Phosphorylation of mycobacterial phosphodiesterase by eukaryotic-type Ser/Thr kinase controls its two distinct and mutually exclusive functionalities

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Phosphorylation-mediated negative feedback regulation of cAMP levels by phosphodiesterase is well-established in eukaryotic cells. However, such a mechanism remains unexplored in prokaryotes. We report here the involvement of eukaryotic-type Ser/Thr kinases, particularly PknA in trans-phosphorylating phosphodiesterase from Mycobacterium tuberculosis (mPDE), that resulted in decreased enzyme turnover rate compared with its unphosphorylated counterpart. To elucidate the role of mPDE phosphorylation in hydrolyzing cellular cAMP, we utilized a phosphodiesterase knock-out Escherichia coli strain, ΔcpdA, where interference of endogenous eukaryotic-type Ser/Thr kinases could be excluded. Interestingly, the mPDE-complemented ΔcpdA strain showed enhanced cAMP levels in the presence of PknA, and this effect was antagonized by PknA-K42N, a kinase-dead variant. Structural analysis of mPDE revealed that four Ser/Thr residues (Ser-20, Thr-22, Thr-182, and Thr-240) were close to the active site, indicating their possible role in phosphorylation-mediated alteration in enzymatic activity. Mutation of these residues one at a time to alanine or a combination of all four (mPDE-4A) affected catalytic activity of mPDE. Moreover, mPDE-4A protein in kinase assays exhibited reduction in its phosphorylation compared with mPDE. In consonance, phosphoproteins obtained after co-expression of PknA with mPDE/S20A/T240A/4A displayed decreased phospho-signal intensities in immunoblotting with anti-phosphoserine/phosphothreonine antibodies. Furthermore, unlike mPDE, phospho-ablated mPDE-T309A protein exhibited impaired cell wall localization in Mycobacterium smegmatis, whereas mPDE-4A behaved similarly as wild type. Taken together, our findings establish mutually exclusive dual functionality of mPDE upon PknA-mediated phosphorylation, where Ser-20/Thr-240 influence enzyme activity and Thr-309 endorses its cell wall localization.

Cyclic-adenosine monophosphate (3′,5′-cAMP), a universal and well-studied secondary messenger, is ubiquitously present among all kingdoms of life. cAMP is critically involved in regulating various signaling pathways, thus maintaining cellular homeostasis. In eukaryotes, cells utilize cAMP for multitude of functions like trophoblast fusion, metastasis regulation, neuro-modulation, and immunoregulation in humans (1–4) and social motility in trypanosomes (5). Prokaryotes, on the other hand, require cAMP for catabolite repression (6, 7), virulence, and pathogenesis (8, 9). To turn “on” or “off” the relay of signals within cells, catalytic machinery involving adenylyl cyclases (enzymes that convert ATP to cAMP) and phosphodiesterases (causing hydrolytic degradation of cAMP to AMP) are present throughout the phylogeny. In this context we concentrated on Mycobacterium tuberculosis, the causative agent of tuberculosis, which has extensively high levels of cAMP as its genome encodes ~16 genes for adenylyl cyclases (10, 11). In contrast, it has single phosphodiesterase (mPDE) encoded by Rv0805, which is a crucial enzyme involved in maintaining intra- and extracellular cAMP levels (12, 13). It has been reported that overexpression of mPDE resulted in an ~30% reduction in cAMP levels in Escherichia coli and Mycobacterium smegmatis (12) but a 50% decrease in M. tuberculosis strain H37Rv (13). Reports have also suggested the role of mPDE in modulating the host signaling pathway in a cAMP-dependent manner (13). We recently reported that mycobacterial eukaryotic-type Ser/Thr kinase, like PknA, phosphorylates a threonine residue (Thr-309) at the C terminus of mPDE and determines its localization to cell wall (14). Phosphorylation-mediated control of phosphodiesterase activity that eventually governs the feedback regulation of this enzyme is well-established in eukaryotes (15), but existence of such an event in prokaryotes demands elucidation.

In this study we report that phosphorylation of mPDE by PknA resulted in a decrease in its enzyme turnover rate. To evaluate the role of mPDE phosphorylation on its functionality, we utilized a phosphodiesterase knock-out strain of E. coli, where interference of endogenous mycobacterial kinases could be excluded. Interestingly, the mPDE-complemented strain showed enhanced cAMP levels in the presence of PknA, whereas this effect was antagonized by its kinase-dead variant, PknA-K42N. Structural analysis of mPDE indicated four puta-

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The abbreviations used are: mPDE, M. tuberculosis phosphodiesterase; Ni-NTA, nickel-nitrilotriacetic acid.
Phosphorylation modulates mPDE activity

Results
Phosphorylation modulates enzyme activity and functionality of mPDE

We investigated if eukaryotic-type Ser/Thr kinase is capable of modulating the functionality of mPDE in terms of any alteration in its enzyme activity. Accordingly, we monitored enzymatic activity of mPDE after its phosphorylation with PknA. Both unphosphorylated (mPDE) and phosphorylated (mPDE-P) proteins were purified from E. coli strain BL21(DE3) transformed with either pET-Duet-mPDE or pET-Duet-mPDE/PknA constructs. His-tagged mPDE proteins (unphosphorylated and phosphorylated, 1–16 μg each) were incubated with cAMP (0.5 mM) at 30 °C for 75 min in the presence of Mn2+. The reaction was terminated by adding Biomol green dye, and A620 was measured. Fig. 1A represents comparative activities of unphosphorylated and phosphorylated mPDE as the function of increasing amounts of protein. Interestingly, phosphorylated mPDE exhibited a significant decrease in its activity compared with that of the unphosphorylated protein at all concentrations tested. Comparison of the kinetic parameters of both the proteins (3 μg/assay) with increasing concentrations of cAMP (0–0.8 mM) also revealed an ~30% reduction in the enzyme turnover rate of the phosphorylated mPDE compared with its unphosphorylated counterpart (Fig. 1B). Western blotting using anti-mPDE or anti-phosphothreonine antibody validated the identity of the proteins used in enzymatic assay (Fig. 1B, left inset). To ensure that the outcome is not an experimental artifact, we utilized an E. coli BL21(DE3) system expressing pET-mPDE with or without pMAL-PknA, which also resulted in loss of enzyme activity upon phosphorylation (Fig. 1B, right inset).

Phosphorylation of phosphodiesterase in eukaryotes is known to influence cellular cAMP levels (15–18). To evaluate if phosphorylation on mPDE affects such functionality, we utilized endogenous phosphodiesterase knock-out E. coli strain JW3000-1 (∆cpcdA) transformed with different constructs using two incompatible vector systems (pGEX-KG and p19kpro) bearing same origin of replication, and colonies were selected on ampicillin and hygromycin plates. We found that mPDE was able to complement phosphodiesterase function in E. coli (third lane, Fig. 1C). Interestingly, as depicted in Fig. 1C, cells expressing both mPDE and PknA (fifth lane) exhibited cAMP levels more than that transformed with either mPDE (third lane) or PknA (fourth lane). Furthermore, co-expression of mPDE and kinase-dead mutant PknA-K42N in the ∆cpcdA strain displayed a decreased level of cAMP compared with the vector control (sixth lane as opposed to the second lane; Fig. 1C), and this is a consistent observation (left inset, Fig. 1C). We observed a slight decrease in cAMP levels in the ∆cpcdA strain transformed with only PknA (Fig. 1C, fourth lane) or PknA-K42N (Fig. 1C, left inset, second lane). However, this decrease was not as noticed with either mPDE (Fig. 1C, third lane) or PknA-K42N (Fig. 1C, sixth lane and left inset, third lane). Expressions of mPDE and PknA/PknA-K42N were confirmed by Western blotting using appropriate antibodies (right inset, Fig. 1C). It needs to be mentioned here that we used E. coli cells to obtain the null background system where the effect of only PknA or PknA-K42N over mPDE can be justified. Thus, our results indicated that PknA-mediated phosphorylation of mPDE affects its enzyme activity, thereby increasing cAMP levels within the E. coli cells, whereas its kinase dead variant antagonizes this effect.

Identification of phosphorylating residues influencing the catalytic activity of mPDE

Because phosphorylation of mPDE by eukaryotic-type Ser/Thr kinase, especially PknA, affected its enzymatic activities, it is intriguing to identify serine/threonine residues involved in the process. Previously, our mass spectrometric data identified Thr-309 as a phosphorylating residue in mPDE (14). We, therefore, compared the enzymatic activities of wild-type, mPDE-T309A, and the phosphorylated form of mPDE-T309A (mPDE-T309A-P) proteins. As expected from the catalytic parameters reported earlier for mPDE and mPDE-T309A proteins (14), we noted no significant differences in their behavior (Fig. 2). Interestingly, as shown in Fig. 2, mPDE-T309A-P exhibited a decrease in its catalytic efficiency (kcat/Km = 0.2 ± 0.03 μM⁻¹min⁻¹), which is similar to the phosphorylated mPDE (kcat/Km = 0.3 ± 0.1 μM⁻¹min⁻¹; Table 1). Western blotting using anti-mPDE antibody showed in the inset (Fig. 2) is a representation of proteins used in the assay.

We performed CD analysis as well as gel filtration chromatography with mPDE and mPDE-P proteins to elucidate any distinction between them. As evident from CD data, no significant variation in the secondary structure of mPDE was observed in phosphorylated protein as opposed to its unphosphorylated form (supplemental Fig. S1A). In gel filtration chromatography also, we observed elution of both the proteins (mPDE-P and mPDE) as dimers (supplemental Fig. S1B), which is unlike a previous report where both monomeric and dimeric forms of mPDE were noticed (12). However, such a variation seems to be minor, and column chromatography results often depend on experimental set up used.

We further carried out structural analysis to identify putative Ser/Thr residues in mPDE, which may affect its catalytic activity upon phosphorylation. Supplemental Table S2 depicts all Ser/Thr residues present in mPDE protein sequence, and the possibility of these amino acids affecting the catalytic activity upon phosphorylation was studied (using PDB ID 3IB8 as the template). The structural analyses revealed that four Ser/Thr

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residues (Ser-20, Thr-22, Thr-182, and Thr-240) close to the active site (5'-AMP-binding or metal-binding sites) might affect the activity of mPDE protein upon phosphorylation (Fig. 3 and inset). Interestingly, multiple sequence alignment by Clustal omega indicated most of these residues of mPDE are well-conserved in other mycobacterial orthologs (supplemental Fig. S2).

To gain an insight on the role of these residues, we mutated them one at a time to alanine (mPDE-S20A/-T22A/-T182A/-T240A) and also generated a multiple mutant, mPDE-4A (mutation of all four residues to alanine). Analysis of kinetic parameters of purified His-tagged mutant proteins compared with the wild-type mPDE exhibited a reduction in their turnover rate and catalytic efficiencies (Fig. 4A).
Phosphorylation modulates mPDE enzymatic activity. Schematic diagram shows the C^- trace of mPDE structure (PDB ID 3IB8). The helices, strands, and loops are shown in cyan, pink, and white, respectively. The Ser/Thr residues (Ser-20, Thr-22, Thr-182, and Thr-240) around the vicinity of the catalytic site and AMP are represented as sticks. The metal atoms Mn^2+ (purple) and Fe^3+ (magenta) bound with the crystal structure are shown as spheres. The inset shows a close view of the Ser/Thr residues around the active site that may influence the catalytic activity of mPDE upon phosphorylation. The distance between atoms is shown in dashed lines along with their values in Å. The figure was generated through PyMol using the PDB ID 3IB8 (39).

Table 1

| mPDE/variants | $K_m$ (mM) | $V_{max}$ (nmol/min/mg) | $k_{cat}/K_m$ (min^-1) | n |
|----------------|------------|-------------------------|-------------------------|---|
| mPDE           | 0.11 ± 0.02| 20.5 ± 0.4              | 11.0 ± 9.8              | 28c |
| mPDE-P         | 0.08 ± 0.01| 6.6 ± 0.3               | 6.9 ± 0.3               | 4  |
| mPDE-T22A      | 0.04 ± 0.01| 0.08 ± 0.3              | 0.23 ± 0.04             | 4  |
| mPDE-T240A     | 0.02 ± 0.04| 0.06 ± 0.2              | 0.2 ± 0.04              | 3  |
| mPDE-S20A      | 0.02 ± 0.06| 0.08 ± 0.4              | 0.3 ± 0.01              | 3  |
| mPDE-T182A     | 0.06 ± 0.3 | 0.11 ± 0.3              | 0.3 ± 0.03              | 3  |
| mPDE-T309A     | 0.11 ± 0.03| 23.9 ± 0.3              | 7.3 ± 0.3               | 3  |
| mPDE-P          | 0.02 ± 0.04| 0.06 ± 0.2              | 0.2 ± 0.04              | 3  |
| mPDE-T240A-P   | 0.02 ± 0.06| 0.08 ± 0.4              | 0.3 ± 0.01              | 3  |
| mPDE-S20A-P    | 0.02 ± 0.06| 0.08 ± 0.4              | 0.3 ± 0.01              | 3  |
| mPDE-T240A-P   | 0.02 ± 0.06| 0.08 ± 0.4              | 0.3 ± 0.01              | 3  |

Figure 2. Phosphorylation-mediated alteration in mPDE activity is independent of Thr-309 modification. Phosphodiesterase enzyme assays were performed for mPDE, mPDE-T309A, and mPDE-T309A-P (3 μg each) using increasing amounts of cAMP (0–0.8 mM). SDS-PAGE (inset) shows the loading control for the proteins used (3 μg/lane) in the assay. LMW, low molecular weight marker.

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| mPDE-T22A      | 0.04 ± 0.01| 0.08 ± 0.3              | 0.23 ± 0.04             | 4  |
| mPDE-T240A     | 0.02 ± 0.04| 0.06 ± 0.2              | 0.2 ± 0.04              | 3  |
| mPDE-S20A      | 0.02 ± 0.06| 0.08 ± 0.4              | 0.3 ± 0.01              | 3  |
| mPDE-T182A     | 0.06 ± 0.3 | 0.11 ± 0.3              | 0.3 ± 0.03              | 3  |
| mPDE-T309A     | 0.02 ± 0.04| 0.08 ± 0.3              | 0.2 ± 0.04              | 3  |
| mPDE-P          | 0.02 ± 0.06| 0.08 ± 0.4              | 0.3 ± 0.01              | 3  |
| mPDE-T240A-P   | 0.02 ± 0.06| 0.08 ± 0.4              | 0.3 ± 0.01              | 3  |
| mPDE-S20A-P    | 0.02 ± 0.06| 0.08 ± 0.4              | 0.3 ± 0.01              | 3  |
| mPDE-T240A-P   | 0.02 ± 0.06| 0.08 ± 0.4              | 0.3 ± 0.01              | 3  |

Figure 3. Bioinformatic prediction of phosphorylating serine and threonine residues influencing mPDE enzymatic activity. Kinetic parameters were obtained from fitting the data of Fig. 4A using the Michaelis-Menten equation.

Phosphorylation modulates mPDE enzymatic activity. Schematic diagram shows the C^- trace of mPDE structure (PDB ID 3IB8). The helices, strands, and loops are shown in cyan, pink, and white, respectively. The Ser/Thr residues (Ser-20, Thr-22, Thr-182, and Thr-240) around the vicinity of the catalytic site and AMP are represented as sticks. The metal atoms Mn^2+ (purple) and Fe^3+ (magenta) bound with the crystal structure are shown as spheres. The inset shows a close view of the Ser/Thr residues around the active site that may influence the catalytic activity of mPDE upon phosphorylation. The distance between atoms is shown in dashed lines along with their values in Å. The figure was generated through PyMol using the PDB ID 3IB8 (39).

To ascertain the involvement of these residues (Ser-20, Thr-22, Thr-182, and Thr-240) in PknA-mediated phosphorylation of mPDE, we performed an in vitro kinase assay, and phospho-signals were adjudged by scanning band intensity. Interestingly, among all mutant proteins tested, only mPDE-4A displayed a significant decrease in the magnitude of its phosphorylation compared with that of the wild-type mPDE, indicating a role of all four predicted residues (Fig. 5). We used Coomassie-stained gel as the loading control in this experiment (Fig. 5, bottom panels). We further co-expressed mutant constructs (in pET-28c vector) one at a time along with PknA (in pMAL-c2 vector) in E. coli BL21(DE3) cells. Expression of both the proteins in cell lysates was ensured in Western blotting using anti-PknA and anti-mPDE antibodies respectively (Fig. 6A, upper and lower panels). Among different Ni-NTA-purified mutant proteins, phosphorylated mPDE-S20A-P, mPDE-T240A-P, and mPDE-4A-P samples were poorly recognized on immunoblotting with phospho-antibodies (anti-phosphoserine and anti-phosphothreonine) compared with that of the mPDE control (Fig. 6B, see the fourth and fifth lanes and the sixth and seventh lanes of the left and right panels, respectively). We further compared phosho-signal intensities of different amounts of wild-type and mutant proteins in Western blotting using anti-phosphoserine/phosphothreonine antibody. On comparing lanes 3, 4, and 5 of different rows of phosphorylated mutant proteins with that of the wild-type control, we concluded that the decrease in phospho-signal intensities was not an experimental artifact (Fig. 6C, left and right panels). Quantitative analyses of phosphorylation intensity of bands from Western blotting recognized by anti-phosphoserine and anti-phosphothreonine antibodies using ImageJ software showed a significant decrease in trans-phosphorylation of mPDE-S20A/-T240A/-4A compared with the wild-type mPDE (Figs. 6, B and C, bottom panels). The Ponceau-S-stained blot served as the loading control for different samples used in this experiment (please see supplemental Fig. S3). The results of the kinase assay with mutant proteins like mPDE-S20A and mPDE-T240A, as opposed to their co-expression experiments with PknA, indicated a discrepancy (please see Figs. 5 and 6B). Among four residues, it is quite likely that phosphorylation of Ser-20 and Thr-240 were sufficient in affecting the process in an in vivo setting. Nonetheless, based on our observation with mPDE-4A, it is apparent that PknA-mediated phosphorylation decreased mPDE activity, and the contributions of Ser-20 and Thr-240 are noteworthy in the process.
Phosphorylation modulates mpDE activity

We previously reported that cell wall localization of mpDE is affected in the mpDE-T309A mutant protein, but its enzymatic activity remains unaltered (14). In this study we observed that either mpDE-S20A or mpDE-T240A or mpDE-4A affected the activity of the protein (Fig. 4 and Table 1). We were therefore intrigued to elucidate whether both the phosphorylation-mediated events, localization and activity regulation of mpDE, are independent or interdependent phenomena. For this, we prepared cell wall fractions from *M. smegmatis* expressing either vector alone or mpDE or mpDE-T309A or mpDE-T240A or mpDE-4A proteins. Expression of each construct in *M. smegmatis* was validated by Western blotting of whole cell lysates with anti-mpDE antibody (Fig. 7A, upper panel). Immunoblotting with anti-mpDE antibody revealed that mpDE-T240A and mpDE-4A variants of mpDE localized similarly as was observed with the wild type, whereas localization of mpDE-T309A was impaired (Fig. 7B, compare lane 4 with lanes 3, 5, and 6). To ensure the purity of different samples, Western blotting using anti-Ag85 (cell wall marker) and anti-RpoB (cytosolic marker) antibodies was carried out (Fig. 7C). To validate this observation, we further mutated Thr-309 to alanine in the mpDE-4A (termed as mpDE-5A), and the pVV2-mpDE-5A construct was transformed in *M. smegmatis*. After its expression (Fig. 7D, upper panel), we observed an impaired localization of

Figure 4. Active site serine and threonine are involved in maintaining mpDE enzyme activity. A, mutation of Ser/Thr alters enzyme activity. Four residues (Ser-20/Thr-22/Thr-182/Thr-240) identified by PyMOL were mutated to alanine by site-directed mutagenesis. Kinetic parameters for the wild-type (mpDE) and its variants (mpDE-P/S20A/T22A/T182A/T240A/4A) were calculated using the protocol described under “Experimental procedures.” The inset represents the SDS-PAGE for depicting the loading of proteins used in the assay. Asterisks indicate the significance of particular data set (*Kcat/Km*) with respect to the wild-type mpDE where *p* < 0.01. B, secondary structure of all mpDE variants remains unaltered. Left panel: CD for mpDE and its point mutants (0.3 mg protein) was performed to calculate the mean residual ellipticity (MRE) using eight accumulations each. Right panel: an independently performed paired CD experiment for mpDE and mpDE-4A. The percentage of α helices and β strands present in all the proteins was calculated using online K2D2 software.

PknA-mediated phosphorylation of mpDE controls its two distinct but mutually exclusive functionalities

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mPDE-5A protein compared with that of either wild-type or mPDE-4A (Fig. 7D, middle and bottom panels; also see supplemental Fig. S4 depicting Ponceau-S stained blots for ensuring similar loading). The impairment in cell wall localization of mPDE-5A protein also led us to examine its enzyme activity. We, therefore, cloned in pET-28c vector, expressed in E. coli strain BL21(DE3), and purified mPDE-5A using Ni-NTA-purified columns. As shown in Fig. 7E, mPDE-5A displayed a reduced enzymatic activity compared with the wild-type protein, and it was very similar to that of the wild-type mPDE protein using ImageJ software. Coomassie-stained radioactive gels provided in lower panels were used as loading controls. Results shown are a representation of three independent experiments from two different preparations. Numbers indicate molecular weight markers in kDa. AU, arbitrary units; LMW, low molecular weight marker.

mPDE-5A protein compared with that of either wild-type or mPDE-4A (Fig. 7D, middle and bottom panels; also see supplemental Fig. S4 depicting Ponceau-S stained blots for ensuring similar loading). The impairment in cell wall localization of mPDE-5A protein also led us to examine its enzyme activity. We, therefore, cloned in pET-28c vector, expressed in E. coli strain BL21(DE3), and purified mPDE-5A using Ni-NTA-purified columns. As shown in Fig. 7E, mPDE-5A displayed a reduced enzymatic activity compared with the wild-type protein, and it was very similar to that of the wild-type mPDE protein using ImageJ software. Coomassie-stained radioactive gels provided in lower panels were used as loading controls. Results shown are a representation of three independent experiments from two different preparations. Numbers indicate molecular weight markers in kDa. AU, arbitrary units; LMW, low molecular weight marker.

Figure 5. Identification of phosphorylating residues of mPDE by in vitro kinase assay. MBP-PknA (1 μg) was briefly incubated with Ni-NTA-purified mPDE and its point mutants (left upper panel; 10 μg each). Samples were processed as described under “Experimental Procedures.” mPDE-4A was generated by a combined mutation of four Ser/Thr residues (Ser-20/Thr-22/Thr-182/Thr-240) in a single protein sequence. In vitro kinase assays were performed for wild type and mPDE-4A using mPDE-T240A as a reference point mutant (right upper panel; 10 μg of protein/slot). Graphs presented in the middle panel compare the phosphorylation intensity of each mutant with the wild-type mPDE protein using ImageJ software. Coomassie-stained radioactive gels provided in lower panels were used as loading controls. Results shown are a representation of three independent experiments from two different preparations. Numbers indicate molecular weight markers in kDa. AU, arbitrary units; LMW, low molecular weight marker.

In consequence, we measured the cellular cAMP levels in E. coli ΔcpdA cells after expression with mPDE or mPDE-4A. Interestingly, we observed a decrease in cellular cAMP levels on expression of mPDE, whereas this phenomenon was reversed with mPDE-4A (Fig. 8). Thus, our results established the functional role of phosphorylating residues like Ser-20 and Thr-240 in regulating mPDE enzyme activity.

Discussion

Cyclic-AMP, as a secondary messenger, is an important regulator of various biological events in both prokaryotes and eukaryotes (19). In bacteria, cAMP-mediated signaling regulates important processes, such as catabolite repression (6), quorum sensing (20, 21), biofilm formation (22), and virulence...
and pathogenesis (9). Because there exists a single phosphodiesterase in *M. tuberculosis*, it seems very likely to be the major regulatory body for maintaining cAMP levels in cells >16 adenylate cyclases. In eukaryotes, phosphorylation-mediated regulation of phosphodiesterase activity is well-known (15). Therefore, it is intriguing to predict the occurrence of such
Phosphorylation modulates mPDE activity

Suspecting the role of some alternative mechanism in the pro-T309A protein behaved similarly to that of mPDE-P (Fig. 2), an earlier report (14). Furthermore, the phosphorylated mPDE enzyme activity (Fig. 2); it is also in consonance with our expectation. Expectedly, Thr-309 did not play any role in modulating the phospho-residue involved in the process of its cell wall localization (14). In this study, we were interested in elucidating functional impact of such phosphorylation on the activity of mPDE.

We obtained phosphorylated histidine-tagged-mPDE protein after its co-expression with PknA using an E. coli-based heterologous expression system. It needs to be mentioned here that E. coli served as a valuable surrogate host in the study, as it is benefitted with the absence of any eukaryotic-type Ser/Thr kinase or phosphatase under normal physiological conditions (23). Assessment of the activity revealed a 30–50% reduction in the enzyme turnover rate of phosphorylated protein compared with its unphosphorylated counterpart (Fig. 1, A and B; Table 1). Under our experimental conditions, we observed mPDE was able to functionally complement E. coli phosphodiesterase activity to the extent of ~50% (Fig. 1C). In fact, comparison of their sequences also revealed an ~44% similarity. Because there are no PknA/phosphatase orthologs in the E. coli genome and a single copy of adenylate cyclase as well as phosphodiesterase is present, we thought that an endogenous phosphodiesterase knock-out E. coli strain JW3000-1 (∆cpdA) would be ideal in monitoring the effect of kinase onto the system. Moreover, the use of the mycobacterial system as such or its mPDE knock-out variant for this purpose seems to be difficult, if not impossible, as it possesses 11 eukaryotic-type Ser/Thr kinases, one phosphatase and 16 adenylate cyclases that might have cumulative and interfering effects. Using the mPDE-complemented ∆cpdA strain after transformation with either PknA (kinase) or PknA-K42N (a kinase-dead variant of PknA), we monitored the cAMP level by a Direct ELISA. Intriguingly, the mPDE-complemented strain showed enhanced cAMP levels in the presence of PknA, whereas this effect was antagonized where PknA-K42N was expressed (Fig. 1C). Taken together, these in vitro and in vivo studies argued that PknA-mediated phosphorylation could modulate mPDE enzyme activity as well as its functionality as evident by high cAMP levels within E. coli cells.

To understand the functional cause of mPDE activity drift upon phosphorylation with PknA, we review the role of Thr-309 that has already been identified by mass spectrometry as a phospho-residue involved in the process of its cell wall localization. Expectedly, Thr-309 did not play any role in modulating mPDE enzyme activity (Fig. 2); it is also in consonance with our earlier report (14). Furthermore, the phosphorylated mPDE-T309A protein behaved similarly to that of mPDE-P (Fig. 2), suggesting the role of some alternative mechanism in the process. It needs to be noted here that in mass spectrometric studies we did not identify any phosphorylating residue(s) other than Thr-309. This may be due to the intensity of phosphorylation of any other residue below the detection limit in our experimental setting. In fact, in M. tuberculosis, phosphoproteome mapping did not detect mPDE as a phosphoprotein (24, 25). CD and gel filtration profiles indicate the absence of the involvement of any secondary or quaternary structure alterations (supplemental Fig. S1). This led us to carry out structural analysis of mPDE for identification of any putative residue(s) that might be involved in phosphorylation-mediated activity regulation (Fig. 3). We identified four serine and threonine residues (Ser-20, Thr-22, Thr-182, Thr-240) that were close (between 4.0 and 8.5 Å) to the vicinity of the active site (Ser-20 and Thr-22 are closer to metal-binding sites, whereas Thr-182 and Thr-240 are near 5'-AMP). We speculated that the addition of phosphate moiety would further reduce the distance even more. Thus, point mutation of these residues to alanine (replacement of one amino acid at a time or all four residues mutated) led to a significant loss in catalytic activity of mPDE (Fig. 4A). In the kinase assays, the multiple mutant protein (mPDE-4A) in the presence of PknA displayed a significant decrease in its phosphorylation ability, signifying it as a phosphorylation-mediated event (Fig. 5). This led us to perform in vivo phosphorylation experiments using an E. coli-based co-expression system, which established Ser-20 and Thr-240 of mPDE as critical phosphorylating residues (Fig. 6). To evaluate the role of these residues in cell wall localization, we cloned different mutant constructs in mycobacterial expression vector (pVV2-mPDE/pVV2-mPDE-T240A/-T309A/-4A/-5A) and introduced non-pathogenic M. smegmatis strain mc^2155, where the mPDE ortholog is absent, but endogenous eukaryotic-type Ser/Thr kinases are present (equivalent to a mycobacterial mPDE knock-out strain) as described elsewhere (14). It needs to be mentioned here that mPDE-S20A mutation was not included in this experiment as it has already been incorporated in mPDE-4A. Cell wall fractionation experiments with anti-mPDE antibodies established that the phosphorylating residues, Ser-20 and Thr-240, were not involved in localization of mPDE to the cell wall unlike the role played by Thr-309 of this protein (Figs. 7, B and D). Additionally, the mPDE-5A protein displayed catalytic behavior similar to either mPDE-4A or mPDE-4A-P (Fig. 7E), indicating dual regulation of mPDE by the existence of phosphorylation wherein Ser-20 and Thr-240 (near to active site) contributed to enzyme activity, whereas the C-terminal Thr-309 contributed to cell wall localization. These findings were further endorsed when we observed that the expression of catalytically deficient mutant protein (mPDE-4A)

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**Figure 6. Identification of Ser-20 and Thr-240 as phosphorylating residues.** A, PknA expression in cells. E. coli strain BL21(DE3) expressing mPDE variants in the presence or absence of pMAL-PknA were allowed to grow and express recombinant proteins. Immunoblotting with anti-PknA antibody was performed to ensure PknA expression in each construct. The lower panel represents Ponceau-S stained blot. B, identification of mPDE phospo-site. Recombinant His-tagged mPDE and its variants were purified using Ni-NTA affinity chromatography from BL21(DE3) cells expressing pMAL-PknA. Top panel: phosphorylation status of the proteins was determined in Western blotting by probing with anti-phosphoserine (left side) and anti-phosphothreonine (right side) antibodies. Purified PknA was loaded as the positive control for anti-phosphoserine antibody (sixth lane, left side). Middle panel: Ponceau-S-stained blot as a loading control for the top panel. Bottom panel: quantification of phosphorylation intensities using the top panel by ImageJ software. C, Ser-20 and Thr-240 identified as major phosphorylating residues. Wild-type and mutant proteins (mPDE-P, mPDE-S20A-P, mPDE-T240A-P, and mPDE-4A-P) were loaded in increasing amounts (1–10 μg) along with their respective unphosphorylated versions (5 μg) and immunoblotted with anti-phosphoserine and anti-phosphothreonine antibodies. Band intensities from these blots were calculated using ImageJ software and are represented as bar graphs (bottom panels). Numbers indicate molecular mass in kDa. AU, arbitrary units; PM, pre-stained marker; U, unphosphorylated; P, phosphorylated.

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17369
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A

B

C

D

E

M. smegmatis Whole Cell Lysate

M. smegmatis Cell Wall

M. smegmatis Cell Wall

Intensity in AU (pg/mg/mg)

Activity (nmol/min/mg)

3', 5' - cAMP (mM)
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Figure 8. Phosphorylation affected mPDE functionality. Intracellular cAMP levels were measured using Direct ELISA for E. coli cells (JW3000-1) expressing either vector control or pGEX-mPDE or pGEX-mPDE-4A. The y axis represents the free cAMP levels (in picomoles) present within ~10^9 bacterial cells. Data (mean ± S.D.) presented are the outcomes of three different experiments from two independent preparations. Inset: Western blotting using anti-mPDE antibody of the whole cell lysate of each strain used in the assay. PM, pre-stained marker.

led to the reversal of intracellular cAMP levels within E. coli strain JW3000-1 as opposed to the cells complemented with the wild-type protein (Fig. 8). Thus, our results convincingly established inhibitory effects of phosphorylation over mPDE activity.

The intracellular concentration of CAMP in cells is usually regulated by negative feed-back mechanism through interaction of synthases and phosphodiesterase. In this scenario it is tempting to speculate that phosphorylation-mediated reduction in mPDE enzymatic activity would definitely benefit mycobacteria in enhancing its intracellular CAMP pools using an additional means. Although the eukaryotic-type Ser/Thr kinase-mediated phosphorylation of mPDE in cell wall localization seems to be distinct and mutually exclusive, their occurrence as parallel events needs to be explored. However, the present study provides a unique opportunity for understanding how phosphorylation of mPDE regulates its enzyme activity and localization in bacterial cells. Furthermore, elucidating how mPDE and PknA would interact and behave under varying physiological conditions would open up new vistas in the horizon of cAMP-mediated signaling. In addition, mPDE is phosphorylated by other eukaryotic-type Ser/Thr kinases as well (14). Therefore, it would be interesting to see their contribution toward the functionality of this protein. Nevertheless, unraveling such an exceptionally unique signaling cascade(s) like eukaryotic-type Ser/Thr kinase-mediated regulation of small molecule-hydrolyzing machinery would definitely aid in conceiving novel drug intervention strategies. Furthermore, the environmental cues responsible for regulating such a cross-talk between independent signaling pathways would offer promising perspectives for rationalizing the development of new antimycobacterials in the years to come.

Experimental procedures

Materials

Restriction/modifying enzymes used in this study were obtained from New England BioLabs and Fermentas. Unless mentioned otherwise, fine chemicals (Tris, NaCl, maltose, MnCl2, MgCl2, and SDS) were purchased from Sigma. Anti-phosphotheonine (Cell Signaling), anti-Ag85, anti-RpoB, anti-FLAG (Sigma), and anti-rabbit IgG (GE Healthcare) were commercially available. Anti-PknA and anti-mPDE antibodies were in-house–generated as mentioned previously (14, 26). Cyclic-AMP complete ELISA (ADI-900-163) and phosphodiesterase assay kits (BML-AK800-0001) were the products of Enzo®Lifesiences. [γ-32P]ATP was obtained from Jonaki Laboratories, Hyderabad, India. Desalted oligonucleotides were custom-synthesized from Sigma. BW25113 (wild type) and JW3000-1 (ΔcpdA; endogenous phosphodiesterase knockout) derived from E. coli strains K12 were obtained from Coli Genetic Stock Center (CGSC, Yale University).

Cloning, expression, and site-directed mutagenesis

Mycobacterial genes used in this study were amplified through PCR using M. tuberculosis genomic DNA as the template. Primers used were designed by incorporating suitable restriction sites based on gene sequences obtained in TuberculList database, and they are enlisted in supplemental Table S1. M. tuberculosis phosphodiesterase gene was cloned in either pET-28c (NdeI/HindIII sites; pET-mPDE) or pET-Duet-1 (EcoRI/HindIII sites at MCS-1; pET-Duet-mPDE) or pGEX-KG (EcoRI/HindIII sites; pGEX-mPDE) depending on the experimental design. Histidine-tagged proteins were expressed and purified from E. coli strain
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BL21(DE3), details of which was mentioned elsewhere (14, 27). On the other hand, GST fusion proteins were expressed in either E. coli BW25113 or JW3000-1 strains. For expression of histidine-tagged mPDE protein in M. smegmatis strain mc²155, it was cloned in pV2 at Ndel/HindIII sites (14, 26, 28). PknA or its kinase-dead variant (K42N) was cloned in p19kpro vector and expressed in E. coli strain BW25113 or JW3000-1 for either its expression or co-expression along with GST fusion protein constructs (14, 26, 28). Co-expression studies in E. coli BL21(DE3) were usually carried out with two incompatible plasmids with the same origin of replication, like pMAL-PknA and pET-mPDE, where different antibiotic selection facilitated expression of interacting mycobacterial proteins. As reported previously, although the level of expression of these interactors varied between experiments, it did not affect their intrinsic properties (29–31). However, in some experiments, PknA was cloned at the Ndel/KpnI sites of MCS-2 of the pET-Duet-mPDE construct (pET-Duet-mPDE/PknA) to carry out co-expression studies maintaining strict control on copy number of individual gene.

Site-directed mutagenesis employing the overlap-extension PCR method was adopted to generate the mPDE mutants (mPDE-S20A/-T22A/-T182A/-T240A/-4A) using pET-mPDE DNA as the template. All mutant constructs were cloned in the appropriate expression vectors (mentioned above), and their DNA as the template. All mutant constructs were cloned in the NdeI/KpnI sites of MCS-2 of the pET-Duet-mPDE construct (pET-Duet-mPDE/PknA) to carry out co-expression studies maintaining strict control on copy number of individual gene.

Kinase assay

In vitro kinase assays of histidine-tagged mPDE and its mutant proteins were performed to detect transphosphorylation by PknA (27). Briefly, PknA (0.5–1 μg/reaction) in 1× kinase buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl containing 10 mM MnCl2) and 2 μCi of [γ-³²P]ATP was incubated with mPDE and/or its mutants (5–20 μg/reaction) at 25 °C for 30 min (total reaction volume = 20 μl). The reaction was terminated by adding 5× SDS dye. Samples were resolved in 10% SDS-PAGE, and gels were stained with Coomassie Brilliant Blue. Gels were analyzed in a phosphorimaging device (Fujifilm model FLA 9000) and exposed to Eastman Kodak Co. X-Omat/AR film for autoradiography.

Western blotting

Immunoblotting of purified proteins (usually 1–2 μg) or whole cell lysates (usually 5–30 μg) was carried out using either anti-phosphothreonine (1:1000) or anti-PknA (1:1000) or anti-mPDE (1:1000) or anti-Ag85 (1:500) or anti-RpoB (1:500) and anti-rabbit IgG (1:5000) as the primary and secondary antibodies, respectively. Protein fractions were separated on 10% SDS-PAGE gels. The resolved proteins were transferred (120 V) to nitrocellulose membrane (0.45 μm) using mini-transblot apparatus (Bio-Rad). Blots were probed with suitable antibodies, and proteins were finally detected with luminata substrate (Millipore) and developed on X-ray films (Kodak) following the manufacturer’s recommended protocol. For monitoring in vivo phosphorylation, pET-mPDE or its mutant proteins cloned in pET-28c were co-transformed with pMAL-PknA in E. coli BL21(DE3) cells, and their expression as a phosphorylated protein was detected employing Western blotting using anti-phosphothreonine antibody as described earlier (32).

Phosphodiesterase enzyme activity

Phosphodiesterase activity of mPDE was determined using cAMP hydrolysis assay, essentially described earlier (14). The catalytic activity of unphosphorylated and phosphorylated (pET-Duet-mPDE and pET-Duet-PknA-mPDE) proteins (3 μg each) was monitored under the at same conditions (75 min incubation) in the presence of Mn²⁺ (200 μM) at 30 °C and 5′-nucleotidase enzyme (0.5 kilounits/μl). Phosphate thus generated was detected at A₅₂₀ by adding BIOMOL GREEN™ dye (30 min incubation at room temperature). A standard curve was prepared by using 5′-AMP (0.25–3 nmol) in PDE assay buffer that catalyzes its cleavage by 5′-nucleotidase enzyme. Enzyme activity was also measured for mPDE and mPDE-P using increasing protein amounts (0–16 μg) with 0.5 mM cAMP substrate. Similarly, mPDE activity was compared with mPDE-T309A with increasing protein amounts (0–16 μg).

Cyclic-AMP ELISA

Cellular lysis for performing cAMP ELISA was accomplished either by sonication over ice (10 s on and 10 s off cycles for 5 min; 20% amplitude) or heating at 100 °C (10 min) and centrifuging at 10,000 × g at 4 °C for 20 min. Briefly, the supernatants were then used for cAMP ELISA using the manufacturer’s guidelines. Expression of mPDE/mutants and PknA were monitored in all samples using Western blotting with anti-mPDE and anti-PknA antibodies, respectively.

CD spectroscopy

CD spectra of wild-type mPDE (unphosphorylated and phosphorylated) along with all point mutants were compared using a Jasco J-810 spectropolarimeter. Protein solutions (0.25–0.30 mg/ml, dialyzed in 20 mM Tris, pH 7.5, 20 mM NaCl) employing a J cell with a path length of 0.1 cm at 25 °C was used for measurements in the far ultraviolet region (250–190 nm). Each spectrum reported is an average of 8 scans, and the mean residue ellipticity (θ) was calculated considering 110 Da as the mean of amino acid residue molecular mass.

Gel filtration chromatography

Unphosphorylated and phosphorylated mPDE proteins (mPDE and mPDE-P) were purified from E. coli strain BL21(DE3) with or without pMAL-PknA expression using Ni-NTA affinity-based column chromatography as described earlier. Proteins were then resolved using size exclusion chromatography using a Superdex-200 (16/60, GE Healthcare) column on an AKTA purifier protein purification system (GE Healthcare). The protein profile in terms of retention volume was extrapolated on the standard curve prepared using markers from GE Healthcare.

Expression, cell wall localization of mPDE, and its mutants in M. smegmatis

M. smegmatis mc²155 cells expressing wild-type mPDE or mPDE-T240A/-T309A/-4A cloned at the Ndel/HindIII sites in pV2 vector (pV2-mPDE/pV2-mPDE-T240A/-T309A/
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