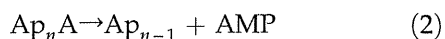


three-dimensional structure of Rv2613c and elucidated its structure–function relationships.

Since the amino acid sequence of Rv2613c has a histidine triad (HIT) motif (His- φ -His- φ -His- φ - φ , where φ represents a hydrophobic amino acid), Rv2613c is thought to belong to the HIT superfamily. This superfamily also includes nucleotide hydrolases and transferases such as adenosine monophosphate (AMP) lysine hydrolases, HIT family diadenosine polyphosphate (Ap_nA) hydrolases, galactose-1-phosphate uridylyltransferases, and Ap_4A phosphorylases.⁷ The amino acid sequence of Rv2613c has both similarities with and differences from the sequences of other Ap_4A phosphorylases in the HIT superfamily. For example, *Kluyveromyces lactis* Ap_4A phosphorylase (Swiss-Prot ID: APA2_KLULA) and *Saccharomyces cerevisiae* Ap_4A phosphorylases 1 and 2 (Swiss-Prot IDs: APA1_YEAST and APA2_YEAST) have a His-X-His-X-Gln motif instead of the HIT motif.^{8,9} In addition, unlike these phosphorylases, which are approximately 330 amino acids long,^{8,9} Rv2613c is only 195 amino acids long; therefore, its length is similar to that of *Schizosaccharomyces pombe* Ap_nA hydrolase (Swiss-Prot ID: APH1_SCHPO), which is 182 amino acids long.¹⁰ Furthermore, the amino acid sequence of Rv2613c is more similar to that of HIT family Ap_nA hydrolases than typical Ap_4A phosphorylases. For instance, the amino acid sequence of Rv2613c is 28.8% identical and 61.9% similar to *Sc. pombe* Ap_nA hydrolase, whereas it is 16.2% identical and 60.7% similar to *S. cerevisiae* Ap_4A phosphorylase 1. Collectively, these observations suggest that the amino acid sequence of Rv2613c is more related to HIT family Ap_nA hydrolases than to typical Ap_4A phosphorylases.

Furthermore, we recently showed that Rv2613c converts Ap_4A into ATP and adenosine 5'-diphosphate (ADP) in the presence of inorganic phosphate (Eq. (1)), so it is a phosphorylase rather than a hydrolase.⁴ In contrast, HIT family Ap_nA hydrolases convert Ap_nA into Ap_{n-1} and AMP (Eq. (2)):⁷



Previous crystallographic studies of HIT superfamily proteins have revealed that the HIT motif is part of the substrate binding site and that the conserved central His residue in the HIT motif is important for catalytic activity.^{11,12} In addition, a Ser residue, which is upstream of the HIT motif, is important for the catalytic activity of AMP-lysine hydrolase¹³ and galactose-1-phosphate uridylyltransferase.¹⁴ However, the three-dimensional structure and catalytic residues of an Ap_4A phosphorylase are not known. Therefore, we aimed to determine the crystal structure of

Rv2613c and to characterize and compare the structural basis of its catalytic activity with those of HIT family Ap_nA hydrolases and typical Ap_4A phosphorylases.

Previously, we also showed that the multimerization of Rv2613c differs from those of HIT family Ap_nA hydrolases and typical Ap_4A phosphorylases.⁴ Specifically, Rv2613c is homotetrameric in solution,⁴ whereas HIT family Ap_nA hydrolases and typical Ap_4A phosphorylases are homodimeric¹⁵ and monomeric,^{16,17} respectively. Therefore, we also investigated the importance of multimerization for the catalytic activity of Rv2613c.

Recently, we reported the crystallization and preliminary X-ray analysis of Rv2613c.¹⁸ Here, we describe the 1.89-Å-resolution crystal structure of Rv2613c and provide insights into the structural basis of its catalytic activity. To our knowledge, this is the first report of the crystal structure of a protein with Ap_nA phosphorylase activity.

Results

Structure determination

Previously, we showed that the Rv2613c crystal belongs to the C2 space group and has unit cell parameters of $a=101.5$ Å, $b=63.6$ Å, $c=79.1$ Å, and $\beta=110.9^\circ$.¹⁸ In addition, we reported that Rv2613c exists as a homotetramer of 25-kDa subunits in solution⁴ and that there are two subunits per asymmetric unit, which are characterized by a V_m of 2.41 Å³ Da⁻¹ and a solvent content of 49.1%.¹⁸ Here, we solved the crystal structure of Rv2613c at 1.89 Å resolution by using single-wavelength anomalous dispersion (SAD). The final R -factor and R_{free} were 17.6% and 20.1%, respectively (Table 1). The final model of Rv2613c consisted of 325 residues, 233 water molecules, 3 phosphate ions, and 2 tetraethylene glycols in the asymmetric unit, which included subunits A and B (Rv2613c-A/-B) (Fig. 1a). Electron density was present for all residues in Rv2613c-A, except for the His tag and for residues 1–13, 36–54, and 171–175. Similarly, electron density was present for all residues in Rv2613c-B, except for the His tag and for residues 1–23. In addition, residues 196–198 in Rv2613c-B were derived from the expression vector. The average B -factors for all atoms of Rv2613c-A, Rv2613c-B, water molecules, phosphate ions, and tetraethylene glycols were 32.7, 26.5, 36.3, 36.6, and 43.8 Å², respectively. Ramachandran plot analysis showed that the φ/ψ angle pairs of most residues (322/325; 99.1%) were in the favored regions, whereas the pairs of the remaining residues (3/325; 0.9%) were in the allowed regions (Table 1).

Table 1. Data collection and structure refinement statistics

	Native	SeMet
<i>Data collection</i>		
Wavelength (Å)	1.00000	0.97901
Resolution range (Å)	50.0–1.89 (1.93–1.89) ^a	50.0–2.50 (2.54–2.50)
Space group	C2	C2
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	101.5, 63.6, 79.1	101.3, 63.5, 79.5
Cell dimension β (°)	110.9	110.6
Unique reflections	37,314 (1826)	16,529 (808)
Average redundancy	7.4 (6.4)	7.6 (7.3)
Completeness (%)	99.8 (98.3)	99.9 (99.9)
Mean $I/\sigma(I)$	42.2 (4.4)	33.6 (6.9)
R_{merge}^b	6.1 (38.0)	8.6 (33.4)
<i>Refinement</i>		
Resolution range	39.4–1.89 (1.94–1.89)	
Number of reflections	36,096 (2327)	
R-factor (%) ^c	17.6 (19.5)	
R_{free} (%) ^d	20.1 (26.6)	
RMSD from ideality		
Bonds (Å)	0.005	
Angles (°)	1.025	
Ramachandran plot		
Favored region (%)	99.1	
Allowed region (%)	0.9	
Outlier region (%)	0	

^a Data for the highest-resolution shells are given in parentheses.

^b $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i th intensity measurement of reflection hkl and $\langle I(hkl) \rangle$ is its average.

^c $R\text{-factor} = \sum_{hkl} |F_{\text{obs}}| - k|F_{\text{calc}}| / \sum_{hkl} |F_{\text{obs}}|$, where k is the scaling factor.

^d This value is based on 5% of the data excluded from refinement at random.

Protein architecture and conformation of prosthetic groups

The overall structure of Rv2613c consisted of a small N-terminal domain (residues 1–39) and a large C-terminal domain (residues 40–195). In Rv2613c-A, the N-terminal domain consisted of two β -strands, whereas in Rv2613c-B, the N-terminal domain consisted of one α -helix and one β -strand (Fig. 1a and b). The C-terminal domain formed an α/β structure in both Rv2613c-A and Rv2613c-B, and was involved in the dimer interaction between Rv2613c-A and Rv2613c-B in the asymmetric unit (Fig. 1a and b). The main structural feature of this dimer was an antiparallel β -sheet with a normal left-handed twist (Fig. 1a). This β -sheet consisted of 10 β -strands, which included strands C, D, E, G, and F from Rv2613c-A, and strands M, N, L, K, and J from Rv2613c-B (Fig. 1a and b). Two long α -helices (α_4 in Rv2613c-A and α_{12} in Rv2613c-B) also were involved in the dimer interaction and were located inside this β -sheet (Fig. 1a and b).

The quaternary structure of Rv2613c was a tetramer consisting of a dimer of dimers (Rv2613c-A/-B + Rv2613c-A'/-B') (Fig. 1c), in agreement with

our previous observation that Rv2613 tetramerizes in solution.⁴ The N-terminal domain was involved in intersubunit contacts (Fig. 1b and c). Specifically, at the A–B' contact, a mixed β -sheet and a parallel β -sheet consisted of three β -strands (A and H from Rv2613c-A and I' from Rv2613c-B') and two β -strands (B from Rv2613c-A and O' from Rv2613c-B'). Similarly, at the B–A' contact, a mixed β -sheet and a parallel β -sheet also were composed of three β -strands (I from Rv2613c-B, and A' and H' from Rv2613c-A') and two β -strands (O from Rv2613c-B and B' from Rv2613c-A') (Fig. 1b and c). In addition, three α -helices (α_6 in Rv2613c-A, and α_8 and α_{15} in Rv2613c-B) were involved in this tetramerization (Fig. 1c).

In Rv2613c-A, one phosphate ion and one tetraethylene glycol were bound to the C-terminal domain, while two phosphate ions and one tetraethylene glycol were bound to the C-terminal domain in Rv2613c-B (Fig. 1a). One phosphate ion was bound to the same binding site in each subunit, while the other phosphate ion in Rv2613c-B was bound to the C-terminal domain (Fig. 1a). In addition, the binding site and conformation of tetraethylene glycol molecule in Rv2613c-A were different from those of the tetraethylene glycol molecule in Rv2613c-B (Fig. 1a).

Structural comparisons

We searched for similar protein structures. As a result, the structure of Rv2613c is similar to those of other proteins in the HIT superfamily such as *Homo sapiens* fragile HIT protein [Fhit; Protein Data Bank (PDB) IDs: 6FIT and 1FHI; Z-score = 16.1 and 15.8, respectively], which is a HIT family Ap_nA hydrolase;^{11,12} Zn-bound HIT family protein from *Mycobacterium smegmatis* (MSMEG5028; PDB ID: 3O0M; Z-score = 16.1); and ADP-glucose phosphorylase from *Arabidopsis thaliana* (Ath; PDB ID: 1Z84; Z-score = 11.5).¹⁹ For example, superimposition of the structures of Rv2613c, Fhit, and MSMEG5028 revealed that the structure of Rv2613c-A/-B is similar to those of the Fhit and MSMEG5028 dimers (Fig. 2a). In particular, the 10-stranded antiparallel β -sheet and inner long α -helices of the Rv2613c dimer were in good agreement with those of the Fhit and MSMEG5028 dimers (Fig. 2a). In contrast, neither Fhit nor MSMEG5028 was structurally similar to the N-terminal domain of Rv2613c (Fig. 2a).

In addition, since this is the first report of the crystal structure of a protein with Ap_nA phosphorylase activity, we compared the structure of Rv2613c with that of Ath in place of a typical Ap_4A phosphorylase. Ath has a His-X-His-X-Gln motif, which is characteristic of typical Ap_4A phosphorylases. In addition, the amino acid sequence of Ath is 36% identical and 56% similar to *S. cerevisiae* Ap_4A phosphorylase 1. Therefore, we considered the

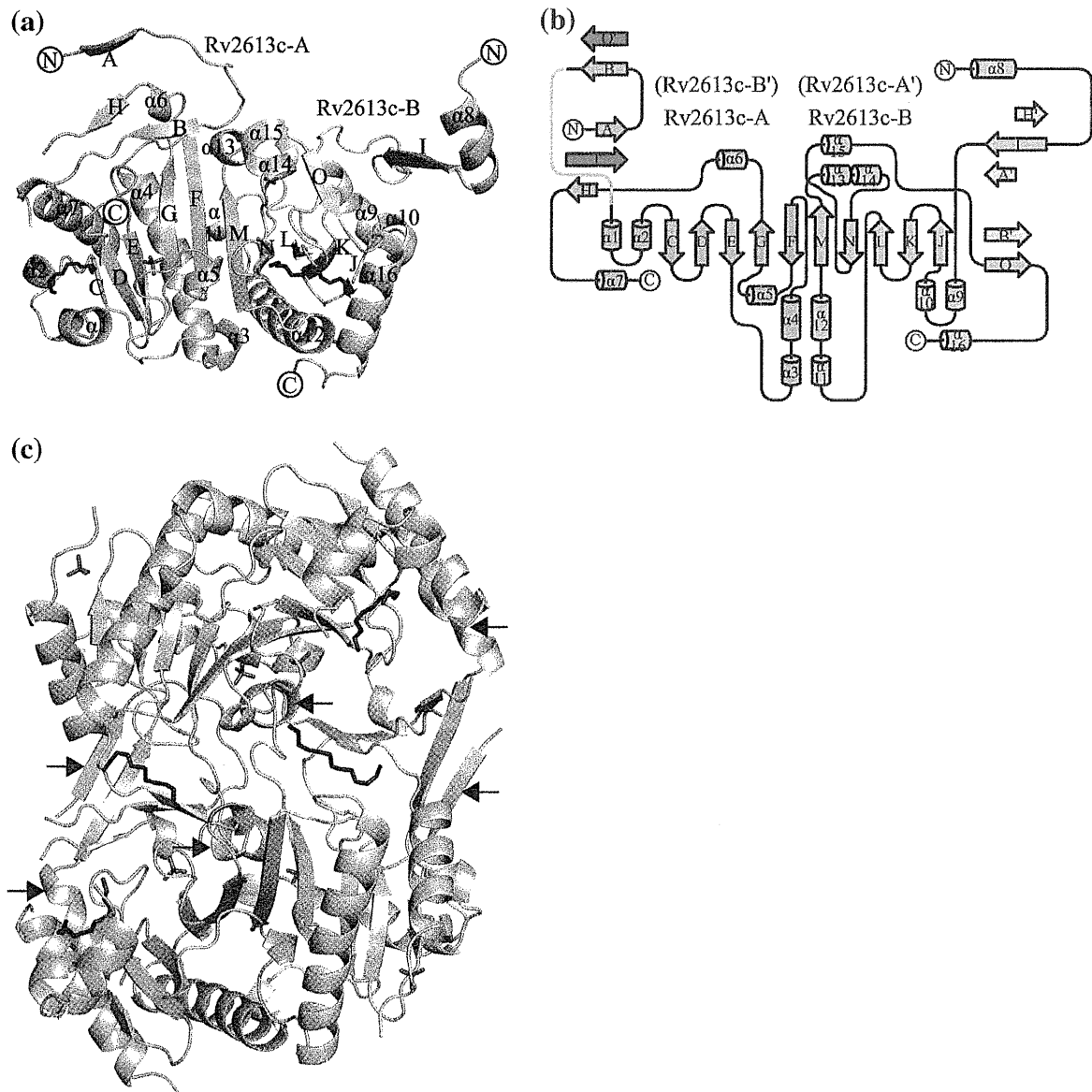


Fig. 1. Structure of Rv2613c. (a) Ribbon diagram of Rv2613c subunit A (green) and subunit B (cyan) (Rv2613c-A/-B) in the asymmetric unit. The N-terminus and the C-terminus are indicated by circled letters. α -Helices are numbered, and β -strands are lettered in order from the N-terminus of Rv2613c-A to the C-terminus of Rv2613c-B. Phosphate ions and tetraethylene glycols are modeled as red and blue sticks, respectively. (b) Secondary structure of the Rv2613c dimer shown in (a). Rv2613c-A and Rv2613c-B are indicated in green and cyan, respectively. The N-terminus, C-terminus, α -helices, and β -strands are labeled as in (a). In Rv2613c-A, the loops, which were not modeled due to poor electron density, are shown as gray lines. The three β -strands from Rv2613c-A' and the two β -strands from Rv2613c-B' are shown in yellow and red, respectively. (c) Ribbon diagram of the quaternary structure of Rv2613c. The asymmetric unit contains two subunits (Rv2613c-A/-B or Rv2613c-A'/-B'). Rv2613c-A, Rv2613c-B, Rv2613c-A', and Rv2613c-B' are shown in green, cyan, yellow, and light brown, respectively. Phosphate ions and tetraethylene glycols are represented as in (a). The arrows show A-B' and B-A' contacts.

structure of Ath an appropriate comparison structure. Superimposition of the structures of Rv2613c and Ath showed that the structure of Rv2613c-A/-B is similar to that of the Ath monomer (Fig. 2b). In particular, the 10-stranded antiparallel β -sheet and inner long α -helices of the Rv2613c dimer were in good agreement with that of the Ath monomer (Fig.

2b). In addition, Ath exhibited structural similarity to the N-terminal domain of Rv2613c (Fig. 2b). Furthermore, the structure of the Rv2613c tetramer was similar to that of the Ath dimer. Moreover, the interaction between Rv2613c-A/-B and Rv2613c-A'/-B' also was similar to the interaction in the Ath dimer. Finally, unlike the crystal

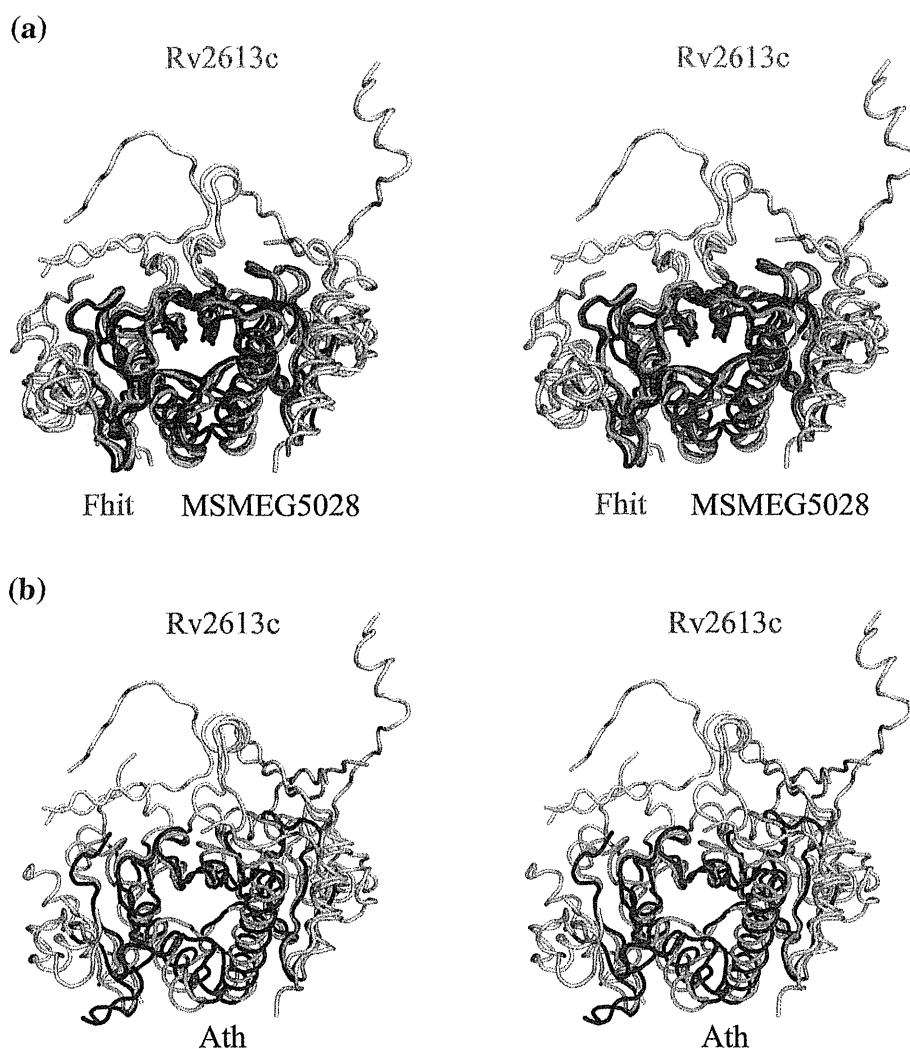


Fig. 2. Stereo representation of structural comparisons between Rv2613c and representative proteins in the HIT superfamily. (a) Superimposition of the structure of Rv2613c-A/-B on those of *H. sapiens* Fhit (PDB ID: 6FIT) and the Zn-bound HIT family protein from *M. smegmatis* (MSMEG5028; PDB ID: 3O0M) dimers. Rv2613c, Fhit, and MSMEG5028 are shown in green, red, and blue, respectively. Regions that are structurally similar are illustrated with darker colors compared to those that are divergent. (b) Superimposition of the structure of Rv2613c-A/-B on that of the ADP-glucose phosphorylase from *A. thaliana* (Ath; PDB ID: 1Z84) monomer. Rv2613c and Ath are shown in green and blue, respectively. Regions that are structurally similar are depicted with darker colors compared to those that are divergent. The structurally similar region of the Ath monomer is probably an internally duplicated subdomain.

structures of MSMEG5028 and Ath, which include a zinc ion, the crystal structures of Rv2613c and Fhit did not include any metal ions.

Catalytically important residues

The binding site of the phosphate ion in Rv2613c (Fig. 3a) corresponded to that of the tungstate ion of adenosine monotungstate (AMW) in Fhit (Fig. 3b) and that of the α -phosphate of AMP in Ath (Fig. 3c). Since HIT superfamily proteins, including Rv2613c, act on the α -phosphate of ribonucleotides,^{4,7} it is likely that the phosphate ion binding site of Rv2613c

is its active site. In this site, Asn139, Ser147, His153, and His155 were bound to the phosphate ion, and Gly146 was positioned near the phosphate ion (Fig. 3a). Thus, these five residues are probably catalytically important for the enzyme activity of Rv2613c.

Furthermore, the multiple sequence alignment of Rv2613c and related HIT superfamily proteins provides additional evidence for the importance of these residues. For example, His153 and His155 were in the HIT motif, whereas Asn139, Gly146, and Ser147 were upstream of this motif (Fig. 4). In addition, the multiple sequence alignment of the region containing these five residues in the

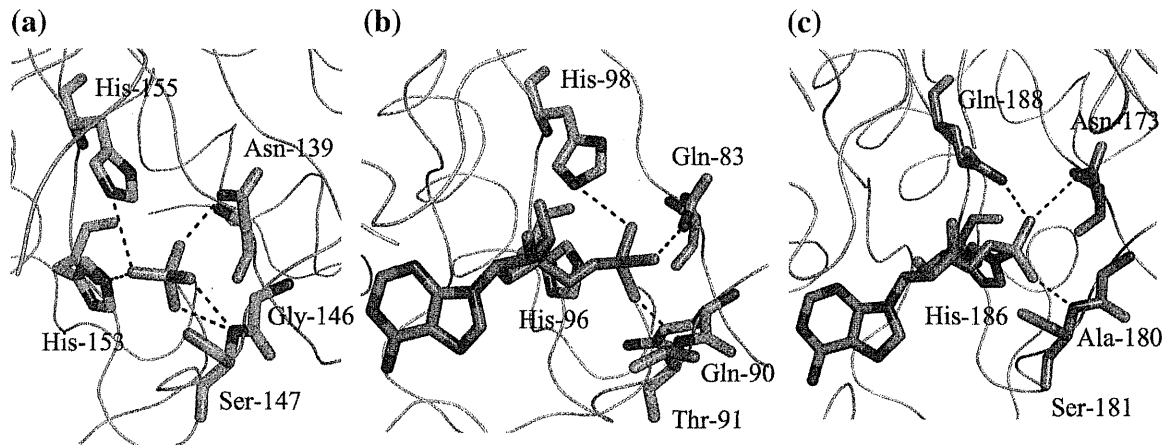


Fig. 3. Comparison of the active sites of Rv2613c, Fhit, and Ath. (a) Active site of Rv2613c. Asn139, Gly146, Ser147, His153, His155, and the phosphate ion are represented by sticks. Carbon, nitrogen, oxygen, and phosphorus atoms are shown in green, blue, red, and orange, respectively. (b) Active site of Fhit (PDB ID: 6FIT). Gln83, Gln90, Thr91, His96, His98, and AMW are represented by sticks. The carbon atoms in the amino acids and adenosine are shown in orange and purple, respectively. Nitrogen, oxygen, and tungsten atoms are shown in blue, red, and cyan, respectively. (c) Active site of Ath (PDB ID: 1Z84). Asn173, Ala180, Ser181, His186, Gln188, and AMP are represented by sticks. The carbon atoms in the amino acids and adenosine are shown in light blue and purple, respectively. Nitrogen, oxygen, and phosphorus atoms are shown in dark blue, red, and orange, respectively.

phosphate binding site of Rv2613c and the corresponding residues in the AMW binding site of Fhit showed that His153 and His155 in Rv2613c correspond to His96 and His98 in Fhit, respectively (Figs. 3a and b and 4). Similarly, Asn139, Gly146, and Ser147 in Rv2613c are equivalent to Gln83, Gln90, and Thr91 in Fhit, respectively (Figs. 3a and b and 4). In addition, the Gln, Gln, and Thr residues, which corresponded to Gln83, Gln90, and Thr91, respectively, in Fhit were conserved among HIT family *Ap_nA* hydrolases (Fig. 4). On the other hand, the

multiple sequence alignment showed that Asn139, Ser147, and His153 in the phosphate binding site of Rv2613c corresponded to Asn173, Ser181, and His186 in the AMP binding site of Ath, respectively (Figs. 3a and c and 4). Similarly, Gly146 and His155 in Rv2613c are equivalent to Ala180 and Gln188 in Ath, respectively (Figs. 3a and c and 4). In addition, the Asn and Ser residues, which corresponded to Asn139 and Ser147, respectively, in Rv2613c were conserved among *Ap₄A* phosphorylases and ADP-glucose phosphorylase (Fig. 4).

	139	146 147	153 155	160
Rv2613c	GLNLGTSAGGSLAE	HLHVHV	VPRWGGDANFI	
APA2_KLULA	FYNCGPNSGSSQ	DHKHLQIL	PLPYKFVPIYQ	
APA1_YEAST	FYNSGPASGSSL	DHKHLQIL	OMPEKFVTFQ	
APA2_YEAST	FYNCGPHSGSSQ	DHKHLQIM	OMPEKFIPFQ	
Fhit	SMQDGPEAGQTV	KHVHVHVL	PRKAGDFHRN	
FHIT_MOUSE	SMQDGPEAGQTV	KHVHVHVL	PRKAGDFPRN	
APH1_SCHPO	GIQDGV DAGQTV	PHVHVHI	PRKKADFSEN	
Ath	FKNQGASAGASM	SHSHSOMMAL	PVVPPTVS	
	: *	: *	* * :::	

Fig. 4. Multiple sequence alignment of the active-site region of Rv2613c and representative *Ap₄A* phosphorylases (APA2_KLULA, APA1_YEAST, and APA2_YEAST) and HIT family *Ap_nA* hydrolases [Fhit; FHIT_MOUSE (*Mus musculus*); and APH1_SCHPO], and ADP-glucose phosphorylase (Ath). The Swiss-Prot (UniProtKB) IDs of these proteins are as follows: Rv2613c (O06201), APA2_KLULA (P49348), APA1_YEAST (P16550), APA2_YEAST (P22108), Fhit (P49789), FHIT_MOUSE (O89106), APH1_SCHPO (P49776), and Ath (Q9FK51). The position of residues in Rv2613c is shown above the alignment. The residues that correspond to Asn139, Gly146, Ser147, His153, and His155 in Rv2613c are highlighted in blue, while those that correspond to Trp160 in Rv2613c are highlighted in red. The HIT and His-X-His-X-Gln motifs are boxed. Identical and highly conserved residues are denoted by an asterisk (*) or a colon (:), respectively. The multiple sequence alignment was generated by CLUSTAL W 1.83.

Table 2. Kinetic values of Rv2613c and derivatives

Enzyme	Activity (U mg ⁻¹) ^a	K _m (mM)	K _{cat} (s ⁻¹)	K _{cat} /K _m (M ⁻¹ s ⁻¹)
Wild type	8.69	0.10±0.001	8.48±0.313	84.82
N139A	ND ^b	—	—	—
N139Q	ND	—	—	—
G146S	2.63	0.08±0.014	1.99±0.115	24.88
G146Q	1.19	0.08±0.019	1.38±0.108	17.25
S147A	0.96	0.07±0.015	0.48±0.036	6.90
S147T	1.92	0.11±0.018	1.49±0.130	13.62
N139Q/S147T	ND	—	—	—
W160A	ND	—	—	—

^a Specific activity determined in the presence of 1.0 mM Ap₄A.

^b ND, not detected (i.e., enzyme activity was not detected using up to 100 μg of purified enzyme for up to 2 h).

To test the hypothesis that Asn139, Ser147, and Gly146 are catalytically important residues in Rv2613c, we examined the catalytic activities of seven Rv2613c mutants, namely N139A, N139Q, G146S, G146Q, S147A, S147T, and N139Q/S147T. Three mutants (N139A, N139Q, and N139Q/S147T) did not exhibit any detectable catalytic activity, while the activity of the other four mutants (G146S, G146Q, S147A, and S147T) was lower than that of wild-type Rv2613c (Table 2). In addition, the K_m value of the G146S, G146Q, S147A, and S147T mutants was similar to that of wild-type Rv2613c; however, the K_{cat} and K_{cat}/K_m values of these four mutants were markedly lower than those of wild-type Rv2613c (Table 2).

We also determined the importance of Gly146 on the production of AMP during catalysis. Wild-type Rv2613c and G146S produced very little AMP (1.2 and 1.5 nmol from 100 nmol of Ap₄A, respectively; Fig. 5a and b), whereas G146Q produced more AMP (11.2 nmol from 100 nmol of Ap₄A; Fig. 5c).

Relationship between tetramerization and catalytic activity

To elucidate the relationship between the tetramerization and the catalytic activity of Rv2613c, we predicted the binding site of Ap₄A and its mode of binding in Rv2613c by superimposing the α-phosphate of Ap₄A on the phosphate ion in the putative active site of Rv2613c-A. As a result, one adenosine binding site corresponded to the tetraethylene glycol binding site in Rv2613c-A, while the other adenosine binding site corresponded to the tetraethylene glycol binding site in Rv2613c-B' (Fig. 6a and b). Furthermore, the putative position of adenine, which was bound to Rv2613c-B', could make π-π stacking interactions with Trp160 in this subunit (Fig. 6b). Therefore, we predicted that the tetramerization of Rv2613c (i.e., A-B' and B-A' contacts) is involved in the formation of the Ap₄A binding site and that Trp160 might participate in

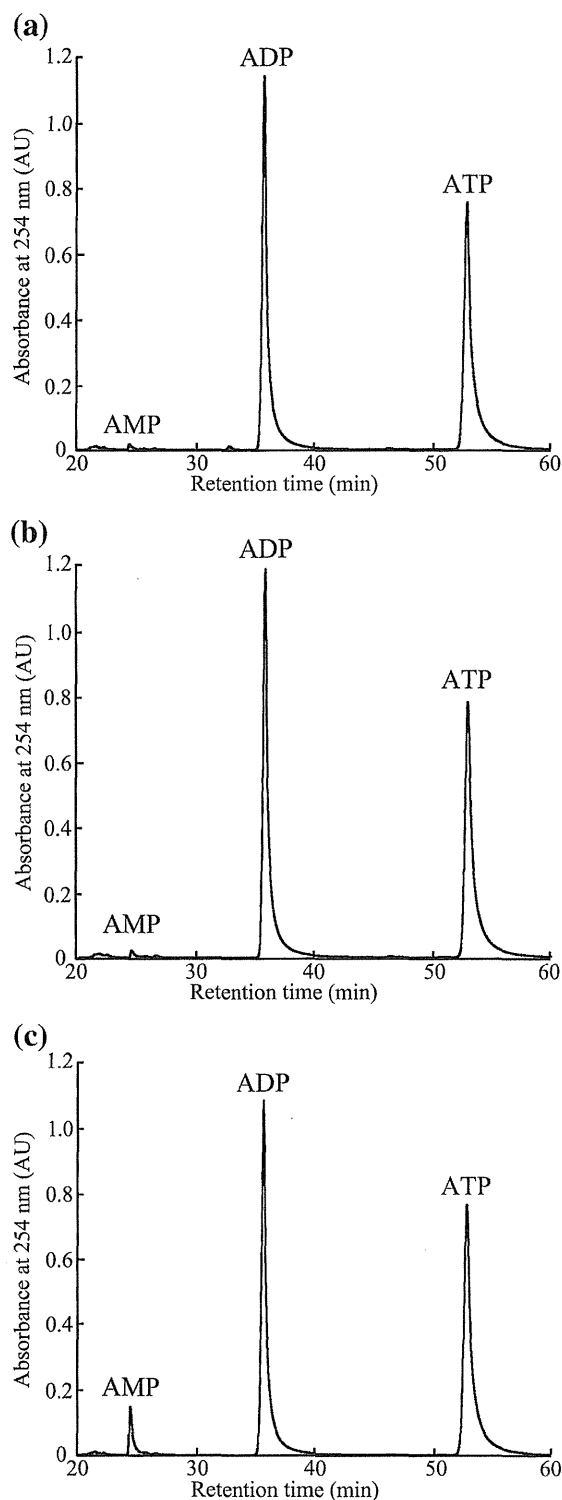


Fig. 5. High-performance liquid chromatography analyses of products formed by the complete degradation of 100 nmol of Ap₄A with purified Rv2613c (a), G146S (b), or G146Q (c). The reaction conditions are described in Materials and Methods.

the binding of Ap_4A . The latter hypothesis is supported by the observation that the W160A mutant of Rv2613c did not have any detectable catalytic activity (Table 2). In addition, the multiple sequence alignment in Fig. 4 showed

that Trp160 in Rv2613c corresponds to a Pro residue in typical Ap_4A phosphorylases and ADP-glucose phosphorylase, and to a Lys residue in HIT family Ap_nA hydrolases. Finally, in the putative Ap_4A binding site of Rv2613c-A, the side

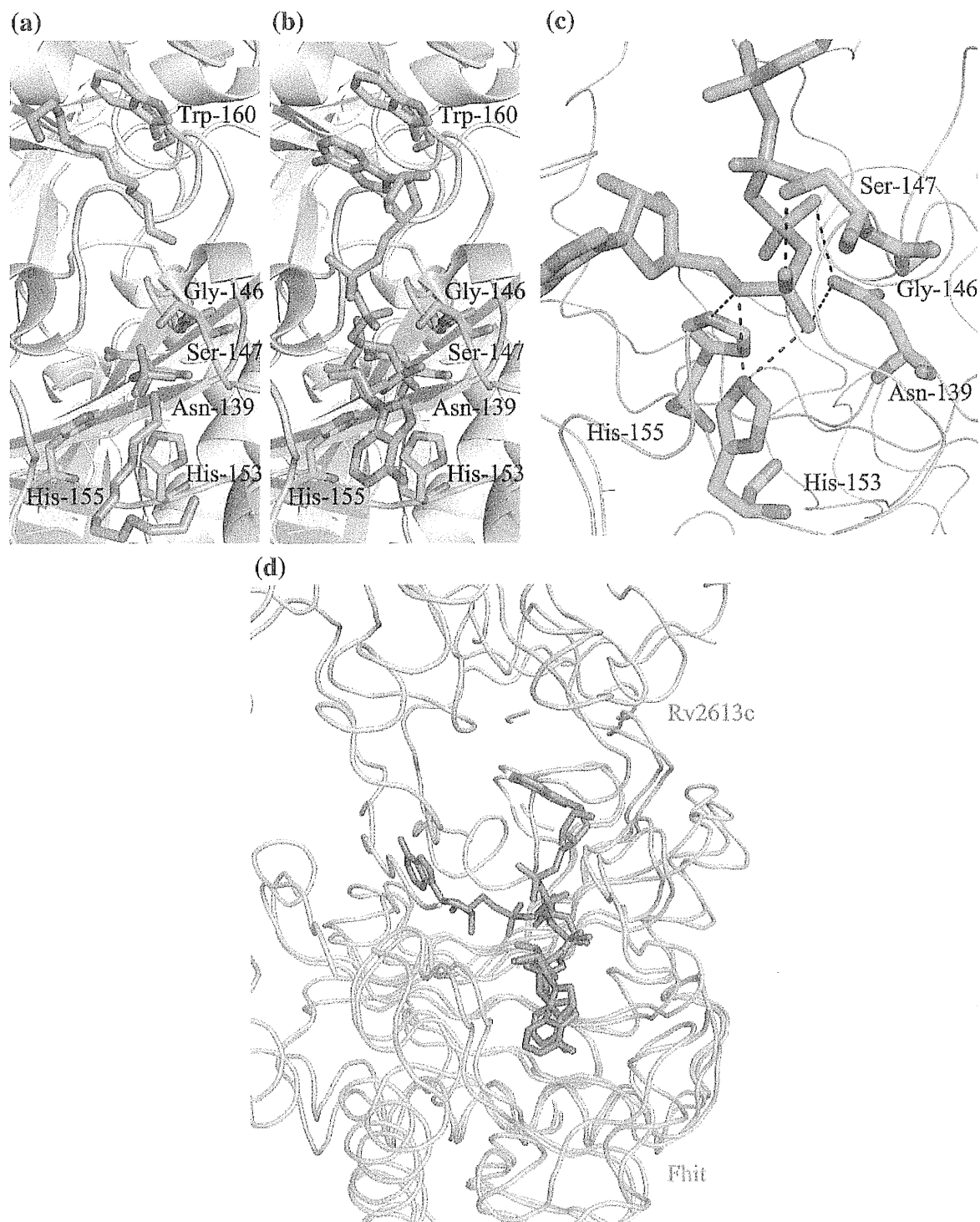


Fig. 6 (legend on next page)

chain of Asn139 would bind to both α -phosphoryl and β -phosphoryl oxygens of Ap_4A , and the side chains of Ser147, His153, and His155 would bind to the α -phosphoryl oxygen of Ap_4A (Fig. 6c).

A comparison of the putative Ap_4A binding site in Rv2613c with the P^1, P^2 -methylene- P^3 -thio-dadenosine triphosphate (IB2) binding site in Fhit¹² revealed that the putative binding site of the adenosine of Ap_4A in Rv2613c-A was the same as the adenosine binding site of IB2 in Fhit. However, the putative binding site of the other adenosine of Ap_4A in Rv2613c-B' was different from the binding site of the other adenosine of IB2 in Fhit (Fig. 6d).

Discussion

Recently, we reported that Rv2613c is a tetrameric Ap_4A phosphorylase, but its amino acid sequence is more similar to HIT family Ap_nA hydrolases than to typical Ap_4A phosphorylases.⁴ Now, we have solved the 1.89-Å-resolution crystal structure of Rv2613c (Fig. 1a and Table 1), which is the first known structure of a protein with Ap_nA phosphorylase activity, and characterized the structural basis of its catalytic activity.

We found that the overall and active-site structures of Rv2613c were similar to those of other proteins in the HIT superfamily (Figs. 2 and 3). Specifically, the structure of Rv2613c-A/-B was in agreement with those of the Fhit dimer, MSMEG5028 dimer, and Ath monomer (Fig. 2a and b). Furthermore, a 10-stranded antiparallel β -sheet and long inner α -helices were common secondary structures in these proteins (Fig. 2a and b). Consistent with our previous results,⁴ we also found that Rv2613c is tetrameric (Fig. 1c) and that its N-terminal domain is involved in intersubunit contacts (Fig. 1b and c). In contrast, Fhit and MSMEG5028 do not have a structural equivalent of the N-terminal domain of Rv2613c; therefore, they are homodimeric rather than tetrameric. In contrast, since Ath has a structural equivalent of the

N-terminal domain of Rv2613c (Fig. 2b), it forms a homodimer, which corresponds to the Rv2613c tetramer. Therefore, a consensus structure of the N-terminal domain of Rv2613c might be required for the multimerization of HIT superfamily proteins.

In Rv2613c, one phosphate ion was bound to the same binding site in each subunit (Figs. 1c and 3a), which is thought to be the active site of Rv2613c. The interaction of Asn139 and Ser147 with the phosphate ion and the close proximity of Gly146 to the phosphate ion (Fig. 3a) suggested that these residues are catalytically important. We evaluated the catalytic importance of these residues by measuring the enzymatic activities of mutant Rv2613c proteins with point mutations in these residues. The lack of catalytic activity in the N139A, N139Q, and N139Q/S147T mutants (Table 2) showed that Asn139 is catalytically important. In addition, the similar K_m values and decreased K_{cat} values of the G146S, G146Q, S147A, and S147T mutants compared with those of wild-type Rv2613c (Table 2) suggested that Gly146 and Ser147 are probably important for the catalytic activity of Rv2613c rather than for Ap_4A binding. In the putative Ap_4A binding site of Rv2613c, the interactions between the side chain of Asn139 and the α -phosphoryl and β -phosphoryl oxygens of Ap_4A , as well as those between the side chain of Ser147 and the α -phosphoryl oxygen of Ap_4A (Fig. 6c), suggested that Asn139 and Ser147 are important to properly orient the α -phosphates and β -phosphates of Ap_4A for catalysis. This hypothesis is in agreement with two previous studies of HIT superfamily proteins, namely AMP-lysine hydrolase¹³ and galactose-1-phosphate uridylyltransferase,¹⁴ which showed the importance of a Ser residue in the active site. Furthermore, the catalytic importance of Asn139 and Ser147 in Rv2613c is consistent with the conservation of Asn and Ser residues at these positions in typical Ap_4A phosphorylases and ADP-glucose phosphorylase, but not in HIT family Ap_nA hydrolases, which have Gln and Thr residues in their active site (Figs. 3b and 4).

Fig. 6. Putative Ap_4A binding site in Rv2613c and comparison with the IB2 binding site in Fhit. (a) The binding sites for phosphate and tetraethylene glycol at the A-B' contact of Rv2613c. Rv2613c subunits are colored as in Fig. 1c. Nitrogen, oxygen, and phosphorus atoms are shown in dark blue, red, and orange, respectively. The carbon atoms of tetraethylene glycol are shown in light blue. The carbon atoms of Asn139, Gly146, Ser147, His153, and His155 in Rv2613c-A are shown in green, and those of Trp160 in Rv2613c-B' are shown in pink. (b) Putative Ap_4A binding site of Rv2613c. To create this model, we superimposed the α -phosphate of Ap_4A onto the phosphate ion in Rv2613c-A as shown in (a), and then we optimized the structure of Ap_4A by using the CNSsolve program. The Rv2613c subunits are colored as in Fig. 1c. The carbon atoms of adenosine are shown in light blue. Nitrogen, oxygen, and phosphorus atoms are colored as in (a). The carbon atoms of Asn139, Gly146, Ser147, His153, and His155 in Rv2613c-A, and those of Trp160 in Rv2613c-B', are also colored as in (a). (c) Close-up view of the putative active sites of Rv2613c. Asn139, Gly146, Ser147, His153, His155, and Ap_4A colored as in (b). (d) Comparison of the putative binding mode of Ap_4A in Rv2613c and the binding mode of IB2 in Fhit (PDB ID: 1FHI). The backbones of the Rv2613c tetramer and the Fhit dimer are shown in green and light brown, respectively. Ap_4A and IB2 are shown in red and blue, respectively.

In addition to the catalytic importance of Gly146, the proximity of this residue to the α -phosphates and β -phosphates of *Ap₄A* in the putative *Ap₄A* binding site of Rv2613c (Fig. 6c) showed that this residue might influence the phosphorylase activity of Rv2613c. Moreover, G146Q produced about 10-fold more AMP than wild-type Rv2613c or G146S (Fig. 5). In addition, Gly146 in Rv2613c corresponds to Ser and Glu residues in typical *Ap₄A* phosphorylases and HIT family *Ap_nA* hydrolases, respectively (Fig. 4).

The structure of the predicted *Ap₄A* binding site of Rv2613c (Fig. 6b) and the lack of catalytic activity of the W160A mutant (Table 2) suggested that the tetramerization of Rv2613c (i.e., A–B' and B–A' contacts) and Trp160 may be essential for the formation of the *Ap₄A* binding site. Furthermore, the ability of Rv2613c to tetramerize, in contrast to the inability of Fhit to multimerize, might account for the difference between the putative *Ap₄A* binding site of Rv2613c and the IB2 binding site of Fhit (Fig. 6d).

The tetrameric structure of Rv2613c is unique among enzymes that use *Ap_nA* as a substrate. In addition, Trp160 in Rv2613c is not conserved in other *Ap₄A* phosphorylases and *Ap_nA* hydrolases in the HIT superfamily (Fig. 4). These differences suggest that Rv2613c may have a unique *Ap₄A* binding site, which may facilitate the design of specific inhibitors against Rv2613c. *Ap₄A* is found in both prokaryotes and eukaryotes,^{20,21} and is involved in virulence and biofilm formation.^{22,23} Furthermore, *Rv2613c* is an essential gene in *M. tuberculosis* H37Rv.⁵ Therefore, the specific inhibitors against Rv2613c be potentially useful anti-TB drugs. As a result, we are currently screening for novel inhibitors of Rv2613c.

Materials and Methods

Selenomethionine substitution and site-directed mutagenesis of Rv2613c

The *Rv2613c* gene from *M. tuberculosis* H37Rv was cloned into the pCold I expression vector (Takara Bio, Inc., Shiga, Japan) to construct the pMS2613c plasmid, as described previously.⁴ To express selenomethionine (SeMet)-substituted Rv2613c (SeMet-Rv2613c), we transformed pMS2613c into *Escherichia coli* B834(DE3) (Merck Novagen, Darmstadt, Germany). In addition, site-directed mutagenesis was used to introduce point mutations (N139A, N139Q, G146S, G146Q, S147A, S147T, N139Q/S147T, and W160A) into Rv2613 by using the Quick-Change Site-Directed Mutagenesis Kit II (Agilent Technologies, Inc., Santa Clara, CA) with pMS2613c as template. The resulting plasmids were sequenced with an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA) to confirm the mutation and were transformed into *E. coli* BL21(DE3)pLysS (Merck Novagen).

Expression and purification of Rv2613c

A single transformed colony of *E. coli* B834(DE3) was inoculated in 5 mL of Luria–Bertani medium and incubated overnight at 37 °C. Subsequently, 2 mL of the overnight culture was transformed into 200 mL of M9 minimal medium supplemented with the following: 1% glucose; 2 mM MgSO₄; 0.1 mM CaCl₂; 50 mg L⁻¹ ampicillin; Kao Vitamins (Sigma-Aldrich Japan, Tokyo, Japan); 5 μ g mL⁻¹ each of L-Trp and L-Tyr; and 50 μ g mL⁻¹ each of L-Ala, L-Arg, L-Asn, L-Asp, L-Cys, L-Glu, L-Gln, L-Gly, L-His, L-Ile, L-Leu, L-Lys, L-Phe, L-Pro, L-Ser, L-Thr, L-Val, and L-SeMet. Then, this culture was grown under aerobic conditions for 7 h at 37 °C until the absorbance at 600 nm (*A*₆₀₀) had reached 1.0. Afterwards, the culture was transferred into 2 L of the same supplemented medium and cultured under the same conditions until an *A*₆₀₀ of 0.4 had been reached. At that time, we added 0.5 mM isopropyl β -D-thiogalactopyranoside (Wako Pure Chemical Industries Ltd., Osaka, Japan) (final concentration) to induce protein expression and continued incubating the culture for 24 h at 15 °C. The Rv2613c point mutants were expressed in the same manner.

To purify the SeMet-Rv2613c and mutant Rv2613c proteins, we centrifuged the resulting *E. coli* cells at 8000g for 10 min at 4 °C, and then resuspended them in buffer A [20 mM sodium phosphate (pH 7.4), 0.5 M NaCl, and 40 mM imidazole]. Subsequently, the cells were disrupted by sonicating them on ice with a UP50H sonicator (Hielscher Ultrasonics, Teltow, Germany). Unbroken cells and cellular debris were removed by centrifugation at 20,000g for 30 min at 4 °C. Then, the clarified supernatant was loaded onto a HisTrap HP column (1.6 cm \times 2.5 cm; GE Healthcare Bio-Sciences, Buckinghamshire, UK) that was equilibrated with buffer A, and the protein of interest was eluted with a linear gradient of imidazole (40–250 mM). Next, the protein was loaded onto a HiPrep 16/60 Sephacryl S-200 HR column (1.6 cm \times 60 cm; GE Healthcare Bio-Sciences) that was equilibrated with buffer B [20 mM sodium phosphate (pH 7.4), 0.5 M NaCl, and 150 mM imidazole]. The sample was separated with buffer B into 1.2-mL fractions. For SeMet-Rv2613c, fractions 24–30 were pooled and then dialyzed against buffer C [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-Na (pH 7.6) and 0.5 mM dithiothreitol] using Slide-A-Lyzer Dialysis Cassettes (10,000 weight cutoff; Thermo Fisher Scientific, Waltham, MA) at room temperature. For the Rv2613c mutants, fraction 25 was dialyzed against buffer C using Slide-A-Lyzer Dialysis Cassettes (10,000 weight cutoff; Thermo Fisher Scientific) at room temperature. These dialyzed proteins were used for all experiments in this study.

Crystallization and data collection

We crystallized Rv2613c and collected the diffraction data as described previously.¹⁸ Briefly, SeMet-Rv2613c was crystallized by hanging-drop vapor diffusion. Specifically, 5 μ L of protein (10 mg mL⁻¹) was mixed with 5 μ L of crystallization solution [0.1 M sodium cacodylate (pH 6.7), 0.2 M lithium sulfate, and 28% polyethylene glycol 400], and then the drop was suspended on a siliconized coverslip over 0.6 mL of the crystallization solution. Prismatic colorless crystals of SeMet-Rv2613c

formed after about 4 weeks at 20 °C and grew to a maximum size of 0.5 mm. The crystals were harvested using a CryoLoop (Hampton Research, Aliso Viejo, CA) and flash cooled to 100 K in a nitrogen gas stream.

The diffraction data for SeMet-Rv2613c were collected at the AR-NW12A station of the Photon Factory (Tsukuba, Japan) at a wavelength of 0.97901 Å with an ADSC Quantum 210r detector (Area Detector Systems Corporation, Poway, CA) located 240 mm from the crystal in 2-s 1° oscillations at 100 K. The diffraction data were processed, merged, and scaled using the HKL2000 (DENZO and SCALEPACK) software package.²⁴

Structure determination and model analysis

The crystal structure of Rv2613c was solved by using SAD. SAD phasing and phase refinement were performed by using the PHENIX software package.²⁵ We built a model of Rv2613c with the Coot program,²⁶ and then refined it with CCP4,²⁷ CNSsolve,²⁸ and PHENIX to 1.89 Å resolution. We validated the model by using MolProbity.²⁹

Ribbon diagrams were generated by PyMOL,³⁰ and protein structure topology cartoons were produced by TopDraw.³¹ The atomic coordinates of crystal structures were downloaded from PDB†. We searched for similar protein structures by using the Dali server‡³² and performed pairwise structure comparisons with the DaliLite program.³³ Sequence alignments were performed by using CLUSTAL W 1.83.³⁴

Enzyme activity assays

The enzyme activity of Rv2613c and its mutants was measured by using a high-performance liquid chromatography system (Shimadzu, Kyoto, Japan) to quantify the amount of remaining substrate after the reaction, as described previously,⁴ with some modifications. The reaction mixture (100 µL) consisted of 50 mM Hepes-Na (pH 7.6), 1 mM MnCl₂, 100 µg mL⁻¹ bovine serum albumin, 1 mM Ap₄A, 5 mM phosphate, and purified enzymes. This mixture was incubated at 37 °C for 30 min. The reaction was stopped by heating at 95 °C for 4 min, followed by centrifugation of the reaction solution at 12,000g for 3 min. The clear supernatant was analyzed using a 4.6 mm × 125 mm Partisphere 5-µm SAX HPLC column (Whatman, Kent, UK), which was equilibrated with pure water. The adsorbed nucleotides, such as AMP, ADP, ATP, and Ap₄A, were eluted with a gradient of water and elution buffer [1.3 M (NH₄)₂HPO₄ with H₃PO₄ (pH 4.8)] as follows: 0–10 min, 0% elution buffer; 10–110 min, 0–50% elution buffer at a flow rate of 0.5 mL min⁻¹. The eluted nucleotides were detected at 254 nm. One unit of enzyme activity was defined as the degradation of 1.0 µmol of Ap₄A in 1 min at 37 °C. *K_m* and *K_{cat}* values were calculated by using GraphPad Prism (GraphPad Software, La Jolla, CA). Results were expressed as the mean (standard error of the mean) of three independent measurements.

Accession codes

The structure factor and atomic coordinates for Rv2613c have been deposited in the PDB under accession code 3ANO.

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‡ ekhidna.biocenter.helsinki.fi/dali_server

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Expression of the *Mycobacterium tuberculosis* PPE37 protein in *Mycobacterium smegmatis* induces low tumour necrosis factor alpha and interleukin 6 production in murine macrophages

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PPE37 is a member of the *Mycobacterium tuberculosis* proline-proline-glutamic acid (PPE) multigene family. Its expression is upregulated in bacteria that are phagocytosed by macrophages and is enhanced even more in bacteria isolated from the lungs of infected mice. This raises the possibility that PPE37 may play a role in the virulence of *M. tuberculosis* and led to this investigation of the function of PPE37. Recombinant bacterial strains, one expressing the *M. tuberculosis* PPE37 protein (*Ms_ppe37*) and another harbouring the vector alone (*Ms_vec*) were generated from the non-pathogenic *Mycobacterium smegmatis*. These bacterial strains were used to infect peritoneal exudate and bone marrow-derived macrophages. It was found that, despite the comparable intracellular survival between the two recombinant *M. smegmatis* strains, *Ms_ppe37* induced a significantly lower level of tumour necrosis factor alpha and interleukin 6 in the infected macrophages compared with *Ms_vec*. Western blot analyses revealed that the activation levels of nuclear factor kappa B, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase and MAPK/p38 were lower in macrophages infected with *Ms_ppe37* than in macrophages infected with *Ms_vec*. These results suggest that PPE37 may have a potential role in interfering with the pro-inflammatory cytokine response of infected macrophages.

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INTRODUCTION

The existence of the proline-proline-glutamic acid (PPE) multigene family was revealed when the decoding of the *Mycobacterium tuberculosis* genome was completed (Cole *et al.*, 1998). Members of this gene family were found to share a highly conserved N-terminal sequence of approximately 180 aa that also contained the conserved PPE motif. In contrast, their C-terminal regions were highly heterogeneous in both sequence and length (Cole *et al.*, 1998). The *ppe* genes are not found outside the genus *Mycobacterium* and are highly distributed among the pathogenic species of mycobacteria (Gey van Pittius *et al.*, 2006). For these reasons, they are speculated to contribute to the pathogenicity of *M. tuberculosis*. A possible functional role has been proposed for the PPE proteins, in which they serve as a source of antigenic variation that promotes antigenic diversity in *M. tuberculosis* (Cole *et al.*, 1998). Indeed, the

PPE proteins reported to date are immunogenic, eliciting either humoral or T-cell immune responses (Choudhary *et al.*, 2003; Khan *et al.*, 2008; Romano *et al.*, 2008; Tundup *et al.*, 2008; Wang *et al.*, 2008).

One of the PPE proteins, PPE37, may have a role in the virulence of *M. tuberculosis*. It has been reported that expression of the *ppe37* gene is upregulated in *M. tuberculosis* during infection of murine bone marrow-derived macrophages (Schnappinger *et al.*, 2003; Voskuil *et al.*, 2004). Moreover, in *M. tuberculosis* isolated from the lungs of infected mice, expression of the *ppe37* gene is enhanced even more (Schnappinger *et al.*, 2003). Earlier studies have shown that an iron-dependent transcriptional regulator that is critical for proper iron homeostasis in *M. tuberculosis* regulates the expression of *ppe37* (Rodriguez *et al.*, 1999, 2002). When *M. tuberculosis* was exposed to iron-limiting conditions in *in vitro* culture, the expression of *ppe37* increased greatly (Rodriguez *et al.*, 2002; Schnappinger *et al.*, 2003). In addition, *in vitro* exposure of *M. tuberculosis* to nitrosative and oxidative growth conditions also increased the expression of *ppe37* (Schnappinger *et al.*, 2003; Voskuil *et al.*, 2004). Both of

Abbreviations: ERK, extracellular signal-regulated kinase; IL, interleukin; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MHC, major histocompatibility complex; NF- κ B, nuclear factor kappa B; TNF- α , tumour necrosis factor alpha; TLR, Toll-like receptor.

these conditions are reported to mimic the macrophage phagosomal environment that contains *M. tuberculosis* (Schnappinger *et al.*, 2003). From these results, it seems that PPE37 is required for the adaptation of *M. tuberculosis* to the intracellular niche in macrophages.

In the present study, as a first step towards evaluating the possible role of PPE37 in the virulence of *M. tuberculosis*, we took advantage of the lack of *ppe* genes in *Mycobacterium smegmatis* and generated two recombinant bacterial strains using this non-pathogenic bacterium. Unlike the *M. tuberculosis* genome, which contains 69 *ppe* ORFs, the *M. smegmatis* genome contains only 2. Furthermore, none of the *M. smegmatis ppe* genes are orthologues of the *M. tuberculosis ppe37* gene (Gey van Pittius *et al.*, 2006). We cloned the *ppe37* gene from the *M. tuberculosis* strain H37Rv and expressed the gene in *M. smegmatis* strain mc² 155 (Ms_ppe37). The ability of Ms_ppe37 to survive inside macrophages was assessed in *in vitro* infection of mouse macrophages. In addition, the effect of PPE37 on the macrophage cytokine response was also investigated.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* DH5 α was routinely grown in Lennox LB medium for use in DNA cloning procedures. *M. tuberculosis* strain H37Rv and *M. smegmatis* strain mc² 155 were grown at 37 °C in Middlebrook 7H9 liquid medium or on Middlebrook 7H10 agar (Difco) supplemented with 0.5% (w/v) albumin fraction V, 0.2% (w/v) glucose, 0.5% (v/v) glycerol and 0.05% (v/v) Tween 80. For the preparation of culture filtrate fraction, recombinant *M. smegmatis* Ms_ppe37 was grown in Sauton medium as described by Rosenkrands & Andersen (2001). When required, 25 μ g kanamycin ml⁻¹ was also added.

Macrophages. C57BL/6 female mice of 7 to 9 weeks old (Japan SLC) were used in experiments according to protocols approved by the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine. Peritoneal exudate cells were harvested from mice 3–4 days after intraperitoneal injection with 2.5 ml 3% (w/v) thioglycollate (Eiken Chemical). Cells were washed, seeded in tissue culture plates and cultured for 2 h in 5% CO₂ at 37 °C in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum. Adherent cells were used as peritoneal exudate macrophages after non-adherent cells had been removed by washing. In other experiments, bone marrow cells were collected from the tibiae of mice. Cells were cultured for 5–7 days in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 20 μ g gentamicin ml⁻¹ and 100 ng mouse macrophage colony-stimulating factor ml⁻¹ (R&D Systems). After removal of non-adherent cells, adherent cells were used as bone marrow-derived macrophages.

Generation of recombinant *M. smegmatis* expressing PPE37. Chromosomal DNA was isolated from *M. tuberculosis*, and the *ppe37* gene was PCR-amplified with the use of forward primer 5'-TTACTAGTcaccatcacACCTTCCCGAT-3' containing an *SpeI* site (underlined) and three His codons (lower-case letters), and reverse primer 5'-CCGTAAGCTTCTTCAACGTTAATCTGACC-3' containing a *HindIII* site (underlined). The PCR product of approximately 1.5 kb was cloned into the pEGFP vector (Clontech Laboratories), generating the recombinant plasmid pEGFP-his3ppe37. Three additional His codons were introduced at the 5'

end of the his3ppe37 sequence with the use of the same reverse primer and a second forward primer, 5'-TGAATTCATGcatcaccatcaccatcacACC-3', containing an *EcoRI* site (underlined) and the His codons (lower-case letters). The resultant PCR product containing six consecutive His codons was inserted in frame into the cloning site of pMV261, a mycobacterial expression vector (Stover *et al.*, 1991), generating pMV261-his6ppe37. The recombinant plasmid or empty pMV261 was electroporated into *M. smegmatis* mc² 155 according to standard procedures (Larsen, 2000). Recombinant *M. smegmatis* expressing 6His-tagged PPE37 (Ms_ppe37) and *M. smegmatis* harbouring empty pMV261 alone (Ms_vec) were selected on Middlebrook 7H10 agar containing 25 μ g kanamycin ml⁻¹.

Detection of *ppe37* gene expression in recombinant *M. smegmatis*. Recombinant *M. smegmatis* strains were cultured until they reached an OD₆₀₀ of 0.6–1.0 in 100 ml Middlebrook 7H9 liquid medium in the presence of 25 μ g kanamycin ml⁻¹. Total bacterial RNA was isolated using SepasolRNA I Super (Nacalai Tesque). All RNA samples were treated with DNase I (Promega) and subjected to PCR to test for the complete removal of genomic DNA. cDNA was synthesized from 1 μ g total RNA in a 40 μ l reaction mix containing reverse transcriptase buffer, 150 ng random primers, 2 μ l 10 mM dNTP mix, 2 μ l 0.1 M DTT and 400 U SuperScript III reverse transcriptase (Invitrogen). PCR was performed with a KOD-Plus enzyme kit (Toyobo) and the following primer pairs: (i) *ppe37* gene – 5'-TGTTGGACTGGTTCATCTCG-3' (forward) and 5'-CAGTCT-TGTTGCTTGTGCTGG-3' (reverse), product size 500 bp; and (ii) aminoglycoside phosphotransferase (*aph*) gene – 5'-AGGTAGC-GTTGCCAATGATG-3' (forward) and 5'-CTCACCAGGAGCAGTTC-CATA-3' (reverse), product size 540 bp.

Detection of His-tagged PPE37. Recombinant *M. smegmatis* strains were cultured to an OD₆₀₀ of 0.6–1.0 in 25 ml Middlebrook 7H9 liquid medium or 50 ml Sauton medium in the presence of 25 μ g kanamycin ml⁻¹. Bacterial pellets were harvested, washed three times with ice-cold PBS and resuspended in extraction buffer containing 20 mM Tris/HCl (pH 6.8), 4 mM EDTA, 0.6% SDS and protease inhibitor cocktail (Nacalai Tesque). Bacterial cells were disrupted and supernatants were collected after centrifugation at 20 000 g for 20 min at 4 °C. For preparation of the culture filtrate fraction, the culture supernatant of bacteria grown in Sauton medium was harvested by centrifugation at 2000 g for 15 min at 4 °C. The supernatant was filtered through a 0.2 μ m syringe filter and concentrated to approximately 150 μ l using a centrifugal filter with a cut-off value of 5 kDa (Millipore). Samples were subjected to SDS-PAGE, and the His-tagged PPE37 protein was detected by Western blotting and mouse anti-penta-His antibody (Qiagen). Chemiluminescent images were captured with a luminescent image analyser LAS-4000mini (Fujifilm).

***M. smegmatis* infection of macrophages.** Macrophages were seeded at 1 \times 10⁶ cells per well in 12-well tissue culture plates or at 3 \times 10⁵ cells per well in 24-well tissue culture plates. Cells were infected with Ms_ppe37 or Ms_vec at an m.o.i. of 20. At this m.o.i., the resulting infection rate was greater than 80% as estimated in preliminary infection assays from microscopy evaluation of slides stained according to the Kinyon method (Chapin & Lauderdale, 2007). Four hours after infection, gentamicin was added to give a final concentration of 5 μ g ml⁻¹. At 6, 24 and 48 h after infection, macrophages were washed and lysed in PBS containing 0.1% (v/v) Triton X-100. Lysates were plated on Middlebrook 7H10 agar plates containing 25 μ g kanamycin ml⁻¹ and the number of intracellular bacteria was enumerated.

Assay for lactate dehydrogenase (LDH) release. Culture supernatants were harvested after infection of macrophages with Ms_ppe37 or Ms_vec for 6, 24 or 48 h. LDH activity in the culture supernatants

was assayed with an LDH cytotoxicity detection kit (Takara Bio). The percentage of LDH release was calculated as: percentage release = $100 \times (\text{experimental LDH release} - \text{spontaneous LDH release}) / (\text{maximal LDH release} - \text{spontaneous LDH release})$. A value of maximal LDH release was obtained from culture supernatants of macrophages that were lysed with 1% (v/v) Triton X-100.

Assay for cytokine productions. Culture supernatants were harvested after infection of macrophages with Ms_ppe37 or Ms_vec for 24 h. The concentrations of cytokines in the culture supernatants were determined using commercially available ELISA kits for tumour necrosis factor alpha (TNF- α), interleukin 6 (IL-6), IL-1 β (eBioscience) and IL-12p70 (Endogen). In some experiments, after infection of macrophages with Ms_ppe37 or Ms_vec for 3, 6, 9, 12 and 18 h, total RNA was extracted with a Nucleospin RNA II kit (Macherey-Nagel). RNA (250 ng) was treated with RNase-free DNase (Promega) and subsequently reverse transcribed into cDNA using a SuperScript VILO cDNA synthesis kit (Invitrogen). cDNAs were diluted tenfold, and a PCR was performed in an equal reaction volume using a KOD-Plus enzyme kit and the following primer pairs: (i) *tnf* gene – 5'-CATGAGCACAGAAAGCATGATCCG-3' (forward) and 5'-TCTGGGCCATAGAACTGATGAGAG-3' (reverse), product size 230 bp; (ii) *IL-6* gene – 5'-TTCCTCTCTGCAAGAGACT-3' (forward) and 5'-TGTATCTCTCTGAAGGACT-3' (reverse), product size 432 bp; and (iii) *Actb* gene – 5'-TGGAAATCCTGTGGCA-TCCATGAAAC-3' (forward) and 5'-TAAAACGCAGCTCAGTAA-CAGTCCG-3' (reverse), product size 350 bp. Equal volumes of the PCR mixtures were electrophoresed in 1.5% agarose gel, and DNA bands were visualized with ethidium bromide staining.

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Flow cytometric analysis. Macrophages were harvested after a 24 h infection with Ms_ppe37 or Ms_vec and treated with anti-CD16/CD32 mAb (clone 93; eBioscience) for 10 min. This was followed by a 20 min incubation on ice with one of the following phycoerythrin-conjugated antibodies against: major histocompatibility complex class I (MHC-I; clone 28-14-8), MHC-II (clone M5/114.15.2), B7.1 (CD80 clone 16-10A1), B7.2 (CD86, clone GL1) or CD40 (clone 1C10), or with an isotype control antibody (all from eBioscience). The intensity of each cell-surface marker was analysed on a FACScalibur flow cytometer equipped with CellQuest software (BD Biosciences).

Assay for nuclear factor-kappa B (NF- κ B), extracellular signal-regulated kinase (ERK) and p38 phosphorylation. Macrophages were infected with Ms_ppe37 or Ms_vec for 0.5, 1, 2, 4, 6, 7 or 8 h. After infection, macrophages were lysed in buffer containing 10 mM Tris/HCl (pH 6.8), 1% (v/v) NP-40 and proteinase/phosphatase inhibitor cocktail (Nacalai Tesque). Cell lysates were harvested and subjected to SDS-PAGE. Phosphorylated and unphosphorylated ERK and p38, as well as the phosphorylated p65 subunit of NF- κ B, were detected in lots with specific antibodies (Cell Signaling Technology). β -Actin was detected with anti- β -actin antibody (Sigma-Aldrich). Chemiluminescent images were captured with a luminescent image analyser LAS-4000mini. In another experiment, macrophages were pre-treated with 20 or 40 μ M U0126 (a MEK1/2 inhibitor; Cell Signaling Technology) or with 10 or 20 μ M Calbiochem SB202190 (a p38 inhibitor; EMD Biosciences). On the basis of preliminary experiments, the inhibitors were used at the concentrations required to inhibit ERK and p38 activities. One hour after treatment, the macrophages were infected with Ms_ppe37 or Ms_vec. The culture supernatants were harvested 24 h after infection, and ELISA was performed to determine the concentrations of TNF- α and IL-6.

Statistical analysis. Data were analysed using Student's two-tailed *t*-test. Statistical significance was defined as a *P* value <0.05. Error bars represent SD.

RESULTS

Ms_ppe37 constitutively expresses *M. tuberculosis* PPE37 protein

In this study, we generated two recombinant *M. smegmatis* strains to investigate the effect of PPE37 on the macrophage response to bacterial infection. The Ms_ppe37 strain was engineered to express a 6His-tagged PPE37 protein from a recombinant pMV261 vector, whilst the Ms_vec strain harboured the vector alone. The pMV261 vector contains the kanamycin resistance gene *aph* for selection of transformed bacteria (Stover *et al.*, 1991). Both Ms_ppe37 and Ms_vec, which were grown in Middlebrook 7H9 medium in the presence of kanamycin, expressed the *aph* gene. However, only Ms_ppe37 was able to express the *ppe37* gene (Fig. 1a). Furthermore, Western blot analysis with anti-penta-His antibody detected a protein band representing PPE37 in the total cell lysate prepared from Ms_ppe37 but not from Ms_vec (Fig. 1b). These results confirmed that the transformation was successful and that *ppe37* gene expression was detectable only in *M. smegmatis* that had been electroporated with the recombinant vector. In addition, Western blot analysis also revealed that a protein band representing PPE37 was detectable in the total cell lysate but not in the culture filtrate fractions prepared from Ms_ppe37 grown in Sauton medium (Fig. 1c). This was not due to the absence of proteins in the culture filtrate fraction, as Coomassie blue staining revealed the presence of many protein bands in both the culture filtrate and the cell lysate fractions. From the result in Fig. 1(c), it could be suggested that PPE37 is not a secretory protein. We also compared the growth kinetics of Ms_ppe37 and Ms_vec in Middlebrook 7H9 medium, as excess production of recombinant protein is known to exert a metabolic burden on recombinant bacteria, sometimes reducing the growth of these cells (Bentley *et al.*, 1990). We observed no marked difference in the growth kinetics (Fig. 1d), indicating that expression of the *ppe37* gene did not influence the growth of Ms_ppe37.

PPE37 does not contribute to the intracellular survival of *M. smegmatis* in macrophages

M. smegmatis is inherently unable to multiply inside macrophages, and the number of intracellular bacteria decreased gradually after infection of macrophages *in vitro*. In order to determine whether PPE37 facilitated the intracellular survival of these bacteria in macrophages, we compared the survival kinetics of Ms_ppe37 and Ms_vec in peritoneal exudate macrophages by conducting a gentamicin protection assay. The results showed no significant difference in the number of bacteria between Ms_ppe37 and Ms_vec up to 48 h after infection (Fig. 2a). Similar results were also observed in bone marrow-derived macrophages (Fig. 2b). These results suggested that the presence of PPE37 was not able to enhance the intracellular survival of *M. smegmatis* in macrophages.

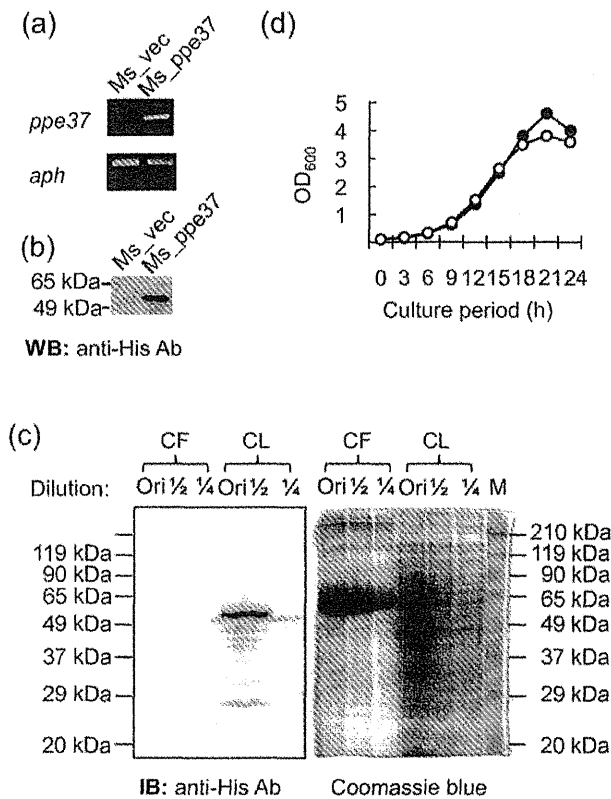


Fig. 1. Expression of *M. tuberculosis* PPE37 by *M. smegmatis* mc² 155. (a) Ms_ppe37 and Ms_vec were grown at 37 °C in Middlebrook 7H9 liquid medium to an OD₆₀₀ of 0.6–1.0. Total bacterial RNA was isolated and subjected to RT-PCR to detect expression of the *ppe37* and *aph* genes. (b) Lysates were prepared from bacterial cells that were cultured as in (a) and subjected to Western blot (WB) analysis to detect His-tagged PPE37 protein using mouse anti-penta-His antibody (anti-His Ab). (c) Lysate and culture filtrate fractions were prepared from Ms_ppe37 grown at 37 °C in Sauton medium to an OD₆₀₀ of 0.6–1.0. Samples were diluted and subjected to Western blot analysis (left panel) as in (b), as well as to Coomassie blue staining (right panel), after SDS-PAGE. (d) Growth of Ms_ppe37 (○) and Ms_vec (●) at 37 °C in Middlebrook 7H9 liquid medium was monitored by determining OD₆₀₀ at intervals of 3 h. Similar results were obtained in two independent experiments. Ori, Original undiluted sample; 1/2 and 1/4, samples diluted twofold and fourfold, respectively; M, protein marker.

PPE37 does not affect macrophage cell death during infection with *M. smegmatis*

One of the consequences of infecting host cells with *M. tuberculosis* is cell death, with the possibility that *M. tuberculosis* manipulates host-cell death as one of the mechanisms of pathogenicity. Recently, emerging new evidence prompted the proposal of a new model on the interaction between *M. tuberculosis* and its host cell (Behar *et al.*, 2010). This model suggests that, as a pathogenic strategy, virulent *M. tuberculosis* inhibits apoptosis whilst actively inducing necrosis in the infected host cell. The

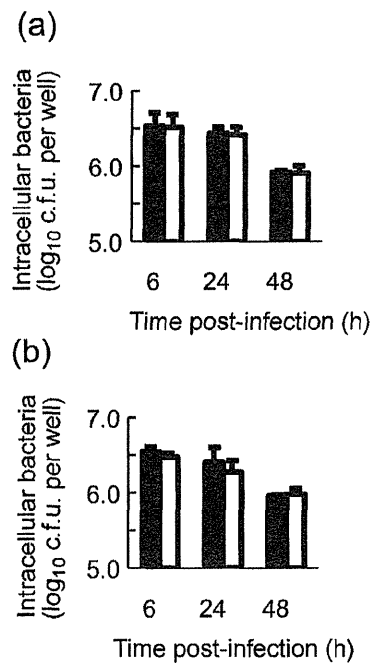


Fig. 2. Intracellular survival of recombinant *M. smegmatis* in macrophages. Peritoneal exudate macrophages (a) or bone marrow-derived macrophages (b) were infected with Ms_ppe37 (white bars) or Ms_vec (black bars) at an m.o.i. of 20. At 6, 24 and 48 h after infection, the macrophages were washed and lysed. Lysates were diluted and plated on Middlebrook 7H10 agar plates containing 25 µg kanamycin ml⁻¹ to determine the number of c.f.u. Data are shown as means ± SD of triplicate wells. Similar results were obtained in three independent experiments.

outcome of this strategy is a reduction in the efficiency of cross-presentation of mycobacterial antigens leading to the impairment in the initiation of T-cell immunity (Behar *et al.*, 2010; Divangahi *et al.*, 2010).

In this experiment, we thus wanted to determine whether PPE37 was able to affect the death of macrophages infected with *M. smegmatis*. Peritoneal exudate macrophages were infected with Ms_ppe37 or Ms_vec, and the amount of LDH released into the culture supernatant (an indicator of cell death) was determined. The result showed that macrophages infected with Ms_ppe37 or Ms_vec released a comparable amount of LDH (Fig. 3). In addition, microscopic examination also revealed no differences in the morphological features of the infected macrophages within the time period of infection (data not shown). Taken together, these results suggested that macrophage cell death was unaffected by PPE37 during infection with *M. smegmatis*.

Ms_ppe37 induces a lower level of pro-inflammatory cytokines in infected macrophages

To deduce the potential role of PPE37 in the virulence of *M. tuberculosis*, we investigated the effect that PPE37 might

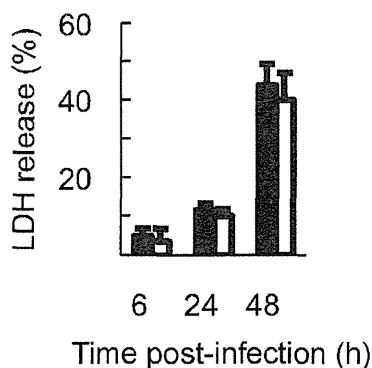


Fig. 3. Assay of cell death in macrophages infected with recombinant *M. smegmatis*. Peritoneal exudate macrophages were infected with Ms_ppe37 (white bars) or Ms_vec (black bars) at an m.o.i. of 20. At 6, 24 and 48 h after infection, culture supernatants were harvested. Release of LDH as a measure of macrophage cell death was estimated by assaying LDH activity in the culture supernatants. Data are shown as means \pm SD of triplicate wells. Similar results were obtained in three independent experiments.

have on the immune response of infected macrophages. The aspect of the immune response that we examined first was cytokine production. Peritoneal exudate macrophages were infected with Ms_ppe37 or Ms_vec for 24 h, and the concentrations of cytokines in the culture supernatants were determined. Consistent with the cytokine profiles reported elsewhere for macrophages infected with *M. smegmatis* (Lee & Schorey, 2005; Post *et al.*, 2001; Roach & Schorey, 2002), peritoneal exudate macrophages infected with Ms_vec or Ms_ppe37 also produced TNF- α , IL-6, IL12p70 and IL-1 β (Fig. 4a–d). However, we discovered that Ms_ppe37 induced a significantly lower level of these cytokines in infected macrophages compared with Ms_vec. We repeated the infection experiment in bone marrow-derived macrophages, and measured the production of TNF- α and IL-6. Similar to infection in peritoneal exudate macrophages, Ms_ppe37 also induced a significantly lower level of TNF- α and IL-6 in the infected bone marrow-derived macrophages compared with Ms_vec (Fig. 4e, f). RT-PCR analyses showed that the expression of TNF- α mRNA in macrophages infected with Ms_ppe37 was delayed compared with the expression in macrophages infected with Ms_vec. The difference in the expression level was observed until 9 h after infection and thereafter became comparable (Fig. 4g). Although expression of IL-6 mRNA occurred much later, from 12 h after infection, the mRNA levels were also lower in macrophages infected with Ms_ppe37 than in macrophages infected with Ms_vec. However, despite the differential cytokine response, flow cytometric analysis revealed no significant differences in the expression levels of MHC-I, MHC-II, CD80, CD86 and CD40 between macrophages infected with Ms_ppe37 and macrophages infected with Ms_vec (Fig. 5). Taken together, these results suggested that PPE37 might possess a functional property that interferes with the pro-inflammatory cytokine

production of macrophages infected with *M. smegmatis* but does not affect the expression of surface markers on these macrophages.

PPE37 alters the activation levels of NF- κ B, ERK and p38 in macrophages infected with *M. smegmatis*

NF- κ B is a major transcription factor responsible for the expression of both TNF- α and IL-6 mRNAs (Collart *et al.*, 1990; Faggioli *et al.*, 2004; Kuprash *et al.*, 1999; Libermann & Baltimore, 1990; Zhang *et al.*, 1994). It has been reported that NF- κ B activation is needed to induce the expression of TNF- α and IL-6 mRNAs in macrophages infected with *M. smegmatis* (Gutierrez *et al.*, 2008). In addition, a previous study showed that *M. smegmatis* infection is also able to induce phosphorylation of the NF- κ B p65 subunit (Lee & Schorey, 2005). The altered TNF- α and IL-6 mRNA expression shown in Fig. 4(g) therefore raised the possibility that NF- κ B activation might be altered in macrophages infected with Ms_ppe37. To clarify this possibility, Western blot analysis was performed and the level of phosphorylated NF- κ B p65 subunit in the infected macrophages was assessed. It was observed that Ms_ppe37 induced a relatively lower level of p65 phosphorylation in the infected macrophages compared with Ms_vec (Fig. 6a).

In addition to NF- κ B, it has been shown that the expression of TNF- α mRNA in macrophages infected with *M. smegmatis* also requires the activation of p38 and ERK (Lee & Schorey, 2005; Roach *et al.*, 2005). Pharmacological inhibition experiments confirmed the requirement for ERK and p38 activities in the production of TNF- α and IL-6 in macrophages infected with Ms_vec (Fig. 6d, e). These results (Figs 4g and 6d, e) thus led us to investigate whether the activation of these mitogen-activated protein kinases (MAPKs) might also be affected in macrophages infected with Ms_ppe37. Western blot analysis was performed and the levels of phosphorylated ERK and p38 in the infected macrophages were assessed. Fig. 6(b, c) showed that Ms_ppe37 also induced a relatively lower level of ERK and p38 phosphorylation in infected macrophages compared with Ms_vec. The lower transcriptional activation of the TNF- α and IL-6 genes in macrophages infected with Ms_ppe37 therefore might have been due to a lower level of activation of NF- κ B, ERK and p38. Taken together, these results further argue for the possibility of PPE37 interfering with the pro-inflammatory cytokine response of macrophages infected with *M. smegmatis*.

DISCUSSION

In the present study, the results suggested the possibility that the *M. tuberculosis* PPE37 protein might interfere with the pro-inflammatory cytokine response in infected macrophages. We found that TNF- α , IL-6, IL-12p70 and IL-1 β were produced at significantly lower concentrations by macrophages infected with Ms_ppe37 compared with

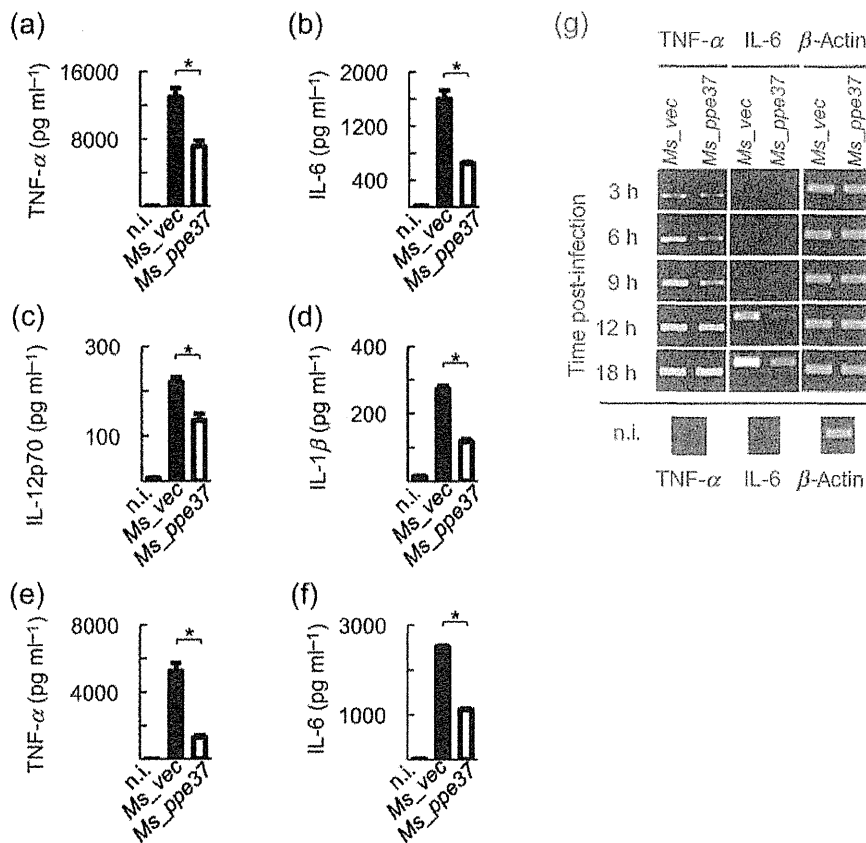


Fig. 4. Production of pro-inflammatory cytokines by macrophages infected with recombinant *M. smegmatis*. Peritoneal exudate macrophages (a–d) or bone marrow-derived macrophages (e, f) were infected with Ms_ppe37 or Ms_vec at an m.o.i. of 20. Culture supernatants were harvested after 24 h of infection and the concentrations of TNF-α (a, e), IL-6 (b, f), IL-12p70 (c) and IL-1β (d) were determined. Data are shown as means ± SD of triplicate wells. *, $P < 0.05$ by Student's two-tailed *t*-test. Similar results were obtained in three independent experiments. (g) Peritoneal exudate macrophages were infected with Ms_ppe37 or Ms_vec at an m.o.i. of 20. At 3, 6, 9, 12 and 18 h after infection, macrophages were washed and total RNA was extracted. Equal amounts of total RNA were subjected to RT-PCR in equal reaction volumes. DNA bands were visualized by ethidium bromide staining after equal volumes of PCR mixture had been electrophoresed. Similar results were obtained in two independent experiments. n.i., No infection.

macrophages infected with Ms_vec. The differential cytokine levels were due to lower transcriptional activation of the cytokine genes, which probably resulted from reduced activation of NF-κB, ERK and p38.

To the best of our knowledge, PPE18 is the only other PPE protein that has been reported to exhibit the property of interfering with the pro-inflammatory cytokine response in infected macrophages (Nair *et al.*, 2009). In the study by Nair *et al.* (2009), phorbol myristate acetate-differentiated THP-1 macrophages were infected with either a recombinant *M. smegmatis* strain that expressed PPE18 or a control strain that harboured the vector alone. It was shown that IL-12p40 production was significantly lower in macrophages after infection with the PPE18-expressing strain than after infection with the control strain. Nair *et al.* (2009) concluded that the decrease in the level of IL-12p40

was due to the anti-inflammatory activity of IL-10. A significantly higher production of IL-10 was concurrently found in macrophages after infection with the PPE18-expressing strain. In contrast to our study, we observed very low levels of IL-10 and found no significant difference in the concentration of IL-10 after infection with Ms_vec and Ms_ppe37 (data not shown). Using a purified recombinant protein, Nair *et al.* (2009) showed that PPE18 stimulated the macrophages to secrete IL-10 by binding to Toll-like receptor 2 (TLR2). A consequence of this binding was an early and sustained activation of p38 MAPK, which has been shown to be critical for the induction of IL-10. Similarly, our findings also implied the involvement of MAPKs. However, our study suggests that the mechanism by which PPE37 might interfere with the pro-inflammatory cytokine response in infected macrophages involves reduced transcriptional activation of the

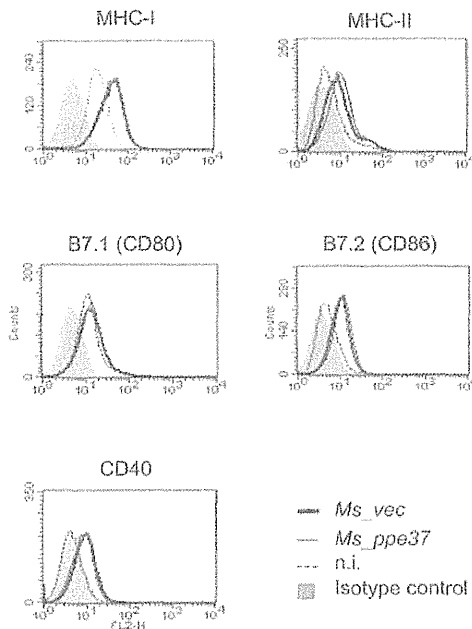


Fig. 5. Expression of cell-surface markers on macrophages infected with recombinant *M. smegmatis*. Peritoneal exudate macrophages were infected with *Ms_ppe37* or *Ms_vec* at a m.o.i. of 20. After 24 h of infection, the macrophages were harvested and the expression levels of MHC-I, MHC-II, CD86, CD80 and CD40 were analysed by flow cytometry. Grey-shaded areas represent the basal fluorescent intensity in macrophages stained with isotype control IgG2a. n.i., No infection.

cytokine genes. This in turn is probably due in part to reduced activation of NF- κ B, ERK and p38.

Although both PPE18 and PPE37 exhibit the similar property of interfering with the pro-inflammatory cytokine response in infected macrophages, there appear to be differences in the mechanisms. This is indicated by the discrepancy in the production of IL-10 and also in the pattern of MAPK activation. One of the possible contributing factors may be attributed to a difference in the intrinsic properties of PPE37 and PPE18. Analysis of their amino acid sequences has led to further classification of PPE37 and PPE18 into the PPE-PPW and PPE-SVP subfamilies, respectively (Adindla & Guruprasad, 2003; Gey van Pittius *et al.*, 2006; Gordon *et al.*, 1999). PPE proteins of the subfamily PPE-PPW are characterized by a conserved 44 aa residue region in the C terminus, which comprises highly conserved Gly-Phe-X-Gly-Thr and Pro-X-X-Pro-X-X-Trp sequence motifs (Adindla & Guruprasad, 2003; Gey van Pittius *et al.*, 2006). Members of the PPE-SVP subfamily, on the other hand, contain the motif Gly-X-X-Ser-Val-Pro-X-X-Trp between position 300 and 350 in their amino acid sequence (Gey van Pittius *et al.*, 2006; Gordon *et al.*, 1999). A systematic functional comparison has yet to be made, but these amino acid sequence motifs may confer distinct properties on the respective PPE proteins.

The other factor that may have contributed to the distinct features in the effect of PPE18 and PPE37 on IL-10 production and the pattern of MAPK activation may stem from differences in the responses between mouse peritoneal macrophages and the human monocytic leukaemia cell line THP-1. Differences in MAPK activation are found to differ considerably depending on the cell type used (Rao, 2001). This has led to the assertion that signalling events associated with MAPK activation cannot be extrapolated from one cell type to another (Rao, 2001). In addition to cell type, it has also been reported that the level of cell maturity also affects the activation of MAPK. Indeed, it was shown that, upon infection with *M. tuberculosis*, the kinetics of p38 MAPK activation in human alveolar macrophages was faster than in human blood monocytes (Surewicz *et al.*, 2004).

Our study showed that the phosphorylation levels of ERK, p38 and NF- κ B p65 were lower in macrophages infected with *Ms_ppe37*. This suggests that PPE37 may be interfering with or inhibiting the activation of these molecules. How does PPE37 achieve this, considering that ERK, p38 and NF- κ B p65 are three different proteins, each associating with three different signalling pathways? A possible mechanism as to how PPE37 might inhibit or interfere with the activation of ERK, p38 and NF- κ B p65 is by inhibiting or interacting with a molecule that is involved in the common activation of these three different proteins. Although the MAPK and NF- κ B signalling pathways are distinct, they are not mutually exclusive. For example, they are known to share some common stretches of the signalling pathways when the TLRs are stimulated (Akira *et al.*, 2003). Among the TLRs, TLR2 is most frequently involved in the recognition of various pathogen-associated molecular patterns isolated from *Mycobacterium* spp. (Jo *et al.*, 2007). Therefore, in the innate immune response to *Mycobacterium* spp. including *M. smegmatis*, the activation of MAPKs and NF- κ B may occur most commonly through the stimulation of TLR2. In general, the stimulation of most TLRs results in the recruitment of the adaptor protein MyD88 to the receptor complex, where it promotes the subsequent interaction of IL-1R-associated kinase with TNF receptor-associated factor 6 (TRAF6). The signalling pathways from TRAF6 then branch out, with one leading to the MAPK pathway and another to the NF- κ B pathway (Akira *et al.*, 2003). This thus makes it very tempting to speculate on the possibility that PPE37 interacts with one of these molecules, including TLR2, that are involved in the common activation of the MAPK and NF- κ B signalling pathways. Although the results shown in Fig. 1(c) suggested that PPE37 is not a secretory protein, computational analysis of the amino acid sequence predicted the subcellular localization of PPE37 to be on the bacterial cytoplasmic membrane (Gardy & Brinkman, 2006; <http://www.psort.org/psortb/>). In line with this, as TLR2 is a cell-surface receptor molecule, it may be more likely to interact with PPE37 than with other molecules in the TLR2 signalling pathways that are involved in the common

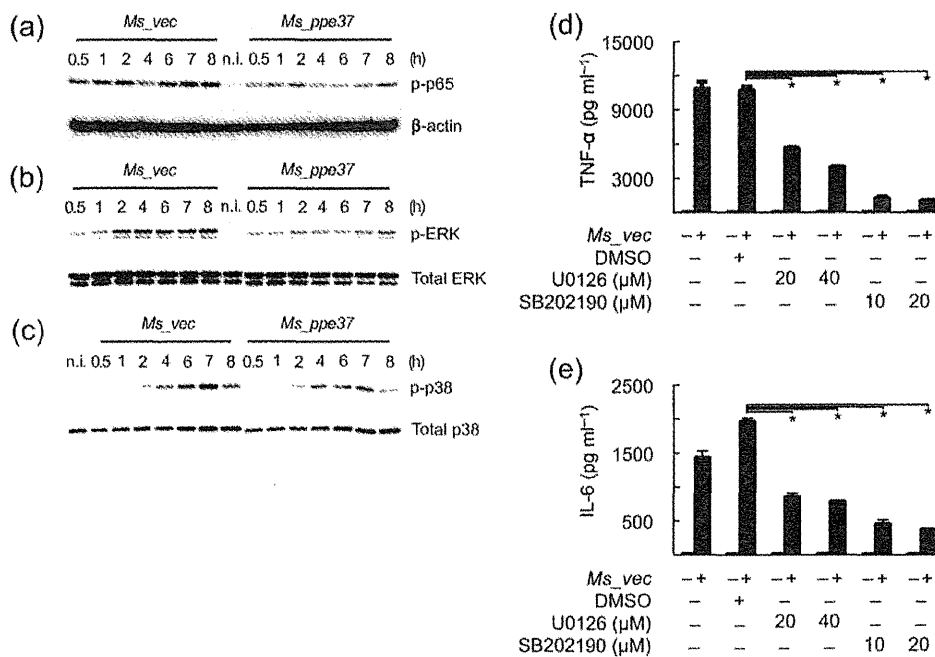


Fig. 6. Phosphorylation of the NF-κB p65 subunit, ERK and p38 in macrophages infected with recombinant *M. smegmatis*. (a–c) Peritoneal exudate macrophages were infected with *Ms_ppe37* or *Ms_vec* at an m.o.i. of 20. Macrophages were washed and lysed after 0.5, 1, 2, 4, 6, 7 and 8 h of infection. Lysates were subjected to Western blot analyses to detect the phosphorylated NF-κB p65 subunit (p-p65) (a), phosphorylated ERK (p-ERK) (b) and phosphorylated p38 (p-p38) (c) with specific antibodies. Detection of β-actin, total ERK and total p38 indicated equal protein loading. Similar results were obtained in three independent experiments. n.i., No infection. (d, e) Peritoneal exudate macrophages were treated with U0126 (a MEK1/2 inhibitor) or SB202190 (a p38 inhibitor) at the indicated concentrations. Treatment with DMSO served as a control for the inhibitor treatments. After 1 h, the macrophages were infected with *Ms_vec* at an m.o.i. of 20. Culture supernatants were harvested after 24 h of infection and the concentrations of TNF-α (d) and IL-6 (e) in the culture supernatants were determined. Data are shown as means ± SD of triplicate wells. *, *P* < 0.05 by Student's two-tailed *t*-test. Similar results were obtained in three independent experiments. –, No infection or treatment; +, with infection or treatment.

activation of MAPKs and NF-κB. However, TLR2 is not the only candidate receptor with the possibility of interacting with PPE37, as the common activation of the MAPK and NF-κB signalling pathways is not limited to this receptor alone.

In the context of *M. tuberculosis* infection, the possible role of PPE37 in interfering with the pro-inflammatory cytokine response in infected macrophages might also be applicable. Manca *et al.* (1999) reported that infection of human monocytes with *M. tuberculosis* clinical isolate CDC1551 induced a higher level of TNF-α, IL-6 and IL-12 than infection with the *M. tuberculosis* laboratory strain H37Rv. It may be possible that this vigorous pro-inflammatory cytokine response induced by CDC1551 was due in part to the loss of PPE37 function. Comparative genome analysis between CDC1551 and H37Rv has revealed that the *ppe37* gene is deleted from the genome of CDC1551 (Gey van Pittius *et al.*, 2006).

In conclusion, the present study suggests that the *M. tuberculosis* PPE37 may have a role in interfering with the

pro-inflammatory cytokine response in macrophages infected with *M. smegmatis*. It is established that pro-inflammatory cytokines such as TNF-α (Elbek *et al.*, 2009; Flynn *et al.*, 1995; Jacobs *et al.*, 2007; Lin *et al.*, 2007; Wolfe *et al.*, 2004) are critical to host immune responses in containing *M. tuberculosis* infection. Subversion and modulation of the host inflammatory response can thus be an advantageous pathogenic strategy for *M. tuberculosis*. In light of the possible role of PPE37 suggested by our study, a hypothesis of the possible contribution of PPE37 to such a pathogenesis strategy is presented. Our results thus provide a basis to investigate and characterize further the role of PPE37 in the context of *M. tuberculosis* infection. Future studies that are needed include the construction and testing of knockout genes in *M. tuberculosis*.

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