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Short Article

Mycobacterium tuberculosis Virulence Is Mediated by PtpA Dephosphorylation of Human Vacuolar Protein Sorting 33B

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SUMMARY

Entry into host macrophages and evasion of intracellular destruction mechanisms, including phagosome-lysosome fusion, are critical elements of *Mycobacterium tuberculosis* (*Mtb*) pathogenesis. To achieve this, the *Mtb* genome encodes several proteins that modify host signaling pathways. PtpA, a low-molecular weight tyrosine phosphatase, is a secreted *Mtb* protein of unknown function. The lack of tyrosine kinases in the *Mtb* genome suggests that PtpA may modulate host tyrosine phosphorylated protein(s). We report that a genetic deletion of *ptp*A attenuates *Mtb* growth in human macrophages, and expression of PtpA-neutralizing antibodies simulated this effect. We identify VPS33B, a regulator of membrane fusion, as a PtpA substrate. VPS33B and PtpA colocalize in *Mtb*-infected human macrophages. PtpA secretion combined with active-phosphorylated VPS33B inhibited phagosome-lysosome fusion, a process arrested in *Mtb* infections. These results demonstrate that PtpA is essential for *Mtb* intracellular persistence and identify a key host regulatory pathway that is inactivated by *Mtb*.

INTRODUCTION

Tuberculosis (TB), caused by the facultative intracellular pathogen *Mycobacterium tuberculosis*, remains a leading cause of death due to infectious disease in the world today and poses a serious challenge to international public health. Ten million new cases of TB arise annually, causing about 2 million deaths each year (Raviglione, 2003).

Entry into host macrophages and evasion of intracellular destruction mechanisms are pivotal to *M. tuberculosis* virulence. Crosstalk between host macrophages and the infecting bacteria is essential for bacterial survival in vivo. Host signaling proteins are modified by mycobacterial infection (Hestvik et al., 2003), and *M. tuberculosis* secretes proteins that contributes to the inhibition of phagosome maturation. For instance, an *M. tuberculosis* lipid phosphatase, SapM, acts by hydrolyzing the host phosphatidylinositol-3- phosphate (PI3P) (Vergne et al., 2005).

Pathogenic bacteria are reported to secrete low-molecular weight tyrosine phosphatases during infection (Bach et al., 2006; Bliska et al., 1992; Fu and Galan, 1998). *M. tuberculosis* PtpA is such a low-molecular weight tyrosine phosphatase (Cowley et al., 2002). The lack of annotated tyrosine kinase genes in the *M. tuberculosis* genome sequence database (Cole et al., 1998) suggests that PtpA may mediate host-pathogen interactions during infection. Here, we report that VPS33B, a host protein involved in vesicle trafficking, is dephosphorylated by PtpA leading to a block of phagosome maturation by *M. tuberculosis*.

RESULTS

Growth of *M. tuberculosis* Δ*ptp*A Mutant Is Attenuated in Human Macrophages

To assess the contribution of PtpA to *M. tuberculosis* virulence and infectivity, we created a *ptp*A knockout strain of *M. tuberculosis* by allelic exchange (Papavinasasundaram et al., 2005) (Figure 1A and Figure S1 available online). Compared to its parental strain, H37Rv, the *Δptp*A mutant showed no differences in its in vitro growth characteristics both in rolling aerobic or semistatic cultures (Figure 1B). However, competitive coinfection of THP-1 macrophages with Δ*ptp*A and its parental strain demonstrated that *ptp*A is required for successful long-term infection (Figure 1C). Although the wild-type and Δ*ptp*A mutant showed similar infectivity with similar colony forming units (CFU) obtained 2 hr postinfection, the *Δptp*A mutant showed a reduction of more than two logs in intracellular survival over 48 hr (Figure 1C). During independent infection of macrophages (Figure 1D), the mutant strain was rapidly cleared; after 72 hr, live bacteria CFUs were reduced by a magnitude of three logs. In contrast, the parental strain and the complemented *Δptp*A mutant established stable infections after 24 hr. The growth of complemented mutants expressing inactive PtpA was also attenuated but to a lesser degree compared to the *ΔptpA* strain.

Survival of *M. tuberculosis* Is Attenuated in Macrophages Expressing αPtpA Single-Chain Antibodies

To identify the location in which PtpA functions, we used the single-chain antibody, sc-007, directed against PtpA (Kd = 0.92 μM). Incubation with sc-007 reduced the catalytic activity of PtpA by 60% as monitored by *p*-nitrophenyl phosphate cleavage (data not shown). Thereafter, the gene encoding sc-007 was transfected and stably expressed to produce intracellular antibodies (intrabodies) in macrophages; the yield was 150 ng intrabodies/10^6 cells (Figure S2). Expression of sc-007 in the macrophage

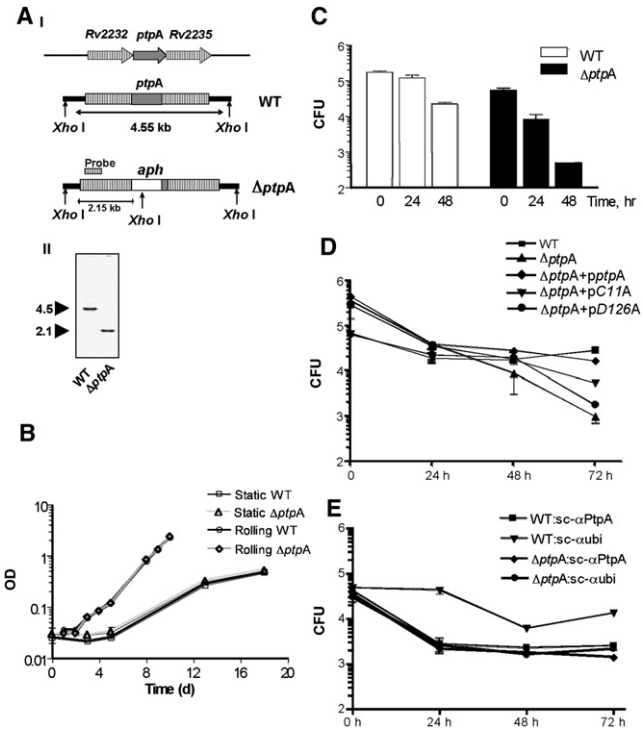

Figure 1. *M. tuberculosis* Δ*ptp*A Construction and Analyses

(A) (I) Map showing the *ptp*A genomic region and modification introduced during the mutant construction. (II) Southern hybridization using α-^{32}P-radioactively labeled probe hybridized to a 4.5 kb *Xho*I fragment for H37Rv wild-type (1) and a 2.1 kb fragment for the Δ*ptpA* mutant (2) as predicted based on restriction map analysis.

(B) In vitro growth of H37Rv wild-type and the Δ*ptpA* mutant strain. The wild-type and Δ*ptpA* mutant strain displayed similar growth rate in 7H9 liquid medium when incubated as rolling cultures. Similar results were observed for wild-type and the mutant in static cultures. In static growth, shown are the mean values (±SD) of three independent experiments.

(C) Competitive coinfection of Δ*ptp*A mutant and its parental strain. Macrophages were infected with an opsonized mixture (1:1) of wild-type and mutant strains at an moi of 1:10 for 2 hr and placed at 37°C and 5% CO_2 after washing away noninternalized bacteria. Macrophages were processed according to Supplemental Experimental Procedures. Shown are the mean values (±SD) of three independent experiments.

(D) *M. tuberculosis* survival in infected macrophages. Δ*ptp*A+p*ptp*A represents the mutant complemented with *ptpA*. Infected macrophages were processed as described in the Experimental Procedures. Shown are the mean values (±SD) of three independent experiments.

(E) *M. tuberculosis* survival in stable-transfected macrophages expressing intrabodies against PtpA. Stable-transfected macrophages were obtained as described in the Experimental Procedures. Shown are the mean values (±SD) of three independent experiments.

inhibited the survival of *M. tuberculosis* as early as 24 hr postinfection. Reduced survival was not observed in cells expressing antibodies against an unrelated protein (anti-bovine ubiquitin) (Figure 1E). As expected, the growth of *Δptp*A in the presence of sc-007 was similar to the parental strain. *M. tuberculosis* growth attenuation provided further evidence that PtpA is required for mycobacterial growth in vivo. The reduction of *M. tuberculosis* survival by inactivating PtpA in the macrophage cytosol outside the mycobacterial phagosome strengthens our hypothesis that PtpA functions within host cells.

Host VPS33B Is a PtpA Substrate

To identify host proteins that interact with PtpA, we used a "substrate trapping" assay, based on methodology used to identify substrates for the *Y. pseudotuberculosis* phosphatase YopH in HeLa cells (Bliska et al., 1992). This mechanism-based approach utilizes a catalytically defective mutant of PtpA to trap substrate complexes. PTPs contain a cysteine nucleophile (Cys11 within the highly conserved sequence C-X_5-R) that forms a phosphocysteinyl intermediate during catalysis (Barford et al., 1998) and a conserved aspartate residue (Asp126) that acts as a general acid/base to protonate the tyrosine phenolic leaving group (Madhurantakam et al., 2005) (Supplemental Data). We hypothesized that mutation of the PtpA catalytic aspartic acid to an alanine (Asp126Ala) would result in a catalytically defective PtpA that, like other similar PTP family mutants, would "trap" host substrate proteins by stabilizing the covalent enzyme-substrate complexes. To test this approach, we constructed and overexpressed the Asp126Ala, Cys11Ala, and Arg17Ala (Supplemental Data and Figure S3A) mutants as well as wild-type PtpA. As expected, only the wild-type PtpA retained phosphatase activity (Figure S3B). After the addition of the Asp126Ala-PtpA protein to a THP-1 macrophage lysate, a protein migrating at a molecular weight of about 70 kDa was trapped (Figure 2AI, lane 4). MALDI-TOF mass spectrometry identified this protein as hVPS33B (human Vacuolar Protein Sorting 33B). Western analysis of the trapped proteins confirmed the identification of VPS33B as the binding partner of Asp126Ala-PtpA (Figure 2AIIb).

PtpA Directly Binds to and Dephosphorylates VPS33B

To ascertain if VPS33B serves as a tyrosine phosphorylated substrate of PtpA, we overexpressed, purified, and checked the phosphorylation properties of VPS33B. We found that VPS33B is a self-phosphorylating kinase. Autophosphorylation of VPS33B occurred at tyrosine residues, as revealed by the extent of γ^{32}P-ATP incorporation in an in vitro kinase assay followed by phospho-amino acid analysis (Figure 2B). Moreover, site-directed mutagenesis of selected tyrosine residues in VPS33B significantly reduced autophosphorylation activity (Figure S7 and Figure 2E).

To demonstrate the interaction of PtpA and VPS33B in vitro, we used three separate approaches: a biochemical assay, protein-protein interaction analysis, and coimmunoprecipitation. The biochemical assay revealed that the extent of γ^{32}P-ATP incorporation into VPS33B was significantly reduced when recombinant active PtpA was included in the reaction mix (Figure 2C). Furthermore, dephosphorylation of VPS33B by active PtpA was dose- and time-dependent, while the catalytic-defective mutants Cys11Ala, Arg17Ala, and Asp126Ala failed to dephosphorylate VPS33B, confirming that the catalytic site is essential for the enzymatic activity of PtpA (Figure S5). The direct interaction of PtpA with VPS33B was confirmed using the amplified luminescent proximity assay. The activity of low-molecular weight phosphatases have been reported to be redox sensitive (Caselli et al., 1998), and two cysteines (C11 and C16) in the active site of PtpA protect the catalytic site serving as a "self-lock" when the redox status changes. The Cys11Ala mutant, which does not form the locked S-S bridge, was used to test the binding of PtpA to VPS33B. As shown in Figure S6, Cys11Ala PtpA bound to VPS33B with Kd of 2.1 nM.

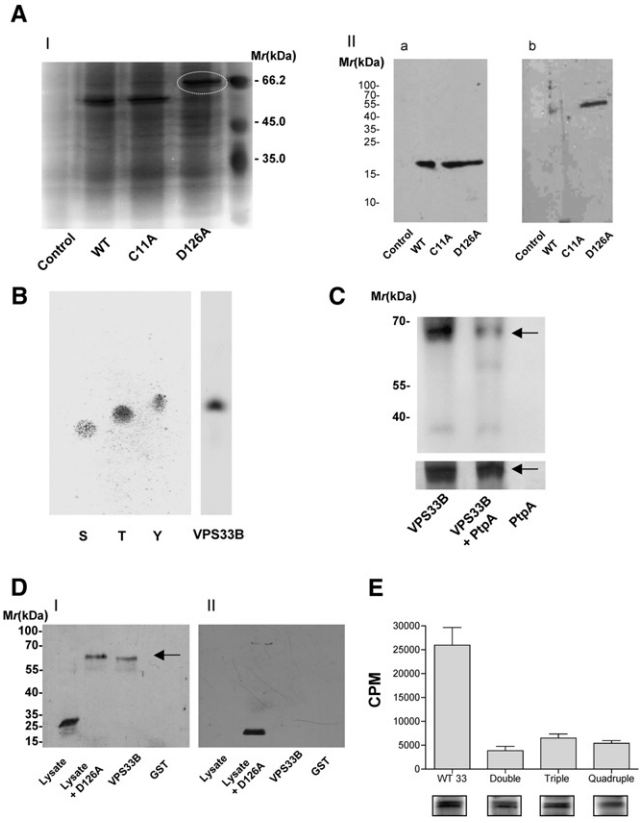

Figure 2. Identification of PtpA Substrates and Analyses of VPS33B

(A) (I) Substrate trapping using phosphatase-active and -defective PtpA. The spot in lane 4 marked with a white dashed ellipse corresponds to VPS33B. The proteins in lanes 2 and 3 are not dephosphorylated by active PtpA. (II) Western blotting of trapping gel showing VPS33B bound to PtpA. (a) Membrane exposed to rabbit αPtpA antibodies. (b) Same membrane exposed to sheep αVPS33B after stripping.

(B) VPS33B is autophosphorylated on tyrosine residues as determined by acidic hydrolysis and separation on TLC. Retention factor: serine (S), 0.40; threonine (T), 0.48; tyrosine (Y) 0.53; VPS33B, 0.51.

(C) Radiolabeling analysis of in vitro dephosphorylation of autophosphorylated recombinant VPS33B by active PtpA (upper image). In VPS33B+PtpA, 0.3 μg of active PtpA was added. Equal loading of VPS33B (lower image) was revealed by silver staining.

(D) (I) Immunoprecipitation of VPS33B by Asp126Ala-PtpA from macrophages lysate. Lysate was coimmunoprecipitated with αPtpA antibodies, resolved on SDS-PAGE, and blotted onto a nitrocellulose membrane. A band matching VPS33B was observed after incubation of the membrane with sheep αVPS33B and donkey αsheep antibodies. (II) Control blot. The same membrane was incubated with rabbit αPtpA and goat αrabbit antibodies after protein stripping. Black arrow indicates VPS33B. The difference in the pattern migration might be due to mammalian posttranslational in THP-1 cells versus *E.coli* which is the host for recombinant VPS33B.

(E) Autophosphorylation activity of site-directed mutated tyrosine residues in VPS33B. Autophosphorylation rates of site-directed mutated VPS33Bs were tested in an in vitro kinase assay. Samples were resolved in a 10% SDS gel and exposed to a screen for radiolabeled band localization. After gel drying, the band corresponding to the phosphorylated spots was excised from the gel, and the radioactive incorporation was measured in a scintillation counter. Images represent the radioactivity of the dried gel. Double is defined as the site-directed mutagenesis of Y133E and Y382E, triple includes the same mutated residues as the double mutant + Y511E, while the quadruple includes the same mutated residues as the triple mutant + Y517E. Results are expressed as mean ± SD.

The interaction of PtpA and VPS33B was further demonstrated in a separate ex vivo experiment. We pulled down VPS33B from a THP-1 lysate using the PtpA Asp126Ala mutant. The complex was immunoprecipitated from the lysate using antibodies against PtpA. Exposure of the immunoblot to αVPS33B antibodies showed a band matching the size of the substrate (Figure 2DI). Taken together, these findings confirm that human VPS33B is a genuine substrate for mycobacterial PtpA.

PtpA and VPS33B Colocalize in *M. tuberculosis*-Infected Macrophages

If VPS33B is an in vivo substrate of PtpA, then these two proteins should colocalize in *M. tuberculosis*-infected macrophages. To test this prediction, we used antibodies directed against PtpA and VPS33B to stain THP-1-derived macrophages infected with live or killed *M. tuberculosis* (Figure 3A). In these experiments, VPS33B and PtpA staining overlapped in the periphery of phagosomes. As expected in the case of a secreted protein, PtpA was detected only in phagosomes containing live, but not killed, bacilli (Figure 3A). Macrophages infected with Δ*ptpA M. tuberculosis* did not show colocalization of PtpA and VPS33B, demonstrating the specificity of the αPtpA antibodies (Figure 3A). Colocalization analyses (Supplemental Data) were statistically relevant with a Mander's overlap coefficient (R) of 0.971 for live bacteria (0.087 for killed) and Pearson's correlation coefficient (Rr) of 0.924 (0.045 for killed); see Supplemental Data for details.

PtpA and VPS33B colocalization was confirmed by electron microscopy. Immunogold labeling of macrophages infected with *M. tuberculosis* H37Rv clearly showed that PtpA closely associated with VPS33B in the macrophage (Figure 3B). An immunoblot analysis of the cytosol/membrane fractionation was performed (Figure 3C). The cytosolic fraction of *M. tuberculosis*-infected macrophages (Figure 3C) show limited visibility of PtpA in macrophage cytosol. However, a band corresponding to PtpA is present in the cytosolic fraction of macrophages infected with a strain expressing PtpA from an extrachromosomal plasmid. These experiments confirmed that (1) PtpA is secreted outside of the mycobacterial phagosome and (2) PtpA and VPS33B colocalize in the macrophage cytosol.

In yeast, the membrane-associated proteins VPS11, VPS16, VPS18, and VPS33 form a protein complex termed Class C. This complex has been shown to be involved in SNARE-mediated membrane fusion (Rieder and Emr, 1997). VPS33B is recruited from the cytosol and interacts with membrane-associated Class C subunits (specifically VPS16) to allow the complex to migrate to the vacuole (Rieder and Emr, 1997). Analysis of *M. tuberculosis*-infected macrophages labeled with antibodies against VPS16 and PtpA clearly demonstrate colocalization of PtpA and VPS16 (Figure 3D).

Overall, these studies support the evidence that VPS33B interacts specifically with secreted PtpA in vivo and are in accordance with the identification of VPS33B as a specific substrate PtpA. Furthermore they suggest that mycobacterial PtpA functions with the Class C complex to influence processes in the phagosome of infected macrophages.

PtpA Inhibits Phagosome-Lysosome Fusion

One of the key mechanisms by which mycobacteria avoid the microbicidal activity of macrophages is the inhibition of fusion

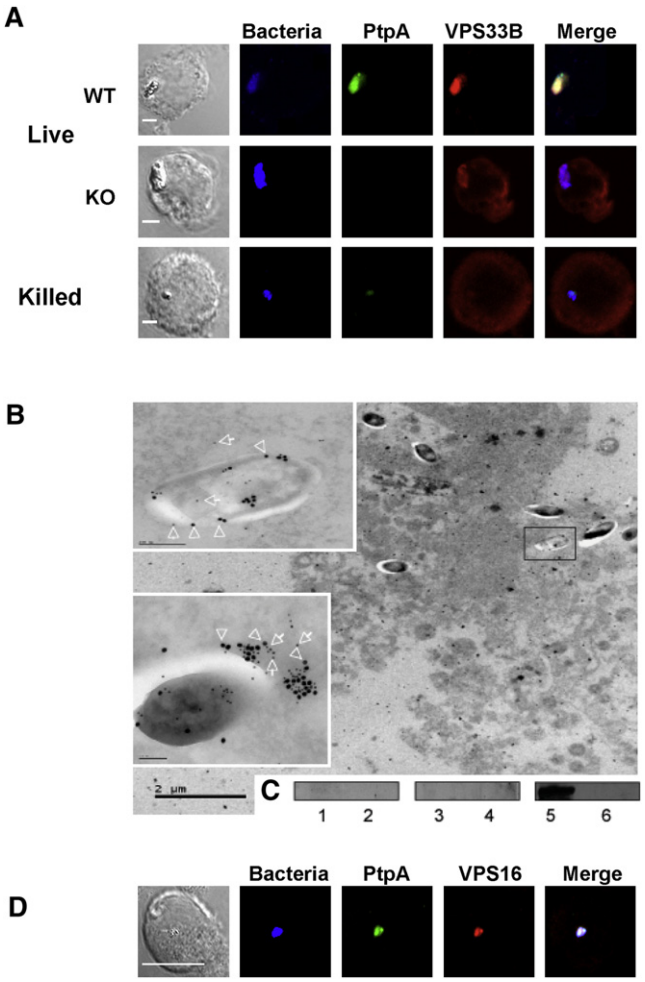

Figure 3. Colocalization of PtpA VPS33B and VPS16 in Infected Macrophages

(A) Macrophages were infected with live or killed bacteria and then processed and immunostained as described in the Supplemental Data. Goat-αrabbit coupled to Alexa 488 was used as to detect αPtpA, while donkey-αsheep coupled to Alexa 633 was used against αVPS33B. Bacteria were stained with Alexa 555. White bar is 20 μm.

(B) Electron microscopy of *M. tuberculosis* H37Rv-infected macrophages. Magnification, 9700×; inserts, 97000×. Bar in the insert is 0.2 μm. VPS33B and PtpA were localized in the macrophage cytosol using 10 nm gold-labeled PtpA (white arrows) and 18 nm gold-labeled VPS33B (arrowheads). The bottom insert shows an independent experiment.

(C) Western analysis of PtpA presence in cytosolic (C) or membrane (M) compartments of infected macrophages. Lanes 1 (C) and 2 (M) WT-infected macrophages; lanes 3 (C) and 4 (M) Δ*ptp*A-infected macrophages; and lanes 5 (C) and 6 (M) Δ*ptp*A+*pptp*A-infected macrophages.

(D) Colocalization of VPS16 and PtpA. Macrophages were infected with the wild-type strain and processed as described in the Supplemental Data. Goat-αrabbit coupled to Alexa 488 was used as to detect αPtpA, while donkey-αsheep coupled to Alexa 633 was used against α VPS16. Bar is 20 μm.

between infected phagosomes and lysosomes. In order to investigate the role of PtpA in blocking phagosome maturation, macrophages were exposed to latex beads non-covalently coated with PtpA, bovine serum albumin (BSA) or one of two phosphatase-defective mutants Cys11Ala, and Asp126Ala. After ingestion of coated beads, phagosome maturation was examined by flow cytometry (FACS) (Hmama et al., 2004). The ability of PtpA to translocate into the cytosol by crossing the phagosome membrane was demonstrated by Western blotting of cytosolic fraction from macrophages ingesting PtpA-coated beads. Moreover, electron microscopy clearly showed that PtpA was detached from the latex beads and crossed the phagosomal membrane toward the cytosol (Figures S8 and S9). There was a 60% reduction in phagolysosomal fusion in macrophages containing PtpA-coated beads (Figure 4A, WT; Figure S10) compared to phagosomes containing BSA-coated beads. In contrast, mutated forms of PtpA had a marginal effect on phagolysosome fusion. Furthermore, beads coated with active PtpA and incubated with sc-007 antibody lost their ability to inhibit phagolysosome fusion (Figure 4A, WT-007). These results were further confirmed by fluorescence (Figure 4B) and electron microscopy (Figure S8) of the PtpA-coated latex beads. These analyses revealed recruitment of VPS33B to the phagosome, but not a complete colocalization to the lysosome, while BSA-coated latex beads recruited VPS33B, which allows them to migrate toward lysosomes (Figure 4B). These data suggest that *M. tuberculosis* PtpA inhibits phagosome maturation.

Influence of PtpA in Phagolysosome Fusion in Presence or Absence of VPS33B

To assess the role of VPS33B in phagosome maturation, we attenuated the expression of endogenous VPS33B expression in THP-1 cells using a siRNA-based approach. Macrophages expressing two independent siRNA against VPS33B sequences showed a significant reduction in their ability to mediate phagosome-lysosome fusion, indicating that VPS33B is essential for this process (Figure S11). No effect was observed in the pattern of phagosome-lysosome fusion when the control scramble RNA was used (Figure S11). Furthermore, macrophages with silenced VPS33B expression failed to distinguish between PtpA-, Arg17Ala-, or BSA-coated latex beads in a phagosome maturation assay (Figure S11), whereas complementing VPS33B-silenced cells by providing active recombinant WT-VPS33B restored the inhibition of phagolysosome fusion only by PtpA-coated beads (Figure 4C and Figure S12). In contrast, equivalent amounts of quadruple-tyrosine-mutated VPS33B (Figure 2E) delivered to the silenced macrophages did not restore the inhibition of phagolysosome fusion by PtpA-coated beads (Figures 4C and Figure S12). Together, these results demonstrate a direct role for phosphorylated VPS33B in PtpA-dependent phagolysosome fusion.

DISCUSSION

Interference with host signaling is a common theme in bacterial pathogenesis. For example, the *Salmonella* and *Yersinia* phosphatases, SptP (Fu and Galan, 1998) and YopH (Bliska et al., 1992), are secreted into host cells where they interact with several adhesion proteins. Here, we provide evidence that *M. tuberculosis* utilizes a similar strategy in that it secretes the protein phosphatase PtpA to promote its survival within the host. The *M. tuberculosis* Δ*ptp*A strain was severely attenuated when invading macrophages but showed no reduction in growth rate compared to the wild-type in axenic culture. A similar reduction in survival was observed when macrophages expressing

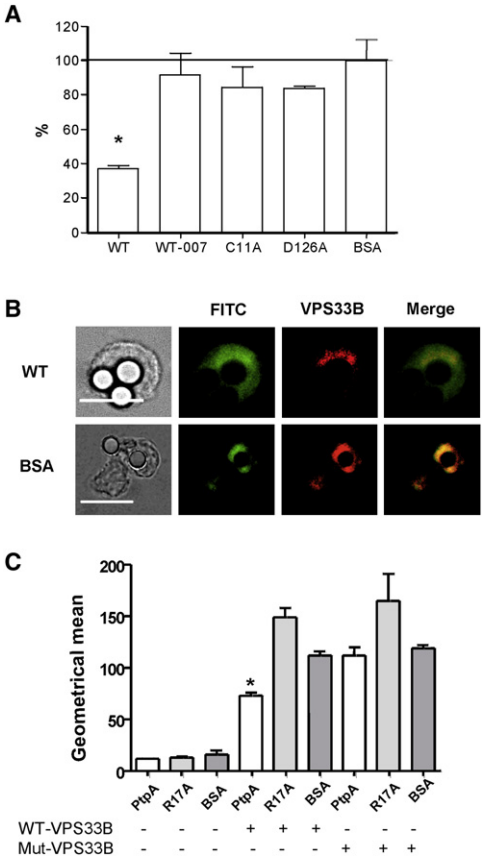

Figure 4. Contribution of PtpA and VPS33B to Inhibition of Phagolysosome Fusion

Inhibition of phagolysosome fusion by latex beads coated with either active PtpA or phosphatase-defective PtpA mutants. Phagolysosome fusion was quantified using FACS on the basis of colocalization of fluorescent FITC-dextran loaded lysosomes and coated beads containing phagosomes.

(A) Percentage of phagosome fusion to lysosome relative to fusion of phagosomes containing BSA-coated beads. WT-007 represents active PtpA exposed to the sc007 prior to bead protein coating. $C^{11}A$ and $D^{126}A$ are catalytic-defective mutants of PtpA. Asterisk represents a statistical mean difference using ANOVA one-way analysis. p value = 0.0073. Shown are the mean values (±SD) of three independent experiments.

(B) Colocalization of active PtpA- (WT) or BSA-coated beads and VPS33B in infected macrophages was investigated using confocal microscopy. Donkey αsheep coupled to Alexa 633 was used as secondary antibody. Bar is 20 μm.

(C) Macrophages depleted of endogenous VPS33B by siRNA (sequence no. J-7261-09) were complemented by delivering exogenous WT or quadruple tyrosine mutant VPS33B using a Profect delivering reagent (Supplemental Data). Complemented macrophages were labeled with FITC-dextran and infected, quantified, and presented as described above. Asterisk represents a statistical mean difference using ANOVA one-way analysis. p value = 0.0001. Shown are the mean values (±SD) of three independent experiments. Geometrical mean is the distribution parameter given by the FACS machine and is obtained by multiplying the "n" individual values of a cluster together and getting the nth root of this product.

a cytosolic single-chain antibody (sc-007) against active PtpA were infected with WT *M. tuberculosis*. Together, these results demonstrate that PtpA is required to establish stable infections in macrophages.

Although it has been shown that PtpA is secreted in different mycobacterial strains both in vitro (Koul et al., 2000) and in vivo (Bach et al., 2006), transit of PtpA into the host cytosol requires passage through the host phagosome membrane. We have clearly demonstrated that PtpA is able to overcome this barrier, as it is present in the macrophage cytosol where it interacts with the host cytosolic protein VPS33B. One report suggests that mycobacterial proteins whose subunit size does not exceed 70 kDa are able to cross the phagosomal membrane (Teitelbaum et al., 1999); as PtpA is 18 kDa, this supports our data.

Usually, microorganisms engulfed by phagocytes are translocated from phagosomes to lysosomes in a process termed phago-lysosome fusion. This is a tightly regulated process involving membrane budding, targeting of vesicles with specific markers, and fusion of distinct compartments of the endocytic pathway. As macrophages infected with WT-PtpA-coated beads caused a pronounced inhibition of phago-lysosome fusion, we conclude that PtpA interferes with this process. This theory is supported by the identification of VPS33B as the host substrate of PtpA. VPS33B is a member of the Class C complex (Banta et al., 1988) ubiquitously expressed in eukaryotic cells and is essential for vesicle trafficking (Huizing et al., 2001). Mutations in this protein cause ARC syndrome, a human genetic disorder causing impaired kidney and liver function followed by early death (Gissen et al., 2004), and the carnation eye-color mutant in *Drosophila* (Sevrioukov et al., 1999). The yeast ortholog of VPS33B, Vps33p, is an ATP-binding protein (Gerhardt et al., 1998) required for vacuole morphogenesis and linked to trafficking of proteins from the Golgi to vacuoles (Banta et al., 1990).

We demonstrated that PtpA colocalizes in vivo with VPS33B when macrophages were infected with live, but not killed bacteria, underlining the need for active secretion (Figure 3A). Electron microscopic analyses of immunolabeled proteins clearly show colocalization of PtpA with VPS33B in the host cytosol (Figure 3B). Furthermore, we demonstrated colocalization of PtpA with VPS16 (Figure 3D), a protein which has been shown to interact with the VPS33B homolog in yeast (Rieder and Emr, 1997). These results suggest that PtpA is recruited to the Class C complex of proteins and interferes directly with vesicular trafficking in the infected macrophage.

Macrophages infected with WT-PtpA-coated beads were impaired in their ability to initiate phagolysosomal fusion in comparison with those given beads coated with BSA or catalytic-defective PtpA. However, siRNA-mediated silencing of VPS33B rendered macrophages incapable of differentiating between the two, although both were phagocytosed with equal efficiency (Figure S11). The loss of VSP33B impairs this process. As expected, when recombinant WT-VPS33B was exogenously delivered into silenced macrophages, WT-PtpA-coated beads regained their ability to arrest phago-lysosome fusion. These findings demonstrate that VPS33B is required for phagosome delivery to the lysosome. To validate these observations, we exchanged predicted phosphorylable tyrosine residues with glutamate by site-directed mutagenesis. As substitution of tyrosine for glutamate mimics constitutive phosphorylation, we expected that this quadruple mutant of VPS33B would be constitutively active, as well as resistant to PtpA activity. Indeed, VPS33B-silenced macrophages complemented with mutant VPS33B showed exacerbated phago-lysosome fusion compared to macrophages expressing WT-VPS33B when infected with

WT-PtpA-coated beads. This is in accordance with the predicated scenario in which the constitutively active mutant VSP33B is rendered resistant to PtpA activity and the resulting impairment of phagolysosomal fusion. Together, these results provide strong evidence that PtpA impairs phagolysosomal fusion in *Mtb*-infected macrophages by desphosphorylation of VPS33B.

To our knowledge, PtpA is the first *M. tuberculosis* enzyme shown to interact directly with an identified host protein substrate. This interaction provides evidence of an *M. tuberculosis* protein disrupting macrophage signaling. PtpA joins two other *M. tuberculosis* proteins that have been linked to phagosome maturation arrest, including a protein kinase, PknG (Cowley et al., 2004; Walburger et al., 2004), with a yet unidentified substrate, and the lipid phosphatase SapM (Vergne et al., 2005). Together, these results imply that *M. tuberculosis* uses multiple molecular mechanisms in parallel to block phagosome maturation in macrophages.

EXPERIMENTAL PROCEDURES

Cells and Antibodies

M. tuberculosis strain H37Rv was grown in 7H9 medium supplemented with 0.05% Tween-80 (Fisher) (7H9T) and 10% OADC (Becton Dickinson). *M. smegmatis* mc^2155 was utilized for protein overexpression and cultured in 7H9T medium supplemented with 1% glucose and 50 μg/ml hygromycin (Calbiochem). THP-1 cells were grown in RPMI 1640 (Sigma) supplemented with 1% L-glutamine, 10 mM HEPES, 100 μg/ml Streptomycin, 100 U/ml Penicillin (Stem Cell Technologies), 0.1% Fungizone (Invitrogen), and 10% fetal calf serum (FCS) (Sigma). Cells were incubated at 37°C supplemented with 5% CO_2. Polyclonal antibodies against VPS33B were kindly provided by Dr. C. MacKintosh (Protein Phosphorylation Unit, University of Dundee, UK). Polyclonal antibodies against PtpA were used according to published protocols (Cowley et al., 2002), and secondary antibodies were obtained commercially.

Construction and Isolation of *M. tuberculosis ptpA* Deletion Mutant

The *ptpA* deletion mutant was constructed by allelic replacement and was isolated by a sequential two-step selection protocol as described earlier (Papavinasasundaram et al., 2005).

Mass Spectrometry Analyses

Resolved proteins were digested and bands from SDS-PAGE were analyzed by MALDI-TOF (Voyager-DE STR work station, Laboratory of Molecular Biophysics, University of British Columbia, Canada).

Substrate Trapping and SDS-PAGE

Substrate trapping was carried out by incubation of a THP-1 lysate with either recombinant wild-type PtpA, Cys11Ala, or Asp126Ala mutants at 4°C overnight. The mixture was purified using Ni-NTA resin (QIAGEN) and resolved in SDS-PAGE.

Immunoprecipitation of Immunocomplexes

Recombinant Asp126Ala-PtpA was incubated overnight with a THP-1 lysate at 4°C and then incubated a further 2 hr with αPtpA polyclonal antibodies. The immunocomplexes were purified (Prosep-G, Millipore), resolved by 10% SDS-PAGE, and immunoblotted onto a nitrocellulose membrane. The membrane was blocked with 3% BSA/PBS, incubated with αVPS33B polyclonal antibodies followed by incubation with secondary antibodies, and developed using chemiluminescence (Pierce).

Immunofluorescence Microscopy

Bacteria were prepared and labeled with Alexa 555 (Invitrogen) as described elsewhere (Bach et al., 2006). Macrophages were infected with bacteria at a multiplicity of infection (moi) of 1:10, fixed with 2.5% *p*-formaldehyde, and permeabilized with saponin (Bach et al., 2006). Confocal images were taken with a Zeiss Confocal Microscope model LSM510 Meta (Bioimaging Facility, University of British Columbia, BC, Canada). Images were collected using the filters required for excitation of the respective secondary antibodies (488 and 633 nm) as well as for bacterium visualization (561 nm). Confocal images were processed with the ImageJ program.

Single-Chain Antibodies

Tomlinson I+J single-chain Fv antibody (scFv) libraries were kindly supplied by Geneservice, Cambridge, UK. Recombinant active PtpA was utilized as antigen for screening. Selected genes coding for scFv-αPtpA were amplified by PCR using oligonucleotides designed with *Xba*I (forward) and *Hind*III (reverse) restriction sites. The amplified genes were subsequently cloned into pMAL-2X (NEB, MA), and amplified again as fused genes to *mal*E (Bach et al., 2001) containing *Nhe*I (forward) and *Hind*III (reverse) restriction sites. *mal*E fused to the scFv genes were cloned into pDSRed2-N1 (Clontech). scFv-αbovine ubiquitin was provided by Geneservice, Cambridge, UK.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, 15 supplemental figures, and one supplemental table and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/3/5/316/DC1/.

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