

# *Mycobacterium tuberculosis* protein tyrosine phosphatase (PtpA) excludes host vacuolar-H<sup>+</sup>-ATPase to inhibit phagosome acidification

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Edited by Carl F. Nathan, Weill Medical College of Cornell University, New York, NY, and approved October 21, 2011 (received for review June 8, 2011)

*Mycobacterium tuberculosis* (Mtb) pathogenicity depends on its ability to inhibit phagosome acidification and maturation processes after engulfment by macrophages. Here, we show that the secreted Mtb protein tyrosine phosphatase (PtpA) binds to subunit H of the macrophage vacuolar-H<sup>+</sup>-ATPase (V-ATPase) machinery, a multisubunit protein complex in the phagosome membrane that drives luminal acidification. Furthermore, we show that the macrophage class C vacuolar protein sorting complex, a key regulator of endosomal membrane fusion, associates with V-ATPase in phagosome maturation, suggesting a unique role for V-ATPase in coordinating phagosome-lysosome fusion. PtpA interaction with host V-ATPase is required for the previously reported dephosphorylation of VPS33B and subsequent exclusion of V-ATPase from the phagosome during Mtb infection. These findings show that inhibition of phagosome acidification in the mycobacterial phagosome is directly attributed to PtpA, a key protein needed for Mtb survival and pathogenicity within host macrophages.

phagocytosis | vesicular trafficking

The etiological agent of tuberculosis (TB), *Mycobacterium tuberculosis*, is one of the most devastating infectious agents in the world. One-third of the world's population is exposed to Mtb, which causes nearly 3 million deaths annually, and is expected to cause an estimated 1 billion new infections by 2020 (1). Mtb primarily infects alveolar macrophages that provide the first line of defense against microbial invasion. Normally, macrophages' engulfment of foreign bodies results in the formation of phagosome, which matures in a process that remodels its membrane and luminal contents through interaction and fusion with the endosomal network (2, 3). These membrane fusions allow the phagosome to acquire antimicrobial properties, including a profoundly acidic lumen, the hallmark of the macrophage maturation process (4).

Macrophages acidify the phagosomal lumen by recruiting V-ATPase, a multisubunit protein-pump complex that actively transports protons across membranes using energy from ATP hydrolysis (5). Structurally similar to ATP synthase, the V-ATPase consists of a membrane-bound domain with five different subunits and a cytosolic domain composed of subunits A–H (reviewed in ref. 6). Delivery of the V-ATPase to the phagosome results in a pH decrease from 6.5 to 5.0 within minutes of phagosome maturation (7). This acidic environment inhibits bacterial growth and enhances the activities of antimicrobial hydrolases. Acidic pH is also crucial for proper vesicular trafficking, directing the fusion of phagosomes with lysosomes or vesicles harboring antimicrobial molecules (8). Therefore, phagosome acidification is a critical event that prompts the destruction of invading particles into constituents for antigen presentation and the initiation of adaptive immune responses (9).

Intracellular pathogens that enter host cells through the phagocytic or endocytic pathway have developed mechanisms to counter the acidic environment within phagosomes. For instance, *Salmonella enterica* adapts to lower pH by activating acid tolerance genes (10). *Yersinia pseudotuberculosis* blocks phagosome acidification by directly inhibiting V-ATPase activity in mouse

macrophages (11), and *Legionella pneumophila* secretes substrates into the host phagocyte to inhibit vacuole acidification through interaction with V-ATPase subunit A (12). In the case of Mtb, lack of acidification in the mycobacterial phagosome is mainly because of the absence of the V-ATPase on the phagosomal membrane (13). However, the mechanism by which Mtb accomplishes this remains undefined.

Mtb is known to be capable of sensing engulfment by macrophages and subsequently interferes with host signaling pathways to promote its intracellular survival (14–16). Mtb possesses a wide repertoire of signal transduction systems, including 11 two-component systems, 11 eukaryotic-like serine/threonine protein kinases (PknA–PknL), two protein tyrosine phosphatases (PtpA and PtpB), and the newly identified protein tyrosine kinase (PtkA) (17–19). These signaling proteins play key roles in bacterial adaptation and response to host defense mechanisms. PtpA, a secreted protein phosphatase, is essential for Mtb pathogenicity, participating in the arrest of phagosome maturation within the host macrophages (14, 20). Earlier, we identified the host vacuolar protein sorting 33B (VPS33B) as the cognate substrate of PtpA (18). VPS33B is a member of the class C VPS complex that regulates membrane fusion within the endocytic pathway (21). PtpA dephosphorylation of VPS33B inactivates this host protein, leading to inhibition of phagosome-lysosome fusion (14).

In this work, we report that Mtb PtpA binds to subunit H of macrophage V-ATPase to block V-ATPase trafficking and phagosome acidification. We further identified a unique role for V-ATPase in the process of phagosome-lysosome fusion. Our results demonstrated that Mtb success in inhibiting phagosome acidification and establishing infection in host macrophages relies on PtpA.

## Results

**Mtb PtpA Binds Subunit H of Human V-ATPase.** Previously, we used a substrate-trapping mutant of PtpA to pull down the catalytic substrate of PtpA, VPS33B, from the Mtb-infected THP-1 cell lysate (14). Interestingly, when we used the WT recombinant PtpA as bait, we were able to pull down another, previously unidentified, 55-kDa macrophage protein (14). We identified this protein by MALDI-TOF mass spectrometry to be subunit H of human V-ATPase (Fig. S1A) and verified its identity by Western blot analysis (Fig. 1A). In vitro kinase assay and phosphoamino acid analysis showed that subunit H was phosphorylated on threonine and could not serve as a catalytic substrate for the tyrosine phosphatase PtpA (Fig. S1B and C). In vitro protein-protein interaction analysis of PtpA and subunit H demonstrated direct contact between mycobacterial PtpA and host V-ATPase subunit H (Fig. 1B and C). A hyperbolic curve fitting

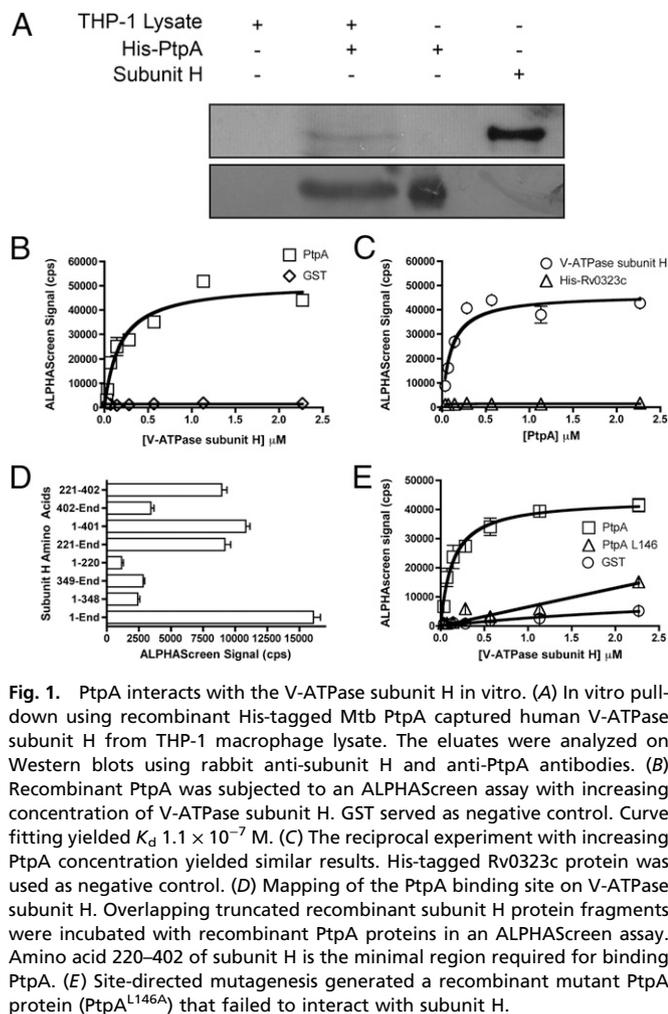
Author contributions: D.W., H.B., Z.H., and Y.A.-G. designed research; D.W. and J.S. performed research; D.W., Z.H., and Y.A.-G. analyzed data; and D.W. and Y.A.-G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1109201108/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1109201108/-DCSupplemental).



**Fig. 1.** PtpA interacts with the V-ATPase subunit H in vitro. (A) In vitro pull-down using recombinant His-tagged Mtb PtpA captured human V-ATPase subunit H from THP-1 macrophage lysate. The eluates were analyzed on Western blots using rabbit anti-subunit H and anti-PtpA antibodies. (B) Recombinant PtpA was subjected to an ALPHAScreen assay with increasing concentration of V-ATPase subunit H. GST served as negative control. Curve fitting yielded  $K_d$   $1.1 \times 10^{-7}$  M. (C) The reciprocal experiment with increasing PtpA concentration yielded similar results. His-tagged Rv0323c protein was used as negative control. (D) Mapping of the PtpA binding site on V-ATPase subunit H. Overlapping truncated recombinant subunit H protein fragments were incubated with recombinant PtpA proteins in an ALPHAScreen assay. Amino acid 220–402 of subunit H is the minimal region required for binding PtpA. (E) Site-directed mutagenesis generated a recombinant mutant PtpA protein (PtpA<sup>L146A</sup>) that failed to interact with subunit H.

the 1:1 Langmuir binding model and a dissociation constant of  $K_d$   $1.1 \times 10^{-7}$  M indicate a high level of affinity between the two proteins.

**PtpA Binds to Amino Acids 220–402 of Subunit H.** We determined the PtpA binding site on subunit H by constructing overlapping polypeptide fragments of subunit H. As shown in Fig. 1D, only fragments that included the linker region (amino acid 220–402) were able to bind PtpA with high affinity. This finding is consistent with previous predictions that the linker region of subunit H is involved in protein–protein interactions (22).

**The C-Terminal  $\alpha$ -Helix of PtpA Binds Subunit H.** Subunit H was reported to interact with dileucine-based motifs on the HIV Nef protein (23). Therefore, we performed site-directed mutagenesis to investigate whether dileucine motifs or other leucine residues on PtpA participate in binding subunit H (Fig. S24). One mutant, PtpA<sup>L146A</sup>, located in the C-terminal  $\alpha$ -helix, was defective in binding subunit H (Fig. 1E). This finding and the previous findings are consistent with the predicted structure of PtpA bound to subunit H using the protein-docking algorithm 3D-Garden (Fig. S2 B and C) (24). Interestingly, the PtpA<sup>L146A</sup> mutant retained its phosphatase activity with kinetics similar to the WT protein (Fig. S2D). This result is in line with our observation that subunit H is not a catalytic substrate but rather a binding partner of PtpA.

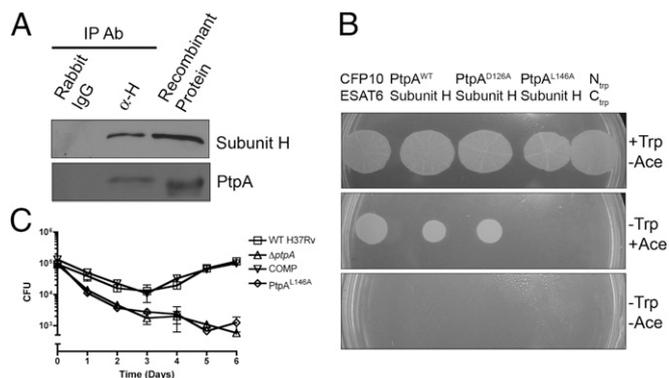
**PtpA Binds V-ATPase Subunit H in Vivo.** Using immunoprecipitation, we investigated the in vivo interaction between PtpA and subunit

H during macrophage infection. Antibodies against subunit H coimmunoprecipitated PtpA from lysates of THP-1 cells infected with H37Rv Mtb expressing WT PtpA, confirming the physiological relevance of PtpA/subunit H interaction during Mtb infection (Fig. 2A).

To further monitor PtpA interaction with subunit H, we used a modified split-Trp mycobacterial assay for detecting protein–protein interactions (25, 26). In this system, a tryptophan auxotrophic strain of *Mycobacterium smegmatis* only grows in the absence of tryptophan if the tested proteins interact with each other. As shown in Fig. 2B, coexpression of subunit H and PtpA fusion proteins restored mycobacterial growth on 7H9 agar plates, confirming protein–protein interaction within a surrogate host. Coexpression with the binding-defective PtpA<sup>L146A</sup> did not lead to mycobacterial growth, suggesting that this phosphatase-active PtpA mutant lost its ability to bind subunit H. The phosphatase-defective PtpA<sup>D126A</sup> retained its subunit H binding ability, further indicating the interaction is independent of the catalytic activity of PtpA (Fig. 2B).

**PtpA Binding to Subunit H Is Required for Mtb Intracellular Survival.**

To test whether PtpA binding to subunit H is required for Mtb pathogenicity, we investigated the ability of the binding-defective PtpA<sup>L146A</sup> Mtb strain to survive in human THP-1 macrophages. As shown in Fig. 2C, a  $\Delta$ ptpA strain complemented with a construct encoding PtpA<sup>L146A</sup> was attenuated within the macrophage in a manner similar to that of the  $\Delta$ ptpA knockout strain, whereas the parental and the complement strains were able to establish a stable infection after 3 d. Expression and stability of the WT and mutant PtpA proteins in these strains were confirmed with Western blot analysis (Fig. S34). At 6 d after infection, the binding-defective strain showed a 2-log reduction in cfu compared with the WT strain. Though the parental and complemented strains entered into growth phase within the macrophage, the  $\Delta$ ptpA and the binding-defective strains were continually cleared, establishing the importance of PtpA interaction with the V-ATPase machinery for Mtb survival within macrophages.



**Fig. 2.** PtpA and V-ATPase subunit H interact in vivo. (A) PtpA coimmunoprecipitated with subunit H from THP-1 macrophages infected with Mtb-expressing PtpA. Immunoprecipitation (IP) was performed with either rabbit anti-subunit H ( $\alpha$ -H) antibodies (Ab) or rabbit IgG as negative control. (B) PtpA and subunit H interacted in the split-Trp protein fragment complementation assay to facilitate N<sub>trp</sub> and C<sub>trp</sub> reassembly required for Trp biosynthesis, thus enabling growth of the *M. smegmatis* Trp<sup>-</sup> strain coexpressing N<sub>trp</sub>-PtpA and subunit H-C<sub>trp</sub> under acetamide (ACE) induction (Middle). N<sub>trp</sub>-PtpA<sup>L146A</sup> failed to interact with subunit H-C<sub>trp</sub> to restore *M. smegmatis* growth. N<sub>trp</sub>-ESAT6 and CFP10-C<sub>trp</sub> were used as a negative control. The negative control consisted of N<sub>trp</sub> and C<sub>trp</sub> fragments alone. (Top) Transformed strains are capable of growing on Trp-supplemented media. (Bottom) No growth is observed in the absence of acetamide induction and exogenous Trp. (C) Loss of PtpA binding to V-ATPase subunit H impairs Mtb survival within host macrophages. V-ATPase binding-defective PtpA<sup>L146A</sup>-expressing Mtb strain showed a 2-log reduction in bacterial load compared with the WT strain at 6 d postinfection.

**PtpA Inhibits Phagosome Acidification.** The in vivo PtpA/subunit H interaction and the impaired intracellular survival of the binding-defective PtpA<sup>L146A</sup> strain suggest that PtpA interferes with the phagosome acidification process. To examine this hypothesis, we used FACS to analyze the pH of Mtb-containing phagosomes. Parental and mutant strains were dually labeled with the pH-sensitive pHrodo fluorescent dye and the pH-insensitive Alexa Fluor 488 while undergoing phagocytosis by THP-1 macrophages. Covalent labeling of the bacteria with fluorescent dyes does not affect PtpA secretion (Fig. S3B). Overlaid FACS histograms showed a clear increase in the mean pHrodo fluorescence intensity for phagosomes harboring  $\Delta ptpA$  Mtb,  $\Delta ptpA$  complemented with the phosphatase-inactive  $ptpA^{D126A}$ , and  $\Delta ptpA$  complemented with the binding-defective  $ptpA^{L146A}$  (corresponding to pH 5.8, 5.6, and 5.55, respectively), compared with phagosome containing parental and complemented Mtb (corresponding to pH 6.7 and 6.4, respectively) (Fig. 3 A and B, Fig. S4, and Table S1). These results indicate a direct functional role for PtpA in the inhibition of Mtb phagosome acidification, which is dependent on both the binding ability and phosphatase activity of PtpA.

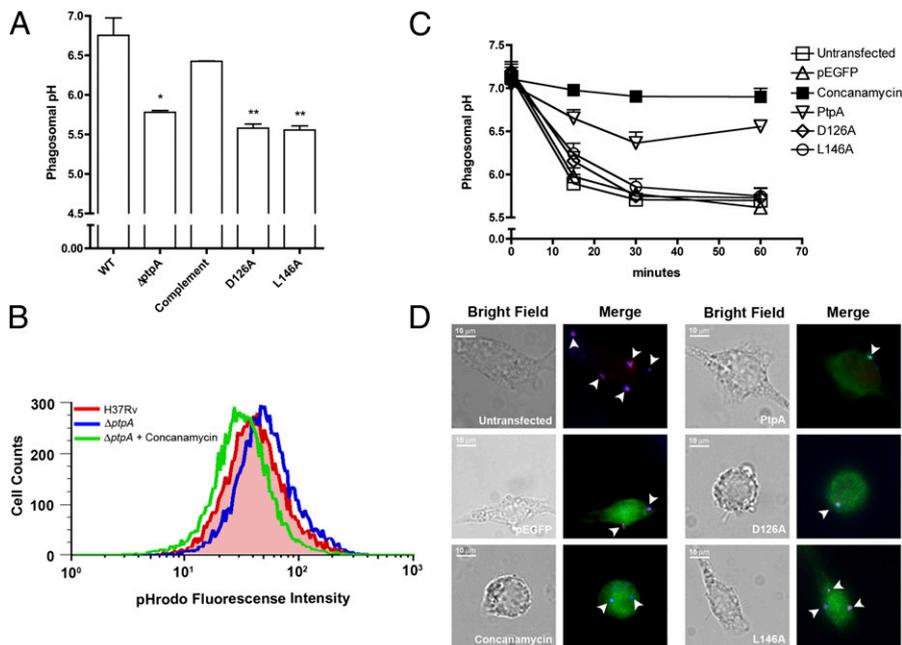
To examine PtpA's ability to modulate phagosome acidification independently of other Mtb proteins, we transfected THP-1 macrophages with GFP-fused constructs of parental and mutated forms of PtpA in a mammalian vector. All constructs were expressed at similar levels, and the survival and phagocytic ability of the macrophages were not impaired by the transfection (Fig. S5 A and B). PtpA-transfected macrophages were infected with *Escherichia coli* labeled with pHrodo and Alexa Fluor 350, and the phagosomal pH was assessed by spectrofluorometry (Fig. S5C). Expression of PtpA within THP-1 macrophages inhibited acidification of *E. coli*-containing phagosomes, whereas both catalytically inactive PtpA<sup>D126A</sup> and binding mutant PtpA<sup>L146A</sup> were unable to block phagosome acidification, showing a marked decrease in phagosomal pH similar to untransfected and empty vector control macrophages (Fig. 3C). Digital confocal microscopy of the infected macrophages confirmed these findings (Fig. 3D and Fig. S5D). These results established that PtpA alone can effectively prevent phagosome acidification in the macrophage. This inhibition depends on both PtpA phosphatase activity and its ability to bind to host V-ATPase machinery.

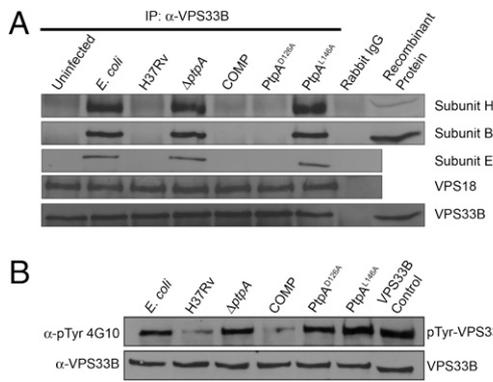
**V-ATPase Machinery Recruits the Class C VPS Complex.** The interaction of PtpA with both subunit H and the previously described VPS33B (14) suggested that the V-ATPase and class C VPS complexes might be in close proximity or even directly interact during phagosome maturation and endosome-lysosome fusion. To investigate this hypothesis, we conducted immunoprecipitation experiments in THP-1 macrophages using antibodies against VPS33B as bait and probed for subunits H, B, and E of the V-ATPase complex and VPS18 of the class C VPS complex by Western blot. In uninfected THP-1 macrophages, faint bands corresponding to subunits of the V-ATPase complex were captured with VPS33B and VPS18 when a higher amount of lysate was used (Fig. S6A). This finding suggests that V-ATPase interacts transiently with the class C VPS to regulate normal endosome-lysosome fusion in resting cells. Furthermore, this interaction is up-regulated upon phagocytosis of *E. coli*, as demonstrated by the prominent bands of V-ATPase subunits captured 2 h post-infection (Fig. 4A and Fig. S6A), further supporting that the class C VPS complex interaction with V-ATPase has a key role in phagosome maturation and fusion with lysosome.

**Mtb PtpA Blocks the Interaction Between Class C VPS and V-ATPase Complexes.** When THP-1 macrophages were infected with Mtb H37Rv, subunit H, B, or E of the V-ATPase machinery was not captured using antibodies against VPS33B (Fig. 4A). Mtb inhibition of V-ATPase and class C VPS interaction is maintained 24 h postinfection (Fig. S6B). However, the V-ATPase subunits coimmunoprecipitated with VPS33B and VPS18 in THP-1 macrophages infected with  $\Delta ptpA$ . Complementation of the  $\Delta ptpA$  mutant strain with WT  $ptpA$  restored the ability of Mtb to inhibit class C VPS interaction with the V-ATPase complex. Furthermore, complementation with  $ptpA^{D126A}$ , but not  $ptpA^{L146A}$ , restored Mtb inhibition of class C VPS and V-ATPase interaction (Fig. 4A). These results indicate that Mtb infection disrupted the V-ATPase and class C VPS association; PtpA is responsible for blocking this interaction; and PtpA binding to subunit H is specifically necessary for this disruption.

**VPS33B Remains Phosphorylated in THP-1 Macrophages Infected with Binding-Defective PtpA.** VPS33B phosphorylation is required for phagosome-lysosome fusion, and Mtb PtpA dephosphorylation of VPS33B blocks this process (14). Because the class C VPS complex is recruited to the V-ATPase, we asked whether PtpA

**Fig. 3.** Mtb PtpA inhibits phagosome acidification. (A) The indicated Mtb strains were labeled with pH-sensitive fluorescent dye (pHrodo) and used to infect THP-1 macrophages. Phagosomal pH of the infected macrophages was measured with FACS. WT Mtb maintained a phagosomal pH of ~6.7, whereas phagosomes of both  $\Delta ptpA$  and PtpA<sup>L146A</sup> strains were acidified to ~pH 5.5. \**P* < 0.05; \*\**P* < 0.01 (significant difference compared with H37Rv by Student *t* test). (B) Overlaid FACS histograms of the pHrodo fluorescence intensity of the WT H37Rv and  $\Delta ptpA$  phagosomes show a shift in phagosomal pH in the presence of PtpA. (C) THP-1 macrophages were transfected with GFP-tagged constructs of WT and mutant PtpA proteins and infected with pHrodo-labeled *E. coli*. Phagosomal pH was measured with spectrofluorometry during the course of infection. WT PtpA inhibits *E. coli* phagosome acidification (pH 6.5), whereas the binding-defective PtpA<sup>L146A</sup> failed to block acidification (pH 5.6). Concanamycin was added as a positive control for inhibition of phagosome acidification. (D) Digital confocal microscopy of the infected macrophages in C confirms the inhibition of phagosome acidification by Mtb PtpA. Green, expression of GFP or GFP-tagged PtpA constructs; red, pHrodo fluorescence in acidified phagosomes; blue, Alexa Fluor 350-labeled *E. coli*.

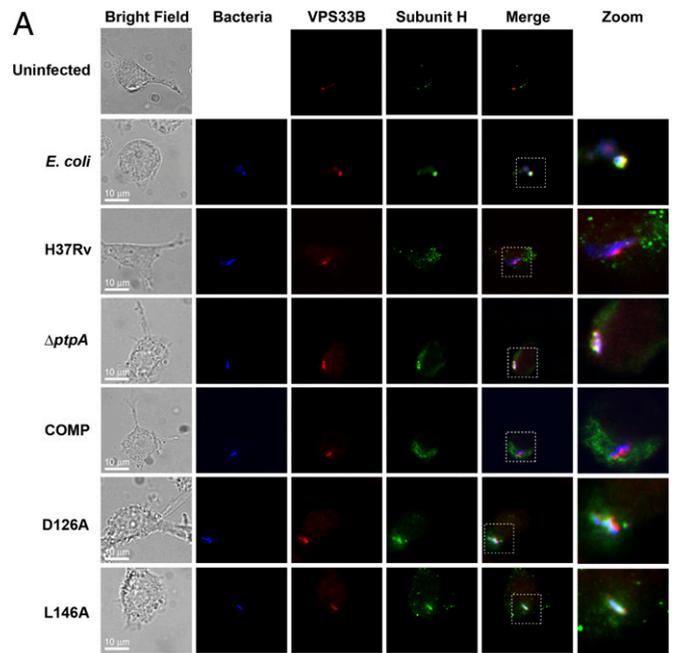




**Fig. 4.** Mtb PtpA disrupts the interaction between the class C VPS and V-ATPase complexes during infection. Immunoprecipitation was performed with anti-VPS33B ( $\alpha$ -VPS33B) using 2 mg of soluble lysate from THP-1 macrophages infected with the indicated strain for 2 h and followed by Western blot analysis with the indicated antibody. Rabbit IgG was used as negative control. (A) The class C VPS (VPS33B and VPS18) interacts with the V-ATPase complexes (subunits B, H, and E) from THP-1 macrophages infected with *E. coli*,  $\Delta$ ptpA, and PtpA<sup>L146A</sup>. The parental H37Rv, PtpA<sup>D126A</sup>, and the complemented strain disrupted the host proteins interaction. (B) Western blot analysis of VPS33B phosphorylation in vivo. VPS33B remained phosphorylated in macrophages infected with the PtpA<sup>L146A</sup>-expressing strain. (Upper) Probed with antiphosphotyrosine antibody 4G10. (Lower) Probed with anti-VPS33B to ensure equal loading.

binding to V-ATPase subunit H affects the phosphorylation status of VPS33B during infection. To address this question, we immunoprecipitated VPS33B and analyzed its phosphorylation with the antiphosphotyrosine antibody 4G10. When *E. coli* was used to infect macrophages, VPS33B was phosphorylated (Fig. 4B). However, as expected, when macrophages were infected with Mtb, down-regulation of VPS33B phosphorylation was observed. The  $\Delta$ ptpA strain did not reduce VPS33B phosphorylation, and this was reversed by complementation with WT ptpA. Interestingly, VPS33B remained phosphorylated in macrophages infected with  $\Delta$ ptpA complemented with either the phosphatase-defective ptpA<sup>D126A</sup> or the binding-defective ptpA<sup>L146A</sup>, despite being catalytically active (Fig. 4B). This finding suggests that the binding of PtpA to subunit H of V-ATPase is a prerequisite for VPS33B dephosphorylation, which is consistent with our findings that the loss of interaction with subunit H renders PtpA non-functional within the host cell.

**PtpA Binding to Subunit H Participates in the Exclusion of the V-ATPase from the Mtb Phagosome.** Disruption of the interaction between class C VPS and V-ATPase by PtpA suggests that PtpA directly impairs V-ATPase trafficking to the Mtb phagosome. To check the effect of PtpA on V-ATPase trafficking, we used digital confocal microscopy to monitor class C VPS and V-ATPase localization within infected macrophages, represented by VPS33B and subunit H, respectively. As illustrated in Fig. 5A, VPS33B and V-ATPase subunit H colocalized to the phagosome of macrophages infected with *E. coli*. In contrast, whereas Mtb-infected macrophage phagosomes were decorated with VPS33B, subunit H was excluded, indicating a lack of V-ATPase recruitment. However, the  $\Delta$ ptpA knockout strain exhibited colocalization of VPS33B and subunit H on the mycobacterial phagosomal membrane. Complementation with WT ptpA reversed this pattern with a similar percentage of phagosomes lacking V-ATPase as the WT strain (Fig. 5B). Corecruitment of subunit H and VPS33B to the bacilli-containing phagosomes was not observed despite complementation with either the catalytically inactive ptpA<sup>D126A</sup> or the binding-defective ptpA<sup>L146A</sup>. Therefore, PtpA binding to subunit H is needed for blocking V-ATPase trafficking to the Mtb phagosome, leading to the inhibition of Mtb phagosome acidification.



**Fig. 5.** Confocal microscopy of THP-1 macrophages infected with *E. coli* or indicated Mtb strains. (A) Localization of VPS33B and V-ATPase subunit H was detected with immunofluorescence staining. V-ATPase was excluded from the Mtb phagosome in macrophages infected with the WT strain, whereas the  $\Delta$ ptpA phagosome acquired V-ATPase. The phagosomes of the binding-defective PtpA<sup>L146A</sup>-expressing strain also failed to exclude host V-ATPase. (B) Quantification of the confocal data shown in A. Values are the mean  $\pm$  SD of phagosome colocalization with V-ATPase subunit H in 50–80 cells from three independent experiments. \*\*\* $P$  < 0.001 (significant difference compared with H37Rv by Student  $t$  test).

Although we have shown that the antibodies against VPS33B and subunit H are highly specific (Fig. S7), to overcome any potential cross-reactivity, we also performed double transfection of the THP-1 macrophages with GFP and DsRed2-fused constructs of subunit H and VPS33B, respectively, allowing direct visualization of host proteins' localization within the macrophage. Colocalization of GFP-subunit H and DsRed2-VPS33B in uninfected macrophages provides further support that V-ATPase and class C VPS functions in the same pathway to regulate normal endosome–lysosome fusion. When the transfected macrophages were infected with the same Mtb strains, similar patterns of subunit H and VPS33B localization was observed compared with the immunofluorescent-stained macrophages (Fig. S8). This result further supports that PtpA binding to subunit H is needed for the exclusion of V-ATPase from the phagosome.

## Discussion

More than a decade ago, Sturgill-Koszycki et al. (13) showed that macrophages fail to acidify phagosomes containing mycobacteria because these phagosomes did not accumulate the V-ATPase responsible for phagosomal acidification. This finding suggested inhibition of the fusion between membrane vesicles harboring

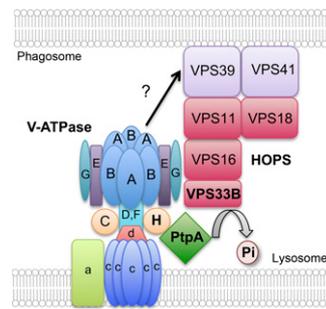
the V-ATPase complex and the mycobacterial phagosome (13). Despite the lack of a mechanistic explanation, the exclusion of the V-ATPase complex during Mtb infection has become a long-established paradigm. Here, we show that the lack of phagosome acidification is directly attributed to the Mtb-secreted protein PtpA, which specifically inhibits V-ATPase trafficking to the mycobacterial phagosome during phagosome maturation.

The exact mechanism by which PtpA is secreted across the phagosomal membrane remains unclear. However, using electron microscopy, neutralizing antibodies, and Western blot analysis, we have previously shown that PtpA is present in the host cytosol milieu (14, 27). There is evidence suggesting that bacterial proteins <70 kDa in molecular size can cross the phagosomal membrane (28). This observation might be related to the more recent discovery that the ESX-1 secretion system with its substrate ESAT-6 can activate the inflammasome and perturb host cell membrane to facilitate the translocation of mycobacterial proteins into the macrophage cytosol (29, 30). Further investigation is needed to elucidate the mechanistic details of PtpA secretion.

We have shown previously that Mtb PtpA dephosphorylates the host macrophage protein VPS33B, a key regulator of membrane fusion, leading to inhibition of phagosome–lysosome fusion (14). In this study, we identified an additional key partner to the process, subunit H of the host V-ATPase machinery. The interaction with V-ATPase subunit H and dephosphorylation of VPS33B are both required for PtpA inhibition of macrophage phagosome–lysosome fusion and phagosome acidification. We were able to identify a catalytically active mutant, PtpA<sup>L146A</sup>, which does not interact with subunit H. Because of its predicted location within the core region of PtpA (Fig. S2 A and B), this mutation most likely alters the conformation of the entire C-terminal  $\alpha$ -helix, leading to defective binding. The loss of either PtpA phosphatase activity or subunit H binding renders PtpA nonfunctional within the host cell.

During phagosome maturation, lysosomal V-ATPase is directly recruited to the phagosomal membrane for luminal acidification (3). The class C VPS complex has been implicated in this process as the membrane-tethering factor. Although studies of *Drosophila* neurons and mammalian renal medulla cells have shown interaction between subunits of V-ATPase and SNARE proteins (31, 32), our studies of PtpA in Mtb demonstrate a direct interaction between the class C VPS and V-ATPase. We further showed that this mechanism is not limited to phagosome maturation, because the same interaction could be detected, albeit at a weaker level, in uninfected macrophages. This finding suggests that class C VPS and V-ATPase interacts transiently in the resting cell to regulate normal endosome–lysosome fusion, and this association is likely up-regulated during phagocytosis to deliver lysosomal contents to the phagosome. Our finding provides compelling evidence that V-ATPase is a key player in the membrane fusion machinery, particularly in cooperating with the class C VPS complex to tether the fusing phagosomal or endosomal membrane with the lysosome (Fig. 6). Disruption by Mtb PtpA further exemplifies the importance of this interaction in host defense mechanism.

With these results, our current model suggests a two-step process for PtpA exclusion of V-ATPase and inactivation of VPS33B. During Mtb infection, PtpA binding to subunit H may first disrupt initial membrane tethering and localize it to the proximity of its catalytic substrate VPS33B (Fig. 6). Subsequent dephosphorylation of VPS33B would then inactivate the entire membrane fusion machinery and its downstream effectors, preventing delivery of V-ATPase to the mycobacterial phagosome. This mechanism would explain the failure of the phosphatase-defective mutant PtpA<sup>D126A</sup>, which retains the capability of binding to V-ATPase and disrupting host complex associations, to shut down the host macrophage pathway completely. Our observation that VPS33B remains phosphorylated in both the phosphatase-defective and binding-defective strain supports such a two-step process, suggesting that PtpA binding to subunit H is



**Fig. 6.** A model for the specific exclusion of V-ATPase and the inhibition of mycobacterial phagosome acidification by PtpA. During infection, the V-ATPase recruits the class C VPS complex, possibly associated with VPS39 and VPS41 as the HOPS complex, aiding the tethering of the fusing phagosomal and lysosomal membranes. PtpA, secreted into the host cytosol by Mtb, binds to subunit H of the V-ATPase complex, disrupting the interaction between the two protein complexes and localizing itself near its catalytic substrate, VPS33B. PtpA then dephosphorylates and inactivates VPS33B, thereby shutting down the membrane fusion machinery. Binding to subunit H, therefore, allows PtpA to specifically inhibit V-ATPase trafficking to the mycobacterial phagosome.

a prerequisite for VPS33B dephosphorylation. This phenomenon is also supported by our confocal data, where both the PtpA<sup>D126A</sup> and PtpA<sup>L146A</sup> strains failed to inhibit V-ATPase trafficking to Mtb phagosomes.

However, the mycobacterial phagosome is not an isolated compartment; rather, it interacts extensively with early endosome to acquire nutrients, such as iron-bound transferrin, to maintain survival within the host cell (33). Vesicle fusion in the endocytic pathway depends on two membrane-tethering protein complexes, class C core vacuole/endosome tethering (CORVET) and homotypic fusion and protein sorting (HOPS) (34, 35). The class C VPS complex serves as the core of both CORVET and HOPS complexes through reversible association with CORVET-specific (VPS3 and VPS8) and HOPS-specific (VPS39 and VPS41) accessory subunits, which mediate early-to-late endosome fusion events and fusions with lysosome, respectively (reviewed in ref. 36). Our results suggest that V-ATPase may specifically interact with the HOPS complex during phagosome–lysosome fusion (Fig. 6). Therefore, by binding to the V-ATPase, PtpA could specifically localize to the phagosome–lysosome interface, and CORVET-mediated early endosome fusions with the phagosome will remain intact. This specific localization mechanism would then allow PtpA to distinguish HOPS from CORVET, thereby specifically excluding V-ATPase to inhibit mycobacterial phagosome acidification. In fact, a link between the V-ATPase and HOPS complexes was observed by a previous study demonstrating that the HOPS-specific subunit VPS41 failed to function in yeast that had mutations in the V-ATPase complex (37). This finding suggests that the HOPS-specific subunits VPS39 and VPS41 might be direct effectors of the V-ATPase complex during phagosome–lysosome fusion (Fig. 6), and the CORVET complex might be recruited to the phagocytic pathway through a different mechanism. As the HOPS-specific subunits are known to interact with activated Rab7 during endosome maturation (38), it is likely that Rab7 is the upstream activator of V-ATPase association with class C VPS complex. Further experiments will be needed to fully characterize these host cellular pathways in the context of mycobacterial infection. Nonetheless, our study clearly demonstrates that the absence of V-ATPase and the lack of mycobacterial phagosome acidification are directly attributed to the Mtb protein tyrosine phosphatase PtpA.

## Materials and Methods

**In Vitro Pull-Down Assay.** In vitro pull-down assay with recombinant His-tagged PtpA was performed as described previously (14).

**ALPHAScreen Protein–Protein Interaction Assay.** An assay using the ALPHAScreen Histidine (Nickel Chelate) Detection Kit was performed according to the manufacturer's protocol (Perkin-Elmer). Purified recombinant protein was biotinylated using the EZ-Link Biotinylation Kit (Pierce) according to the manufacturer's protocol.

**Mycobacterial Split-Trp Protein–Protein Interaction Assay.** DNA encoding PtpA and subunit H were cloned into pJC10 and pJC11 vectors, generating translational fusion constructs with the N<sub>trp</sub> and C<sub>trp</sub> fragments, respectively, of the *N*-(5'-phosphoribosyl)-anthranilate isomerase under the control of the acetamidase promoter. The split-Trp assay (26) was carried out as described previously (25).

**Infection of THP-1.** THP-1 human-derived monocytes (ATCC; TIB-202) were grown in RPMI medium 1640 (Sigma) supplemented with 10% FBS, 1% L-glutamine (StemCell). THP-1 cells ( $1.0 \times 10^6$  or  $5.0 \times 10^5$ ) were seeded onto 12- or 24-well tissue culture plates, respectively, and differentiated with 40 ng/mL 12-phorbol 13-myristate acetate in RPMI 1640 supplemented with 10% FBS and 1% L-glutamine. Infection of THP-1 macrophages was performed using human serum-opsonized Mtb or *E. coli* DH5 $\alpha$  at a multiplicity of infection (MOI) of 10:1. For Mtb intracellular survival studies, infected macrophages (MOI 1:1) were harvested at defined time points, lysed with 0.025% SDS, serially diluted, and plated on 7H10 agar medium supplemented with appropriate antibiotics.

**Immunofluorescence Microscopy.** THP-1 macrophages were seeded on coverslips at  $5.0 \times 10^5$  cells/well in 24-well tissue culture plates. Infection of THP-

1 macrophages and immunostaining was performed as described previously (14, 39) (more details in *SI Materials and Methods*).

**Measurement of Phagosomal pH.** Assessment of the phagosomal pH of *E. coli* and Mtb phagosomes was performed according to previous literature (40, 41). Bacteria were labeled with the pH-sensitive pHrodo probe (Invitrogen), which emits red fluorescence in acidic environment, and the indicated pH-insensitive probes [Alexa Fluor 350 (Blue) and Alexa Fluor 488 (Green); Invitrogen] according to established protocols (41). Phagosomal pH was measured with either spectrofluorometry in the Fusion-Alpha HT Microplate Reader (Perkin-Elmer) or with FACS in the FACSCalibur Flow Cytometer (BD Bioscience). The calculated fluorescence ratios of the pH-sensitive and pH-insensitive probes were used to determine phagosomal pH according to a calibration curve. pH calibration was performed by incubating infected THP-1 macrophages in 10 mM phosphate-citrate buffer of predetermined pH (5.0–8.0) containing 145 mM KCl, 1 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>, 10 mM glucose, 20  $\mu$ M nigericin, and 4  $\mu$ M monensin (Figs. S4 and S8). Concanamycin (50 nM) was added to the macrophages to inhibit V-ATPase proton transport in the indicated samples (more details in *SI Materials and Methods*).

**ACKNOWLEDGMENTS.** We thank the British Columbia Centre for Disease Control for providing access to a containment level 3 facility and Mary Ko and Joseph Chao for technical assistance. D.W. is a recipient of the Canadian Institutes of Health Research Canada Graduate Scholarships Doctoral Award. This study was funded by Canadian Institute of Health Research Operating Grant MOP-106622 (to Y.A.-G.).

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