Protein tyrosine phosphatase PtpA is not required for Mycobacterium tuberculosis growth in mice

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Abstract

Mycobacterium tuberculosis (Mtb) alters the host response to infection by secreting protein factors. Mtb produces two secreted protein tyrosine phosphatases, PtpA and PtpB, which are thought to interfere with host signaling. Deletion of ptpA or ptpB attenuates bacterial growth in activated macrophages. To address the in vivo function of PtpA, we generated a genetic deletion mutant, ΔptpA. The mutant was not defective when grown in vitro, consistent with the presumed role of PtpA in the host. The ptpA mutant, however, also showed no growth defect in a mouse infection model. The absence of a growth defect in mice suggests that the requirement for PtpA differs in mouse and human infections, and that mice are not a suitable infection model for the study of PtpA.

Introduction

The Mycobacterium tuberculosis (Mtb) protein tyrosine phosphatases (PTPs) PtpA and PtpB are thought to manipulate host cell signaling (Koul et al., 2000). Consistent with functions within host cells, no cognate tyrosine kinase has been identified in the Mtb genome. Moreover, PtpA and PtpB are secreted proteins (Koul et al., 2000). Deletion of ptpA attenuates bacterial growth in human macrophages (Bach et al., 2008), and deletion of ptpB reduces Mtb growth in activated mouse macrophages and guinea pigs (Singh et al., 2003). Taken together, these data support the hypothesis that the Mtb PTPs play crucial roles in mycobacterial survival during infection.

Sequence analysis and the crystal structure identify PtpA as a low molecular weight (LMW) PTP (Madhurantakam et al., 2005). PtpA shows 37% sequence identity and high structural similarity (Cα root mean square deviation = 0.99 Å) to the human LMW PTP, but there are notable differences in active-site residues of these enzymes. PtpA overexpression in murine macrophages leads to slight changes in actin polymerization and decreases phagocytosis (Castandet et al., 2005). Vacuolar protein sorting protein 33B (VPS33B), was recently identified as a human substrate of PtpA. These data implicated PtpA in the manipulation of normal vesicle maturation (Bach et al., 2008), but the physiologic functions of PtpA are poorly understood.

The potential role of PtpA as a secreted virulence factor, akin to the Yersinia PTP YopH (Viboud & Bliska, 2005), has prompted attempts to design specific PtpA inhibitors (Weide et al., 2003; Manger et al., 2005; Nören-Müller et al., 2006). Targeting secreted proteins that are important for infection outside of the bacterium circumvents bacterial resistance mechanisms and eliminates the need to traverse the bacterial cell envelope, a particularly difficult hurdle for the treatment of Mtb infection. This interest in PtpA as an inhibitor target magnified the importance of establishing the effects of attenuating PtpA activity in vivo.

Here we examine the effects of a ptpA deletion on Mtb growth in vitro, and in the mouse model of Mtb infection. We find that the ptpA deletion mutant grows like the parental wild-type strain in all of these conditions, implying that the murine model fails to recapitulate effects reported in infections of human macrophages (Bach et al., 2008).

Materials and methods

The ΔptpA deletion mutant was generated by homologous recombination using a specialized transducing phage (Glickman et al., 2000). The ptpA gene was replaced by a
hygromycin expression cassette, preserving the 8-bp overlap of the upstream Rv2232 gene with the PtpA ORF. The allelic exchange substrate was constructed by amplifying the flanking regions of *ptpA* by PCR from *Mtb* genomic DNA. The flanking regions were amplified to contain restriction sites for SpeI and HindIII at the 5’ and 3’ end of the upstream flank, and AflII and KpnI sites for the downstream flanking region, cloned with the TA cloning kit (Invitrogen), and sequenced. The flanking regions were cloned successively upstream and downstream of the hygromycin expression cassette of pJSC407, also containing a λ cos site and a unique PacI site. The PacI-digested pJSC407 was ligated into the unique PacI site of phAE87, a temperature-sensitive mutant of the mycobacteriophage TM4, and packaged using the Gigapack XL kit (Stratagene). High titer transducing phages were prepared in *Mycobacterium smegmatis* at 30 °C and used to infect *Mtb* at 37 °C. Recombinants were selected on medium containing hygromycin, and correct insertion was confirmed by Southern blot analysis using alkaline transfer onto Hybond N membrane (Amersham). Radioactive probes for Southern blotting were prepared with Ready To Go DNA labeling beads (Pharmacia).

All strains were cultured in 7H9 medium containing 0.05% Tween 80, 10% oleic acid albumin dextrose catalase, and 0.5% glycerol. The *in vitro* growth rate was monitored by measuring the cultures at OD600 nm. For aerosol mouse infections, bacteria were grown to mid-log phase, sonicated using a Branson Sonifier 250 at 90% power for 15 s, spun for 5 min at 50 g to pellet clumpy bacteria, and diluted to the desired inoculum in phosphate-buffered saline (PBS). Female C57BL/6 mice were infected via nebulization for 15 min using a custom built aerosolization chamber (Mechanical Engineering Shops, University of Wisconsin, MA). For CFU assays, the upper lobe of the right lung, the right lobe of the liver, and half of the spleen were harvested and homogenized in PBS containing 0.1% Tween-80. The homogenates were plated in serial dilutions on 7H10 plates supplemented with 10% oleic acid albumin dextrose catalase, 0.5% glycerol, 100 μg mL⁻¹ ampicillin and 50 μg mL⁻¹ cycloheximide, and colonies were counted after 3.5 weeks. Mice were housed under pathogen-free conditions, and mouse experiments were conducted according to a University of California, San Francisco, Institutional Animal Care and Use Committee-approved protocol.

**Results and discussion**

To explore the roles of PtpA in growth *in vitro* and in a mouse infection model, we generated a *ptpA* deletion mutant in the virulent Erdman strain of *Mtb* by allelic exchange (Glickman *et al*., 2000). Southern blotting of mutant and wild-type genomic DNA confirmed the correct insertion of the hygromycin-resistance cassette into the PtpA site (Fig. 1). Replacement of *ptpA* by the gene coding for hygromycin produced a 600-bp larger Xbal fragment when compared with the wild-type chromosome. A probe specific for the region directly upstream of *ptpA* (probe A) hybridized to a 2.6-kb fragment in genomic wild-type DNA and a 3.2-kb fragment in the mutant genomic DNA. Insertion of the hygromycin-resistance cassette was also detected by Southern

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**Fig. 1.** Chromosomal replacement of the *ptpA* gene with a hygromycin-resistance cassette by homologous recombination. Southern blot showing the correct insertion of the hygromycin cassette. (a) Recombination generates the predicted Xbal fragment of 3.2 kb in the *ptpA* deletion strain compared with the wild-type Erdmann parent. (b) A probe for *ptpA* generates a 2.6-kb fragment only in the parent. (c) A probe for the hygromycin-resistance cassette generates a 3.2-kb fragment only in the mutant.
blotting using a hygromycin-specific probe (probe C). The expected 3.2-kb fragment was detected in the ΔptpA mutant, but not the wild-type sample. Conversely, a 2.6-kb fragment was detected in the wild type, but not the mutant sample using a probe specific for ptpA (probe B).

No difference in the growth rates of the mutant and the wild-type strains was observed in liquid 7H9 culture medium (Fig. 2a). The ptpA deletion mutant showed a slightly higher propensity to form clumps in liquid culture, suggesting that PtpA might have an effect on cell surface properties. This qualitative difference did not lead to a difference in growth rates, suggesting that PtpA does not have a vital function in intrinsic bacterial signaling.

To test the effect of PtpA on Mtb survival in vivo, we infected female C57BL/6 mice with wild-type and the ptpA deletion mutant by aerosol infection. Between 200 and 250 bacteria were deposited in the lungs of infected animals, as determined by a CFU assay of whole lungs of five animals per group on the day of infection. At weeks 1, 2, 3, 5, and 10 after infection, groups of five mice were sacrificed and lungs, livers, and spleens were harvested for CFU assays. In the lung, the numbers of bacteria recovered were similar for wild type and the ptpA deletion mutant (Fig. 2b). Mtb dissemination to the liver and the spleen was observed starting at week two after infection. No difference in bacterial loads in liver and spleen were observed between wild-type- and ΔptpA-infected mice (Fig. 2c and d).

Several lines of evidence point to a role of PtpA in the manipulation of the host response to Mtb infection. The lack of an in vitro growth phenotype of the PtpA deletion mutant also supports this notion. Surprisingly, no growth defect was observed in mice, raising questions about the true in vivo function of PtpA. Cowley et al. (2002) showed increased expression of a PtpA-GFP fusion upon infection of the human macrophage-like cell line THP-1 and Bach et al. (2008) showed reduced virulence of a ptpA deletion mutant in THP-1 cells. These results preclude the possibility that the lack of an in vivo phenotype in the deletion mutant results from a lack of PtpA expression even in wild-type Mtb during infection.

Instead, the mouse model may not faithfully reveal the role of PtpA in human tuberculosis. A number of factors could contribute to the absence of an in vivo growth defect in mice. The human substrates of PtpA, for example, might not be conserved in mice. Alternatively, higher levels of reactive oxygen and nitrogen species in mouse macrophages compared with human cells (Liu et al., 2007) would be expected to selectively reduce the role of PtpA by oxidizing the catalytic cysteine to levels higher than that found in a human infection. In the absence of full wild-type PtpA function, differences caused by the ptpA deletion mutant might be obscured. PtpB, on the other hand, resists oxidative inactivation, because the active site is buried in the protein (Grundner et al., 2005).

Although the normal growth characteristics of the ptpA deletion mutant in vitro and in mice provide little insight into the in vivo functions of PtpA, the results suggest that the mouse is not an appropriate infection model for the study of PtpA function. Further development of PtpA inhibitors hinges on the discovery of a suitable animal model in which to assay for the attenuation of PtpA activity.

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**Fig. 2.** Growth of ptpA deletion strain compared with the parental wild-type Erdmann strain. (a) In vitro growth of wild-type Mtb and ΔptpA mutant in 7H9 medium. (b) CFU recovered from lungs of mice infected with wild-type Erdmann and ptpA mutant. (c, d) CFU recovered from spleen and liver. The ptpA deletion does not affect in vitro or in vivo growth of Mtb. Error bars indicate the SD of data from five animals.
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References


