 3RL3) are shown in orange. Core phosphodiesterase domains are shown in green. In the case of Rv0805 and GpdQ the second protomer of the dimer is colored gray. Active site Fe³⁺/Fe²⁺ and Mn²⁺ ions are shown as cyan and magenta spheres, respectively. Cap domains are located at the C terminus in a–c or at the N terminus in d.
Functions for the Cap Domain of the Rv0805 MPE

In Vitro Activity of Rv0805—Activity of Rv0805 and its mutants against bispNPP and 2',3'-cyclic adenosine monophosphate (2',3'-cAMP) was monitored as described earlier (17). Michaelis-Menten constants $K_m$ and $V_{max}$ were determined using Graphpad Prism Version 5.00 (GraphPad Software) and data from three independent experiments, with each assay carried out in duplicate. Phosphodiesterase activity in lysates of *M. smegmatis* was measured as described earlier (17) using 50 μg of total lystate protein and 10 mM bispNPP as substrate.

Expression of Rv0805 in *M. smegmatis*—Rv0805 and its mutants were expressed under the Rv0805 gene promoter in *M. smegmatis* using derivatives of the pMV-10–25 plasmid (23). Generation of plasmid constructs for expression of Rv0805, Rv0805N97A, Rv0805Δ40, and Rv0805Δ40N97A have been described earlier (19). Similar strategies were used for cloning Rv0805H207A, Rv0805, Rv0805N97A, Rv0805Δ40, and Rv0805Δ20.

For expression of Rv0805 under the hsp60 gene promoter, the Rv0805 sequence was amplified by PCR using Rv0805-Nhel_fwd (5'-AGGGCGTCAAGTGCATTAGACTATGGGCGC-3') and Rv0805Cry-HindIII_rev (18) primers and digested with Nhel and SacI. The GFP sequence from pMV-10–25 vector was cloned Rv0805H207A, Rv0805, Rv0805N97A, Rv0805Δ40 using derivatives of the pMV-10–25 plasmid as substrate.

For the generation of glutathione S-transferase (GST)-tagged cap domain of Rv0805, the SmaI-NotI fragment from pPRO-Rv0805 (8) plasmid, coding for residues Pro278-Asp318 of cap domain of Rv0805, the SmaI-NotI fragment from hsp60 Rv0805 (8) plasmid. The C terminus coding sequence from the amplicon and the SacI-XbaI fragment derived from pPRO-NheI and SacI. The GFP sequence from pMV-10–25 vector was cloned Rv0805H207A, Rv0805Δ40, and Rv0805Δ20.

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Functions for the Cap Domain of the Rv0805 MPE

the bacterial pellet was analyzed for presence Rv0805, Rv0805Δ40, or CRP by Western blot analysis.

Interaction of Rv0805 with Mycobacterial Cell Wall Fraction—Crude cell wall was prepared from *M. smegmatis* or *M. bovis* bacillus Calmette-Guérin as described earlier (25). Crude cell wall and mycolyl-arabinogalactan-peptidoglycan complex (mAGP) from *M. tuberculosis* H37Rv were obtained from Colorado State University (REI resources, TBVTRM Research Institute). Cell wall deproteination was performed by proteinase K treatment for 1 h at 50 °C followed by extraction with sodium dodecyl sulfate (SDS; 2%) for 1 h at 95 °C. Delipidation of the cell wall was achieved after deproteinization by extraction with chloroform:methanol (2:1) for 1 h at room temperature with gentle agitation.

Purified recombinant Rv0805 or its mutants (1 μg) were centrifuged at 13,000 × g for 5 min before use to remove precipitate if any. Fractions were then incubated with crude cell wall or purified mAGP in Rv0805 lysis buffer for 1 h at 4 °C with gentle mixing. Nucleotides 5'-'AMP, 2',3'-cAMP, and 3',5'-cAMP were incorporated at 10 mM final concentration where required. The interaction mix was centrifuged at 16,000 × g for 10 min to separate the particulate cell wall. The pellet was washed once with cold TBS, and cell wall-bound Rv0805 was extracted by boiling samples in 1× SDS-PAGE gel loading buffer and then subjected to Western blot analysis.

Phenotypic Characterization of Rv0805 Expressing *M. smegmatis*—Sensitivity of *M. smegmatis* expressing Rv0805 or Rv0805Δ40 to isoniazid, SDS, and crystal violet were performed as described earlier (17). SDS and crystal violet were used at 0.01% and 10 μg/ml concentrations, respectively. Isoniazid was used at a concentration of 5 μg/ml for Rv0805 and Rv0805N97A expressing *M. smegmatis* and 2.5 μg/ml for Rv0805Δ40 and Rv0805Δ40N97A expressing *M. smegmatis*.

RESULTS

Expression of Rv0805 and C-terminal Truncation Mutants in *M. smegmatis*—We reported earlier that Rv08051–278 that lacks 40 amino acids from the C terminus is the proteolytically stable, catalytically active core MPE domain of this protein (18). Furthermore, only residues Leu266, Asp298 of the Rv0805 C terminus were structured in the full-length enzyme. We, therefore, used phylogenetic comparison with homologous proteins to define the cap domain of Rv0805. Sequences of Rv0805-like MPEs from *M. leprae* and *M. marinum* were selected as representative closely related proteins, whereas those from *N. farcinica*, *R. rhodochrous*, and *A. aurescens* (being Actinobacteria, like Mycobacteria) were used as relatively distant homologs. The sequence of CpdA from *E. coli* was used as an “out-group” protein as it shares only ~35% sequence similarity with Rv0805. The phylogenetic relationship between these proteins was reminiscent of the phylogenetic relationships between the selected bacteria (Fig. 2a). The catalytic domains of these enzymes, containing five sequence blocks required for activity, aligned relatively well (Fig. 2a). However, variation was found among their C termini. The C terminus of Rv0805 showed significant sequence similarity with mycobacterial homologs only until residue Thr309 and with actinobacterial homologs only until residue Ile320. No identifiable stretches of similarity were observed between the C terminus of Rv0805 and CpdA (Fig. 2a).

Taken together with structural studies, we concluded that the cap domain of Rv0805 consisted of at least three segments that may modulate different aspects of the protein function. These were Leu266–Asp298 of a defined structure and conserved among actinobacterial homologs, a flexible part comprising residues Asp299–Leu308 conserved only among mycobacterial homologs, and Leu308–Asp318 unique to Rv0805.

In light of the above analysis we chose to express full-length Rv0805 (residues 1–318) and its mutants lacking 10 (Rv0805Δ10, 20 (Rv0805Δ20), or 40 (Rv0805Δ40) amino acids of the CTE in *M. smegmatis*, which does not code for a close homolog of Rv0805. All four proteins were expressed using a multicopy plasmid under the Rv0805 gene promoter, and their expression was confirmed by immunoblotting with an Rv0805-specific monoclonal antibody. Interestingly, we found a progressive increase in steady-state expression levels of Rv0805 upon deletion of the C terminus, Rv0805Δ40 being expressed to the highest level (Fig. 2b). Increased expression of CTE truncation mutants of Rv0805 in *M. smegmatis* was concomitant with higher phosphodiesterase activity in lysates, indicating that the expressed proteins were active when expressed in *M. smegmatis* (Fig. 2b).

To rule out the possibility of promoter-specific regulation resulting in enhanced expression of the mutants, we assessed expression of Rv0805 and Rv0805Δ40 under the *M. bovis hsp60* promoter. Here as well Rv0805Δ40 showed greater levels of expression than the wild type protein (Fig. 2c). Furthermore, the levels of mRNA of Rv0805 and C-terminal truncation mutants were comparable (data not shown), indicating that the CTE of Rv0805 may regulate its steady-state levels of the protein post-translationally.

We next generated translational fusions of GST to various regions of the CTE of Rv0805 to test the role of the C terminus in regulating levels of expression of a heterologous protein. As shown in Fig. 2d, a GST fusion protein containing residues 278–318 was expressed to far lower levels than a fusion of GST with residues 278–298 or GST alone. Thus the CTE of Rv0805 was able to regulate steady-state levels of proteins in a context-independent manner.

Reduction in intracellular cAMP levels in *M. smegmatis* expressing Rv0805 or its mutants were commensurate with protein expression levels (Fig. 2e). To ensure that truncation at the C terminus did not affect the utilization of cAMP as a substrate, we measured the activity of purified Rv0805 and truncation mutants *in vitro* against representative linear (bispNPP) and cyclic (2',3'-cAMP) phosphodiesterase substrates. Although the V<sub>max</sub> of Rv0805 was higher than truncation mutants, similar K<sub>m</sub> values were obtained for Rv0805, Rv0805Δ10, Rv0805Δ20, and Rv0805Δ40 *in vitro* against both kinds of substrates (Table 1), indicating that deletion of the CTE of Rv0805 did not compromise substrate affinity. Thus CTE-modulated expression levels of Rv0805 translated to changes in the levels of at least one physiologically relevant substrate of this MPE, namely cAMP.

The CTE of Rv0805 Modulates Its Subcellular Localization in Mycobacteria—We reported earlier that in *M. tuberculosis* a significant fraction of Rv0805 is localized to the cell wall and cell...
membrane fractions, representing the envelope of the cells (17). However, no classical secretion signal is identifiable in the sequence of Rv0805. We noticed that the CTE of Rv0805 contains several positively charged residues clustered among hydrophobic and non-polar residues, including glycine (Fig. 3a). A similar arrangement is thought to allow membrane docking in other mycobacterial proteins such as FadD13 (26), making the C terminus of Rv0805 a putative localization domain. We, therefore, tested the subcellular localization of Rv0805 and CTE truncated mutants in *M. smegmatis*. MSMEG_3780, an

functions for the cap domain of the Rv0805 mpe

FIGURE 2. Expression of wild type and C terminus deletion mutants of Rv0805 in *M. smegmatis*. a, sequence alignment of Rv0805 and its homologs from *M. leprae* (Mlep; gi 15828187), *M. marinum* (Mmar; gi 183984854), *N. farcinica* (Nfar; gi 54026843), *A. aurescens* (Aaur; gi 119962173), *R. rhodochrous* (Rrho; gi 515042053), and *E. coli* (E. coli; gi 85675835). Conserved residues required for catalysis in metallophosphoesterases are highlighted in gray. Aligned proteins show similar phylogenetic relationships as the bacteria that code for them (lower panel). b, expression of Rv0805, Rv0805/H900410, Rv0805/H900420, and Rv0805/H900440 in *M. smegmatis* driven by the Rv0805 gene promoter. Equal protein (25 μg) from lysates of each strain was subjected to immunoblotting using an Rv0805-specific monoclonal antibody. A strain carrying plasmid alone was used as a control. Values (pmol/min/mg of protein) below each lane represent the phosphodiesterase activity in lysates using bispNPP as a substrate. c, expression of Rv0805 and Rv0805/H900440 in *M. smegmatis* under the heterologous *M. bovis* hsp60 gene promoter. Equal protein (25 μg) from lysates of each strain was subjected to immunoblotting using an Rv0805-specific monoclonal antibody. A strain carrying plasmid alone was used as a control. d, expression of GST tagged to varying lengths of the Rv0805 cap domain in *M. smegmatis*. Equal protein (50 μg) from lysates of each strain was subjected to immunoblotting using GST-specific polyclonal antisera. CRP was used as a protein loading control. e, intracellular cAMP levels in exponentially growing *M. smegmatis* expressing Rv0805 or cap domain deletion mutants normalized to control cells. The means ± S.D. from three biological replicates are plotted.
Table 1

| Protein          | bispNPP Km (μM) | 2’3’-cAMP V_max (μmol/min/mg) | bispNPP Km (μM) | 2’3’-cAMP V_max (μmol/min/mg) |
|------------------|-----------------|-------------------------------|-----------------|-------------------------------|
| Rv0805           | 295 ± 30        | 537 ± 63                      | 34.8 ± 0.74     | 6.9 ± 0.21                    |
| Rv0805Δ10        | 331 ± 92        | 469 ± 102                     | 44.2 ± 2.6      | 6.6 ± 0.37                    |
| Rv0805Δ20        | 261 ± 40        | 494 ± 101                     | 23.9 ± 0.75     | 2.2 ± 0.11                    |
| Rv0805Δ40        | 285 ± 81        | 667 ± 111                     | 9.2 ± 0.5       | 1.2 ± 0.06                    |
| Rv0805H207A      | 3338 ± 665      | 2148 ± 558                    | 11.2 ± 0.77     | 0.93 ± 0.09                   |

Functions for the Cap Domain of the Rv0805 MPE

These data suggested that not only was Rv0805 localized to the mycobacterial membrane, but it was also partially exposed to the cell wall. We argued that Rv0805 may interact, by means of its CTE, with components of the cell wall that allowed it to be anchored to the cell envelope. To test this, we performed in vitro interaction experiments between crude cell wall preparations from M. smegmatis and purified Rv0805. Rv0805Δ40 was also tested, as it was found not to localize to the cell membrane when expressed in M. smegmatis (Fig. 3b). We found that indeed Rv0805, but not Rv0805Δ40, interacted with the particular cell wall fraction of M. smegmatis in vitro (Fig. 4c). Both Rv0805Δ10 and Rv0805Δ20 also interacted with the cell wall, albeit to slightly lower extents than the full-length protein (Fig. 4c). Rv0805 could also interact with cell wall fractions from M. tuberculosis and M. bovis Calmette-Guérin (Fig. 4d), which are slow-growing mycobacteria that endogenously express Rv0805.

Next, we attempted to identify the component of the cell wall to which Rv0805 bound. Deproteinization or delipidation of the mycobacterial cell wall fraction did not affect its interaction with Rv0805 (Fig. 4e), excluding cell wall protein and the majority of cell wall lipids from being the possible interacting partners of Rv0805. Furthermore, Rv0805, but not Rv0805Δ40, interacted in vitro with purified cell wall mAGP from M. tuberculosis (Fig. 4f), demonstrating that Rv0805 could associate with the core polymer of mycobacterial cell walls and that residues Pro278–Asp298 were critical for the interaction.

The Rv0805 Active Site and C Terminus Together Participate in Cell Wall Interaction—Residues Pro278–His292 of the CTE lie over the active site of Rv0805 and are swapped between two promoters of a dimer of the protein (17) (Fig. 1a). Based on the proximity between the active site of Rv0805 and these residues, we asked if the active site also contributed to the ability of Rv0805 to associate with the cell wall. To answer this question, in vitro cell wall interaction experiments were performed in the presence of 5’-AMP. This nucleotide is known to bind in the active site of Rv0805 but not alter the structure of Rv0805 significantly (17). 5’-AMP inhibited interaction between Rv0805 and the mycobacterial cell wall (Fig. 5a). Similar observations were also made when 2’,3’-cAMP and 3’,5’-cAMP were incorporated into the interaction buffer (Fig. 5a), providing evidence that occluding the active site of Rv0805 prevented association with the cell wall.

We next performed cell wall interaction experiments with Rv0805 harboring mutations in the active site. Earlier work from our laboratory has shown that these mutations compromise catalysis to varying extents (8, 17, 18). As seen in Fig. 5b, mutations at the active site also affected the ability of Rv0805 to interact with the cell wall (Fig. 5b). Residues Asp271, His233, His207, and His209 were the most crucial for interaction with the cell wall (Fig. 5b). Interestingly, Asn277, which when mutated to Ala abrogates the catalytic activity of Rv0805 (17), contributed only marginally to cell wall interaction (Fig. 5b).

Because mutation of His207 to Ala almost completely abrogated interaction with the cell wall in vitro, thus phenocopying Rv0805Δ40, we characterized the catalytic properties of this mutant. In vitro biochemical analysis of Rv0805H207A demonstrated reduced catalytic activity and lower affinity (i.e. an
functions for the cap domain of the Rv0805 MPE

(a) Surface representation of the two protomers of Rv0805 colored in gray and green is presented. The CTE (266–298) of the green protomer is colored in orange. Hydrophobic and non-polar residues including glycine in the cap domain are colored yellow. Basic residues are colored dark blue. Metal ions bound in the active site are colored cyan (iron) and magenta (manganese). The sequence of the C terminus (Leu266–Asp318) is shown below. Apolar residues are in yellow, whereas positively charged residues are in blue.

(b) Equal protein from cytosolic, cell membrane, and cell wall fractions of wild type M. smegmatis strains expressing Rv0805 or its mutants or M. smegmatisΔsecA2 strain expressing Rv0805 were subjected to immunoblotting using Rv0805-specific monoclonal antibody. The quantity of lysates used (10–25 µg) was adjusted according to expression level of Rv0805 to prevent saturation of the blot. Data shown are representative of three independent experiments. MSMEG_3780, a cell envelope-localized adenylyl cyclase (24), was used to assess purity of the subcellular fractions prepared for these analyses.

(c) Relative distribution of Rv0805 and its mutants between cytosolic (c), cell membrane (m), and cell wall (w) fractions was estimated from three replicates by densitometric quantitation of Western blots and plotted as fraction of total. Means ± S.D. are plotted. p values < 0.05 were considered to be not significant (ns). Variations in the distribution in the cell wall fraction were not significant for all mutants compared with wild type. a, solubilization of Rv0805 from the membrane fraction of M. smegmatis. Membrane fraction from M. smegmatis expressing Rv0805 was treated with buffer supplemented with Triton X-100 (0.1%) or NaCl (1 M), and solubilized proteins were collected by filtration through 0.45-µm pore size filters. The filtrate was subjected to immunoblotting using Rv0805-specific monoclonal antibody. Filter from the membrane fraction treated with buffer alone was used as control. An equivalent amount of untreated membrane is represented as input. Data shown are representative of experiments performed three times with three independent membrane preparations.

(d) Solubilized fraction

Increase in $K_m$) for both linear and cyclic substrates, confirming that the active site architecture of this mutant deviated significantly from the wild type protein (Table 1). Rv0805H207A also phenocopied Rv0805Δ40 in vivo, as it was enriched significantly in the cytosolic fraction in comparison with the wild type protein (Fig. 5c). Rv0805N97A, however, showed similar subcellular localization as Rv0805 (Fig. 5c). Indeed, we have reported earlier that the active site of the N97A mutant is very similar to that of the WT protein except for the absence of metals in the active site (18). Collectively, these observations indicated that the active site of Rv0805 together with its CTE modulate the ability to interact with the mycobacterial cell envelope both in vitro and in vivo.

The Cap Domain of Rv0805 Modulates Its Biological Functions—We have so far demonstrated that the CTE of Rv0805 modulates multiple properties of this MPE, such as expression levels and cell envelope association. We next asked if these properties altered the biological roles of this protein in mycobacteria. The identities of the in vivo substrate(s), apart from cAMP, of Rv0805 are unclear at present. We, therefore, compared the sensitivity of M. smegmatis cells expressing Rv0805 to a variety of cytotoxic compounds, including clinically relevant anti-mycobacterials, as a measure of the functions of the protein in vivo. M. smegmatis expressing Rv0805 was hypersensitive to cell wall perturbants SDS and crystal violet, suggesting perturbation of the cell wall (Fig. 6a). However, expression of Rv0805Δ40 did not result in this increased sensitivity (Fig. 6a).

Additionally, enhanced growth of Rv0805 expressing M. smegmatis on isoniazid-containing plates was observed in comparison with control bacteria (Fig. 6b). In stark contrast, however, Rv0805Δ40 led to hypersensitivity to isoniazid (Fig. 6c). Rv0805N97A, a catalytically inactive mutant that could interact with the cell wall (Fig. 5b), continued to confer isoniazid tolerance to M. smegmatis (Fig. 6b), indicating that this phenotype was independent of catalysis. However, Rv0805Δ40N97A expression did not lead to hypersensitivity to isoniazid (Fig. 6c), demonstrating that the effect of Rv0805Δ40 expression was strictly dependent on catalysis. Expression levels of both N97A
Functions for the Cap Domain of the Rv0805 MPE

**FIGURE 4. Interaction of Rv0805 with the mycobacterial cell wall.** a, M. smegmatis cells expressing Rv0805 or Rv0805Δ40 were treated with proteinase K (PK) for the indicated times, and cell pellets were subjected to immunoblotting using an Rv0805-specific monoclonal antibody. CRP was used as a representative intracellular protein. Cells incubated at 37 °C without proteinase K treatment (−PK) were used as control. b, M. smegmatis cells expressing Rv0805 or Rv0805Δ40 were treated with PBS or PBS supplemented with 1% OBG. Cell pellets were subjected to immunoblotting using Rv0805-specific monoclonal antibody. CRP was used as a representative intracellular protein. c, a crude cell wall fraction from M. smegmatis was incubated with purified Rv0805 or cap domain deletion mutants. The presence of Rv0805 in cell wall pellets was monitored by immunoblotting using an Rv0805-specific monoclonal antibody. d, interaction of Rv0805 with cell wall fractions from M. tuberculosis H37Rv or M. bovis bacillus Calmette-Guérin. − and + refer to the addition of recombinant Rv0805 to the interaction mix. e, Western blot analysis showing in vitro interaction between Rv0805 or Rv0805Δ40 (Δ40) and crude cell wall (1) or deproteinated (2) and delipidated (3) cell wall from M. smegmatis. f, Western blot analysis showing in vitro interaction between Rv0805 or Rv0805Δ40 and purified mAGP from M. tuberculosis H37Rv. Data shown are representative of experiments performed three times. In panels c–f, input refers to 2% of the total protein used for interaction experiments.

Mutant proteins were similar to their respective active forms of Rv0805 (Fig. 6d). Thus, the C terminus cap domain altered the cellular effects of Rv0805 expression, providing evidence for the critical importance of this domain in shaping the functions of Rv0805 in vivo.

**DISCUSSION**

Despite high structural conservation, MPEs are known to perform diverse functions. One of the contributors to this functional diversity of MPEs is thought to be a structural feature that has been called the cap domain. In this study we have dissected in detail the functions of the C terminus of the cap domain of Rv0805, an MPE from *M. tuberculosis*. Our analyses revealed that this region of Rv0805 regulates several in vivo properties of this protein including levels of expression, cellular localization, and its modulation of cell wall properties.

Many bacterial phosphoesterases are known to localize to the cell envelope in Gram-negative as well as Gram-positive bacteria, exemplified by CpdB in *Yersinia pestis* (30), YkuE (31), and PhoD (32) in *Bacillus subtilis*. In our analyses too, we found that Rv0805 was present in the cell envelope of mycobacteria (Fig. 3, b and c). Additionally protease sensitivity and detergent extraction demonstrated that a large region of the protein may be exposed to the outer mycobacterial membrane by a cap domain-dependent mechanism (Fig. 4, a and b). Such cellular localization is typically attributed to SecA1-dependent or TAT (twin-arginine transport) protein export systems that have been described in mycobacteria (22, 33). Indeed, these secretion pathways have been implicated in the transport of the CpdB (30), YkuE (31), and PhoD (32) phosphoesterases. Mycobacteria additionally code for functional Type VII secretion systems, a general secretion signal for which was recently identified (34). However, localization of Rv0805 to the cell envelope is most likely independent of these pathways, as the sequence attributes required by these systems are not present in Rv0805. We also found that the localization of Rv0805 to the cell envelope was independent of the SecA2 system (Fig. 3, b and c), the only system known to export proteins independent of a signal sequence in mycobacteria (27). Therefore, the exact mechanism by which Rv0805 is exported remains unknown. Examples of other mycobacterial proteins that localize in a secretion-system independent manner to the cell envelope include FadD13 (26) and Rv1818c (35). In the case of Rv1818c, the first 30 amino acids of its PE (proline glutamate-rich) domain were both necessary and sufficient for localization to the cell envelope (35). In the case of FadD13, however, docking to the cell membrane was determined by the distribution of positively charged and hydrophobic residues on the protein rather than a specific targeting domain (26).

The CTE of Rv0805 is necessary for its cell envelope localization (Fig. 3, b and c). However it may not be sufficient, as Rv0805H207A was enriched in the cytosol despite the presence of the CTE (Fig. 5c). Based on these observations we propose...
that Rv0805 may localize to the cell envelope in a manner similar to FadD13, and the determinants for targeting are constituted by a specific structural organization of the protein cap domain rather than its sequence alone.

The Rv0805 CTE also mediates its interaction with the core cell wall polymer (Fig. 4. c–f). The mycobacterial core cell wall is a covalently linked complex of arabinogalactan, peptidoglycan, and mycolates (36). So far the only mycobacterial protein shown to interact directly with the core cell wall is ArfA, which recognizes and binds to peptidoglycan (37, 38). However, interactions with peptidoglycan and other core cell wall components have been suggested for other mycobacterial proteins as well, such as Erp (39) and PknB (40), both of which are implicated in the virulence of M. tuberculosis. As in the case of Rv0805, the
hydrophobic C terminus of Erp (39) was necessary for anchoring to the cell wall, suggesting that interactions with the cell wall may provide an important mechanism of tethering cell envelope proteins in mycobacteria. Additionally, in a manner similar to that seen for Erp (39), phenotypic analyses reported by us earlier (17) and in the present study (Fig. 6) strongly suggest roles for Rv0805 in modulating cell wall physiology in a cell wall interaction-dependent manner. Thus, although the cell wall may provide an anchor for proteins in mycobacteria, these interacting proteins may also alter the properties of the cell wall. The core polymer of the cell wall, i.e. the mAGP complex, is a target for two important antibiotics used for the treatment of tuberculosis, isoniazid and ethambutol. Rv0805 expression mildly increased the tolerance of *M. smegmatis* cells to isoniazid in a cell wall interaction-dependent manner (Fig. 6b), indicating that the interaction between Rv0805 and mAGP does have relevant functional outcomes. In the context of cell wall physiology as well as antibiotic resistance in mycobacteria, the roles of cell wall-interacting proteins has not yet been explored and may provide important leads into the biology of these bacteria.

Inhibition of interaction between Rv0805 and the cell wall by nucleotides (Fig. 5a) or by mutational perturbation of the active site of Rv0805 (Fig. 5b) clearly demonstrates the importance of the active site of Rv0805 in associating with the cell wall. Furthermore, we found that Asn⁹⁷, an active site residue absolutely critical for catalysis and metal coordination (17, 18), played only a marginal role in interaction with the cell wall (Fig. 5b). On the other hand, residues Asp²¹, His²³, His²⁰⁷, and His²⁰⁹ that are also involved directly in metal coordination, the mutation of which showed milder effects on catalysis (17, 18), were crucial for interaction with the cell wall (Fig. 5b). Thus, the CTE may not be isolated from the core MPE domain in Rv0805, but communicate with it, allowing cross-talk between their functions. These observations are substantiated by our earlier findings that the addition of Fe³⁺ and Mn²⁺ during expression of Rv0805 in *E. coli* led to greater structuring of the cap domain and reduced proteolysis (17, 18).

Although not explicitly tested, the above possibility has been alluded to for other members of the MPE family as well. For instance, complex formation between the Mre11 MPE from *Schizosaccharomyces pombe* with its interacting partner Nbs1 was modulated by binding of Mn²⁺ ions to the active site of Mre11 (41). Similarly, mutations in the MPE motifs of Dbr1 that coordinate metals are also known to compromise RNA splicing in *S. pombe*. Interestingly, mutation of residues in Dbr1 analogous to His²⁰⁷ and His²⁰⁹ in Rv0805 resulted in phenotypes similar to the Δdbr1 strain despite only a marginal compromise of activity in vitro (4). These data are in line with our observations and point to the crucial importance of the fourth MPE motif (His²⁰⁷ and His²⁰⁹ in Rv0805) in modulating interactions of this class of proteins with other cellular components.

The structure of Rv0805 provides evidence for a relay of hydrogen bond interactions involving both main chain and side chain groups, which may anchor the CTE of one protomer of Rv0805 dimer to the side chains of His²⁰⁷ (Fig. 7a) and His²⁰⁹ (Fig. 7b) of the other protomer. The imidazole side chain of His²⁰⁷ forms a hydrogen bond with the main chain carbonyl of Val₁⁸₃ in helix a₅ of the same protomer, which in turn makes a stacking interaction to helix a₇ in the C terminus of the other protomer (Fig. 7a). Therefore, a loss of the hydrogen bond after the H207A mutation could loosen the stacking interaction between the helices of the protomers, disturbing the functional orientation and conformation of the CTE. In the case of His²⁰⁹ (Fig. 7b), a web of hydrogen bonds is formed between the side chain of His²⁰⁹ and the root of the CTE of the other protomer (V272, Fig. 7b). Mutation of His²⁰⁹ to Ala may disturb this chain of interactions and, therefore, anchoring of the cap domain, resulting in decreased interaction with the cell envelope.

Given the above differences in the properties of Rv0805 and its C terminus-truncated mutant Rv0805Δ⁴₀, it is not surprising that expression of the two proteins resulted in very different phenotypic outcomes for *M. smegmatis*. Experiments with catalytically inactive mutants showed that reduced sensitivity to isoniazid upon expression of full-length Rv0805 was independent of catalysis and likely to be dependent on interaction with the cell envelope (Fig. 6b). Hypersensitivity to isoniazid upon expression of Rv0805Δ⁴₀ was, however, dependent on catalysis (Fig. 6c) and perhaps a result of significantly lower intracellular cAMP levels in *M. smegmatis* expressing Rv0805Δ⁴₀ than control or Rv0805 expressing *M. smegmatis* (Fig. 2e). Indeed, although Rv0805 lowered intracellular cAMP levels by ~30%, Rv0805Δ⁴₀ lowered cAMP levels in *M. smegmatis* by ~75% (Fig. 2e). The greater reduction in intracellular cAMP upon expression of cap domain-less Rv0805 is most likely a combination of two different phenomena. The first is the higher expression levels of Rv0805Δ⁴₀ than Rv0805 (Fig. 2b). The second is the enrichment of Rv0805Δ⁴₀ in the cytosol (Fig. 3, b and c), where most of the cellular CAMP is expected to be present. Regardless of the mechanism, it is clear that the CTE of Rv0805 reduced the extent of hydrolysis of cAMP in cells. Indeed, the extent of reduction of intracellular cAMP levels upon Rv0805Δ⁴₀ expression is similar to that observed when other bacterial cAMP phosphodiesterases such as CpdA from *E. coli* (42) or *Pseudomonas aeruginosa* (43) are overexpressed in their respective systems, supporting the above argument.

Our study demonstrates multiple roles for the C-terminal cap of Rv0805 ranging from destabilization of Rv0805 and, hence indirectly, utilization of soluble substrates in vivo, to membrane targeting (Fig. 7c). Once localized in the cell membrane, the Rv0805 CTE together with its active site may also mediate interaction with the cell wall polymer (Fig. 7c) and modify envelope properties independent of catalysis (Fig. 6, a and b). These factors together are crucial in determining the physiologically relevant roles of Rv0805 in mycobacteria. Not surprisingly the extent of conservation between Rv0805 and its homologs in other bacteria is highest in the core catalytic domain and is markedly lower in the C terminus of the protein that dictates more niche-specific functions (Fig. 7d). To the best of our knowledge this is the first systematic dissection of the cap domain of any MPE protein and identifies hitherto unknown communication between the cap domain and the active site of...
MPFs. It will hence provide an important benchmark for the study of other proteins of this class.

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