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CHARACTERIZATION OF A NOVEL ACETYLTRANSFERASE FOUND ONLY IN PATHOGENIC STRAINS OF *MYCOBACTERIUM TUBERCULOSIS*

by

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A DISSERTATION

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MICROBIOLOGY

ABSTRACT

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is still a major threat to the human population. Roughly one third of the world population is asymptomatically infected and two million people die each year of TB. Effective treatments are available as a multi-drug regimen, but there is an ever increasing problem with the rise of multi-drug resistant strains that newer drug targets and a better understanding of how *M. tuberculosis* affects the host need to be elucidated. The primary target of *M. tuberculosis* in the human host is the macrophage where *M. tuberculosis* secretes virulence factors that inhibit various signal transduction pathways in order for intracellular survival. One such virulence factor is MPtpB, a protein tyrosine phosphatase. Tyrosine phosphorylation is an important factor in regulating various host cell processes. To better understand how MPtpB affects the host, we wanted to determine the targets of MPtpB in the macrophage. We also looked at what other virulence factors of *M. tuberculosis* MPtpB might interact with to help increase its virulence upon the macrophage. MPtpB was shown to interact with enhanced intracellular survival (Eis) protein. To delineate the function of Eis, a BLAST search was performed and revealed Eis to have a putative acetyltransferase site. We determine that Eis is capable of acetylating free histones but not histones found in a nucleosomal complex. Eis was also shown to inhibit the phosphatase activity of MPtpB *in vitro* using pNPP as a substrate.

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CHARACTERIZATION OF AN ACETYLTRANSFERASE AND ITS INTERACTION WITH A TYROSINE PHOSPHATASE IN *MYCOBACTERIUM TUBERCULOSIS*

LIST OF ABBREVIATIONS

AAC	aminoglycoside N-acetyltransferase
AANAT	arylalkylamine N-acetyltransferase
AcCoA	acetyl coenzyme A
AgNAT	aminoglycoside N-acetyltransferase
Ara-LAM	arabinose lipoarabinomannan
CaMKII	calmodulin-dependent protein kinase
CDC	Centers for Disease and Control
CR	complement receptor
DSP	dual-specificity phosphatase
EEA1	early endosomal antigen 1
Eis	enhanced intracellular survival
FOA	5-fluoroorotic acid
GNAT	GCN5-related N-acetyltransferases
HAT	histone acetyltransferase
HRP	horseradish peroxidase
IFN-γ	Interferon-γ
IL	Interleukin
JAK/STAT	Janus kinase/signal transducer and activator of transcription
LAMP1	lysosome-associated membrane protein 1
LB	Luria-Broth

LMW	low-molecular weight
Man-LAM	mannose lipoarabinomannan
MAPK	mitogen-activated protein kinases
MBP	myelin basic protein
MDR	multi-drug resistant
PAMPs	pathogen-associated molecular patterns
PIM	phosphatidylinositol monomannoside
PI3K	phosphatidylinositol-3-kinase
PI3P	phosphatidylinositol-3-phosphate
PMSF	phenylmethylsulfonyl fluoride
PTPases	protein tyrosine phosphatases
ROS	reactive oxygen species
SC	synthetic complete
SK	sphingosine kinase
SNAT	serotonin N-acetyltransferase
S1P	sphingosine-1-phosphate
TACO	tryptophan aspartate-containing coat protein
TB	tuberculosis
TLRs	Toll-like receptors
TNFR2	tumor-necrosis factor receptor type 2
TNF-α	tumor-necrosis factor α
WHO	World Health Organization
XDR	extensively drug resistant

INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis, is considered to be a major player in the death of millions of people each year. Death by *M. tuberculosis* is exacerbated with the ever growing widespread of AIDS in the world. Treatment of tuberculosis consists of a multidrug regimen of antibiotics which must be administered faithfully over a long period of time. Unfortunately, these drugs are not completely effective, because the mycobacterium has developed resistance against most of the antibiotics used for the treatment of tuberculosis. These drug resistant mycobacterium are classified as either multidrug resistant (MDR) or extensively drug resistant (XDR) strains. There is an ever growing need for better treatments, diagnosis and prevention due to these new strains. In order to meet these needs, there needs to be a better understanding of the host-pathogen interaction, because our knowledge of how *M. tuberculosis* enters the host cell, inhibits host defenses and infects neighboring cells is lacking.

For *M. tuberculosis* to survive in the host, it has developed ways to circumvent the hostile environment of the macrophage. These include both suppressing the antibacterial response and anti-apoptotic response and also arresting the maturation of the phagosome. *M. tuberculosis* has been shown to secrete virulence factors which help it evade these various host processes in order to survive and replicate. One such secreted virulence factor is MPtpB, a protein tyrosine phosphatase.

MPtpB has been shown to exhibit protein tyrosine phosphatase activity, but there are no known tyrosine kinases in the *M. tuberculosis* genome (2, 5). When the *mptpB*

gene was deleted from *M. tuberculosis*, the mutant strain caused accelerated mycobacterial cell death in the lung and spleen of guinea pigs (8). This would suggest that MPtpB is an important virulence factor that plays a role in dephosphorylating host proteins to allow for the mycobacterium to survive.

Another virulence factor from *M. tuberculosis* is enhanced intracellular survival (Eis). Eis was shown to significantly enhance intracellular survival of *Mycobacterium smegmatis* containing *eis* in the human macrophage cell line U937 (3, 9). Eis has also been shown to modulate the secretion of IL-10 and TNF-α in primary human monocytes in response both to infection with *M. tuberculosis* and to stimulation with recombinant Eis protein (7).

In this dissertation, the first chapter discusses the findings of MPtpB. I showed that MPtpB was capable of being secreted from the non-pathogenic strain *M. smegmatis* when expressed in an episomal plasmid, indicating that the machinery involved in secreting the phosphatase was conserved in both strains. I also determined by domain mapping the region of MPtpB that is involved in its secretion, since MPtpB lacks a known secretion signal peptide.

The next chapter demonstrates the interaction between MPtpB and Eis. I found that MPtpB interacted with Eis in a yeast two hybrid screen of a *M. tuberculosis* genome library and proved its interaction by building an operon with both MPtpB and Eis and expressing them in *M. smegmatis* and performing immunoprecipitations on the lysates. Eis is capable of acetylating free histones but not histones found in a nucleosomal complex, indicating that Eis is indeed an acetyltransferase, but that histones are not its primary target. Also, to determine what role Eis might play on MPtpB, I showed that the tyrosine phosphatase activity of MPtpB was decreased when Eis was present indicating that

Eis might be playing some sort of regulatory role in keeping MPtpB in check when inside the mycobacterium.

CHARACTERIZATION OF A NOVEL ACETYLTRANSFERASE FOUND ONLY IN PATHOGENIC STRAINS OF *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is still a major threat to the human population. Roughly one third of the world population is asymptomatically infected and two million people die each year of TB. Effective treatments are available as a multi-drug regimen, but there is an ever increasing problem with the rise of multi-drug resistant strains that newer drug targets and a better understanding of how *M. tuberculosis* affects the host need to be elucidated. The primary target of *M. tuberculosis* in the human host is the macrophage where *M. tuberculosis* secretes virulence factors that inhibit various signal transduction pathways in order for intracellular survival. One such virulence factor is MPtpB, a protein tyrosine phosphatase. Tyrosine phosphorylation is an important factor in regulating various host cell processes. To better understand how MPtpB affects the host, we wanted to determine the targets of MPtpB in the macrophage. We also looked at what other virulence factors of *M. tuberculosis* MPtpB might interact with to help increase its virulence upon the macrophage. MPtpB was shown to interact with enhanced intracellular survival (Eis) protein. To delineate the function of Eis, a BLAST search was performed and revealed Eis to have a putative acetyltransferase site. We determine that Eis is capable of acetylating free histones but not histones found in a nucleosomal complex. Eis was also shown to inhibit the phosphatase activity of MPtpB *in vitro* using pNPP as a substrate.

INTRODUCTION

Mycobacterium tuberculosis is the main causative agent of tuberculosis (TB) and is a major cause of death in the world. According to the Centers for Disease and Control (CDC) and the World Health Organization (WHO), TB kills about two million people

annually, and approximately one-third of the world's population (or 1.7 billion people) are asymptomatically infected with *M. tuberculosis*. *M. tuberculosis* was the cause of the "White Plague" of the 17th and 18th centuries in Europe. The entire population of Europe was infected with TB and roughly a quarter of them died. Effective treatments are available and consist of a regimen of multiple drugs in order to prevent the emergence of tubercle bacilli resistance. A four drug regimen is highly effective and consists of the drugs rifampin, isoniazid, pyrazinamide and ethambutol or streptomycin which must be taken faithfully for an extended period of time. Even though effective treatments are available, there is an increasing dissemination of drug-resistant mycobacteria which indicates there is an increasing need for the development of new drug therapies to combat TB.

M. tuberculosis is a filamentous Gram-positive bacterium with complex surface lipids. There are two families of *Mycobacterium*: pathogenic and non-pathogenic. The non-pathogenic family consists of *Mycobacterium smegmatis* and *Mycobacterium vaccae*. These can be isolated from environmental sources such as soil and water. The pathogenic family causes TB in humans or in animals and consists of *M. tuberculosis* and *Mycobacterium bovis*. *M. tuberculosis* is an obligate pathogen and must live inside humans where the primary target cells are macrophages.

In order for pathogenic mycobacteria to survive inside macrophages, mycobacteria must be able to inhibit several host-cell processes, whereas non-pathogenic species are not capable. The host processes inhibited by pathogenic bacteria include the fusion of phagosomes with lysosomes, antigen presentation, apoptosis and the stimulation of bactericidal responses dues to the activation of pathways involving mitogen-activated protein kinases (MAPKs), Interferon-γ (IFN-γ) and calcium (Ca^{+2}) signaling (23).

In order for mycobacteria to affect phagosome maturation, mycobacteria need to gain entry into the macrophage. There are several cell-surface molecules, including members of the integrin family (i.e. complement receptors (CRs) 1, 3 and 4), mannose receptors and Fcγ receptors that mycobacteria utilize to gain entry into macrophages (12). Even though these receptors are important for internalization of *M. tuberculosis*, mycobacteria are still able to grow and survive intracellularly. Mycobacteria utilize other methods that protect them from subsequent attack by antimicrobial components in the phagosomal maturation pathway.

Phagosomal maturation involves a series of sequential fusion events with various vesicles from the endocytic pathway, by which nascent phagosomes attain microbicidal properties and become phagolysosomes. Phagolysosomes are acidic organelles rich in hydrolytic enzymes and digest engulfed bacteria and other ingested particles. Upon phagocytosis, the nascent phagosome (pH 7.4) acquires the GTPase Rab5 either from the plasma membrane or by fusion with early endosomes. In turn, Rab5 recruits phosphatidylinositol-3-kinase (PI3K), which generates phosphatidylinositol-3-phosphate (PI3P). PI3P recruits early endosomal antigen (EEA1) from endosomes. Rab5 and EEA1 direct the fusion of phagosomes with late endosomal vesicles. As the phagosome matures, Rab5 and EEA1 are lost and a second GTPase, Rab7 is acquired. These late phagosomes acquire lysosomal markers (i.e. lysosome-associated membrane protein 1 (LAMP1)), acid hydrolases (i.e. cathepsin D), and vacuolar proton-ATPase molecules which acidifies the phagolysosome (47). The entire phagosomal maturation process takes less than one hour.

When pathogenic mycobacteria are phagocytosed by macrophages, the mycobacteria are capable of preventing the phagosome from fusing with lysosomes. These phagosomes have a pH of 6.3 and do not undergo further acidification due to the absence of

proton-ATPase molecules from the vacuolar membrane, and this reduced level of acidification allows the intracellular survival and growth of mycobacteria (47). Vacuoles containing mycobacteria are characterized by the presence of certain cellular proteins on their membranes, including an actin-binding protein, tryptophane aspartate-containing coat protein (TACO) (13) and the small GTPase, Rab5 (11). Rab7 and EEA1 are not recruited to vacuoles containing pathogenic mycobacteria. Phagosomes containing non-pathogenic mycobacteria are unable to retain TACO and Rab5, allowing the phagosome to mature by recruiting EEA1 and Rab7 and thereby fusing with the lysosome. Pathogenic mycobacteria are able to survive and replicate in the mycobacterial phagosome, whereas non-pathogenic mycobacteria are readily killed in phagolysosomes.

Another host process that pathogenic mycobacteria are capable of inhibiting is Ca^{+2} and PI3K signaling. Intracellular changes of Ca^{+2} and PI3K activity are key regulators for maturation of phagosomes. Ca^{+2} concentration modulates the fusion between the phagosome and the lysosomal vesicles by regulating the activity of two Ca^{+2}-dependent effector proteins: calmodulin and calmodulin-dependent protein kinase (CaMKII), a multifunctional serine/threonine kinase (27). An increase in the intracellular concentration of Ca^{+2} leads to a change in the conformation of calmodulin, which in turn induces autophosphorylation and the subsequent activation of CaMKII (36). CaMKII is required for the recruitment of PI3K and EEA1 to the phagosomal membrane and for the regulation of bilayer fusion between endosomal vesicles (34). Inhibition of the increase in cytosolic Ca^{+2} concentration by *M. tuberculosis* is mediated by the lipid effector molecule mannose lipoarabinomannan (Man-LAM), which is able to inhibit ionophore-induced increases in Ca^{+2} concentration in macrophages (39). This inhibition is unique only to LAM from pathogenic mycobacteria and not from non-pathogenic mycobacteria, which consists of

arabinose-LAM (Ara-LAM). It was recently shown that *M. tuberculosis* prevents an increase in cytoplasmic Ca^{+2} concentration by the inhibition of a lipid kinase, sphingosine kinase (SK) (28). SK phosphorylates a host lipid, sphingosine, to form sphingosine-1-phosphate (S1P) (43), which is a ligand for specific G-protein coupled receptors and also regulates intracellular Ca^{+2} homeostasis by releasing Ca^{+2} from cytoplasmic organelles. Inhibition of SK activity is caused by live *M. tuberculosis* resulting in decreased production of S1P causing a reduced cytosolic Ca^{+2} concentration.

Pathogenic mycobacteria are also capable of altering the host apoptotic pathway through several mechanisms. Macrophages containing mycobacteria activate their apoptotic program to resolve the infection, although infection of macrophages with virulent strains of *M. tuberculosis* induces much lower levels of apoptosis than do infections with attenuated strains (19). As mentioned above, Man-LAM can also inhibit apoptosis by preventing the increase of cytosolic Ca^{+2} concentrations. It is thought that Ca^{+2} facilitates apoptosis by increasing the permeability of mitochondrial membranes, thereby promoting the release of pro-apoptotic elements such as cytochrome c (48). It has also been shown that Man-LAM stimulates the phosphorylation of the apoptotic protein Bad through activation of the Akt (protein kinase B) cascade (6, 26). Phosphorylated Bad is unable to bind to the anti-apoptotic proteins Bcl-2 and Bcl-X_L. Since Bcl-2 isn't bound by Bad, Bcl-2 prevents the release of cytochrome c from the mitochondria and is able to inhibit caspase activity. *M. tuberculosis* also limits macrophage apoptosis by inducing the production of the immunosuppressive cytokine, interleukin-10 (IL-10) (3). IL-10 blocks the synthesis of tumor-necrosis factor α (TNF-α), which is an apoptotic stimulator. When TNF-α is stimulated, it binds to the death receptors to activate the apoptotic program. To inhibit TNF-α activity, IL-10 induces the release of the soluble TNF receptor type 2 pro-

tein (TNFR2), which complexes with TNF-α making it inactive, thus preventing the induction of TNF-mediated apoptosis.

When invading bacteria are detected, the macrophage initiates the activation of various host-cell signaling cascades, such as the MAPK or Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways. This activation results in the production of pro-inflammatory cytokines and chemokines, such as IL-1, IL-6, TNF-α and interferons. Only pathogenic mycobacteria have developed ways to suppress these signal transduction cascades and thereby attenuate the cytokine-induced immune response. Macrophages infected with non-pathogenic mycobacteria activates MAPK signaling which leads to the synthesis of various microbicidal molecules, including TNF-α, whereas pathogenic mycobacteria suppress this host response by inhibiting the activation of p38 and ERK1/2 (37). By inhibiting p38, pathogenic mycobacteria have an enhanced survival rate.

JAK/STAT tyrosine phosphorylation is essential for an antibacterial response. When IFN-γ binds to its cell surface receptor, JAK1/2 and STAT become phosphorylated, which in turn activates a strong bactericidal response, such as the production of reactive oxygen and nitrogen intermediates, and the synthesis of cytokines, such as IL-12 and TNF-α (41). Pathogenic mycobacteria are able to suppress the IFN-γ and JAK/STAT signaling pathway by decreasing the levels of IFN-γ receptor, thereby inhibiting JAK/STAT phosphorylation and reduces the DNA-binding activity of STAT (18).

Toll-like receptors (TLRs) are another type of receptor that pathogenic mycobacteria utilize to suppress the immune response. TLRs are phylogenetically conserved receptors that recognize pathogen-associated molecular patterns (PAMPs) to establish innate immunity and activate immune cells against these microorganisms. TLRs are in-

volved in the production of inflammatory cytokines and they are also linked to the NF-κB and MAPK pathways. It is thought that TLR2 and TLR4 are involved in recognition of mycobacteria. Mycobacterial lipoproteins, such as phosphatidylinositol monomannoside (PIM), Man-LAM and the 19-kDa antigen, stimulate TLR2 to produce a pro-inflammatory response, which can either promote mycobacterial killing or induce apoptosis (2, 25, 49). Man-LAM is sufficient in the inhibition of the TLR signaling cascade and induction of secreting the anti-inflammatory and immunosuppressive cytokine IL-10. IL-10 has been shown to block the production of the pro-inflammatory cytokines IL-12 and TNF-α, and IL-10 is capable of reducing the expression level of MHC class II on the cell surface (35).

Interfering with antigen processing and presentation in macrophages is another way for pathogenic mycobacteria to survive. There are several different tactics that mycobacteria use to suppress antigen presentation: 1) sequestering mycobacterial antigens from molecules that are required for T-cell activation (32), and 2) downregulation of the expression of MHC class II molecules and co-stimulatory molecules like CD1 (29, 44, 52). An example of this type of interference is the 19-kDa lipoprotein, which can down-regulate the expression of MHC class II thereby interfering with the presentation of antigens (30, 31).

For bacterial pathogens to evade the host immune responses, many bacteria secrete virulence factors into the host causing modification of host proteins that would allow for the bacteria to survive in the host. Tyrosine phosphorylation is extremely important in eukaryotic cells. An example of tyrosine phosphorylation is reversible phosphorylation of tyrosine residues was shown to represent a key mechanism for the transduction of signals that regulate cell growth, differentiation, mobility, metabolism, and survival

(53). The level of phosphorylation on tyrosine residues required for normal cell function is maintained by the opposing actions of tyrosine kinases (46). For some bacteria, protein phosphorylation has been shown to play an important role in sensing extracellular signals by a pathogen and coordinating intracellular events (20). Protein tyrosine phosphatases (PTPases) have been identified as virulence factors in a number of bacterial species and are essential for their development and pathogenesis. For example, several pathogenic bacteria have tyrosine phosphatases that act as virulence factors: *Yersinia pseudotuberculosis*, *Salmonella enterica* serovar Typhimurium, and enterophatogenic *Escherichia coli*. The extracellular pathogen *Y. pseudotuberculosis* secretes the PTPase YopH. YopH has been shown to dephosphorylate host focal adhesion proteins, such as $p130^{cas}$, paxillin, and focal adhesion kinase. This dephosphorylation leads to the destabilization of focal adhesions that are involved in phagocytosis of bacteria (5, 33). This inhibition of phagocytosis by YopH allows the bacteria to replicate extracellularly and not be internalized by the macrophage. In the same way, when *S. typhimurium* is internalized, it uses a PTPase called SptP, which mediates the reversal of the actin cytoskeleton reorganization that is caused by bacterial entry. SptP interacts with two small GTPase-binding proteins, Cdc42 and Rac1 which restores the normal actin cytoskeletal architecture of the host cells despite the uptake of a large number of internalized bacteria (14).

The genome of *M. tuberculosis* codes for two PTPases, MPtpA (Rv2234) and MPtpB (Rv0153c), but no known tyrosine kinases have been found (9). Both MPtpA and MPtpB exhibit protein tyrosine phosphatase activity (22). Both contain the defining consensus sequences of all PTPases, the active site P loop motif, $CysX_5Arg$, and an essential Asp that functions as a general acid in the catalytic reaction (4). MPtpA is classified as a low-molecular weight (LMW) phosphatase, and MPtpBs sequence falls into the conven-

tional PTP or dual-specificity phosphatases (DSP) class. Since mycobacteria lack tyrosine kinases, it suggests that these phosphatases likely dephosphorylate components of the host cell signaling pathway. In order to support this idea, Koul et al. showed that MPtpB is secreted from *M. tuberculosis* into the culture medium, even though MPtpB lacks any known export signal peptide (22). Southern blot analysis revealed that *mptpB* was present only in the slow-growing species such as *M. tuberculosis* $H_{37}Rv$, *M. tuberculosis* $H_{37}Ra$ and *M. bovis* BCG but absent from the fast-growing species such as *M. smegmatis*. They also demonstrated that both phosphatases were able to dephosphorylate the phosphotyrosine residue of myelin basic protein (MBP), but that these two phosphatases were unable to dephosphorylate serine/threonine residues of MBP. Also, the pH optimum of 5.5 would allow MPtpB to function in low-pH compartments (i.e. phagosomes) of macrophages. When the *mptpB* gene was deleted from *M. tuberculosis*, the mutant strain caused accelerated mycobacterial cell death in the lung and spleen of guinea pigs (42). This group demonstrated that infection of guinea pigs with the mutant strain resulted in a 70-fold reduction in the bacillary load of spleens in infected animals six weeks after infection compared with the bacillary load in animals infected with the parental strain. In addition, wild-type and mutant *mptpB* strains were equally able to survive in resting macrophages, but the ability of *mptpB* mutants to survive in macrophages activated with IFN-γ was highly impaired. This is indicative that MPtpB might mediate mycobacterial survival in host cells by dephosphorylating proteins involved in the IFN-γ signaling pathways.

To determine the mechanisms of regulation and substrate recognition, Grundner et al. determined the 1.7 Å resolution crystal structure of MPtpB in complex with the product phosphate (15). Comparing MPtpB to other conventional PTPases revealed that

MPtpB is deeply diverged and simplified structurally. Even though MPtpB displays the signature core motif of all PTPase structures, which is a four-stranded parallel beta sheet that connects to an α-helix through the catalytic P-loop, MPtpB does not show significant similarity to other PTPases. MPtpB shows two unusual elaborations: a disordered, 31-residue acidic loop, and a flexible, two-helix lid that covers the active site, which are solely specific to mycobacterial orthologs. When the lid is closed, Phe222 protrudes into the active site, effectively blocking access to the active site cleft. This arrangement resembles the phosphotyrosine substrate position expected from the superposition of MPtpB with PTPase structures with substrates or inhibitors bound (15). This group hypothesized that MPtpBs lid opening and closing may be regulated in vivo. Due to the reactivity of the cysteine nucleophile in tyrosine phosphatases, PTPases are generally sensitive to regulation or inactivation by reactive oxygen species (ROS) (40, 50). It might be possible then that MPtpB might encounter ROS in the host macrophage. When treated with increasing amounts of H_2O_2, MPtpB showed extraordinary resistance to inactivation (15). For MPtpB to resist oxidative inactivation supports the idea that MPtpBs lid may work as a special filter that excludes ROS from the active site.

Another important secreted virulence factor protein involved in the intracellular survival of *M. tuberculosis* is enhanced intracellular survival (Eis, Rv2416c), even though Eis does not have a known export signal peptide. Eis from *M. tuberculosis* was shown to significantly enhance intracellular survival of *M. smegmatis* containing *eis* on a multicopy plasmid in the human macrophage cell line U937 compared to that of the vector control, ranging from 2.4- to 5.3-fold at both 24 and 48 hours after infection (51). This group demonstrated by Southern blot analysis that Eis is present only in the slow-growing species such as *M. tuberculosis* H_{37}Rv and *M. tuberculosis* H_{37}Ra but absent

from the fast-growing species such as *M. smegmatis*. Fractionation studies and immunoblot analyses performed on culture-grown *M. tuberculosis* found that the Eis protein appeared mainly in the cytoplasmic fraction but also in the membrane, cell wall and culture supernatant fractions as well (10). Other studies have shown that *eis* is differentially expressed in a clinical strain of *M. tuberculosis* upon infection of activated human macrophages (8). The promoter of Eis is a group A/SigA-like mycobacterial promoter that contains both a core promoter region and an upstream region required for transcriptional activity (38).

Although the function of Eis is unknown, BLAST analysis revealed that Eis is a putative member of the GCN5-related N-acetyltransferases (GNAT) superfamily. GNATs catalyze the transfer of the acetyl group from acetyl coenzyme A (AcCoA, the "donor") to a primary amine (the "acceptor). The GNAT superfamily can be subdivided into three functional families: serotonin N-acetyltransferase (SNAT), aminoglycoside N-acetyltransferases (AgNATs), and histone acetyltransferases (HAT). The first of the three family members, SNAT, also referred to as arylalkylamine N-acetyltransferases (AANAT) catalyzes the penultimate step in the biosynthesis of melatonin, a hormone proposed to play roles in circadian rhythms, and human mood and behavior (21). The circulating levels of melatonin are correlated with the light-dark cycle, with high levels of melatonin occurring only at night. The second family member, AgNATs, catalyze the regioselective acetylation of one of the four amino groups found on a diverse set of aminoglycosides with antibiotic properties. Acetylation reduces the affinity of these compounds for the acceptor tRNA site on the 30S ribosome by four orders of magnitude (24), effectively making pathogenic bacteria expressing these genes resistant to the antibiotic. These modifications are carried out by a class of bacterial aminoglycoside N-

acetyltransferases (AAC). The *M. tuberculosis* genome consists of an AAC, 2'-N-acetyltransferase (AAC(2')-IC). AAC(2')-IC was identified and shown to have aminoglycoside 2'-N-acetyltransferase activity with a broad range of 4,6-and 4,5-substituted aminoglycosides (1, 16). The last family member of the GNAT superfamily, HATs, are mainly involved in the acetylation of histones at specific lysine residues. HATs not only acetylate histones, but they are also capable of acetylating non-histone proteins, general transcription factors and the tumor suppressor p53 (45). HATs are able to acetylate the amino terminal "tails" of histones removing the positive charge, thus likely resulting in a weaker binding of the nucleosome core particles, composed of histones, to DNA. These loci are then free to interact with the complex transcriptional machinery, resulting in enhanced transcription and translation of protein products (7). The GNAT superfamily has four conserved motifs in the order C, D, A, and B. These four motifs are collectively known as the N-acetyltransferase domain. Motif C is involved in substrate recognition, and Motif D is thought to do the same. Both Motif C and D are the least conserved in the GNAT superfamily. The most conserved across the superfamily is in motifs A and B. Motifs A and B forms the essence of the AcCoA binding site with Motif A consisting of the AcCoA contacts and Motif B consisting of the catalytic region. In Motif B, a key tyrosine residue is important for AcCoA contact. One group showed the importance of this tyrosine by creating a Tyr-to-Phe mutation in AANAT and this mutation had a substantial negative effect on catalytic efficiency (17). Out of all the point mutations that were made in the vicinity of the AANAT active site, the effect of this mutation was the most detrimental.

In the present study, MPtpB is shown to interact with Eis using a yeast two hybrid analysis and in *M. smegmatis* containing a multicopy operon of MPtpB and Eis. Addi-

tionally, the interaction between MPtpB and Eis are mapped to determine the region of MPtpB that is involved for secretion into the culture filtrate, and to determine the region where MPtpB interacts with Eis. The function of Eis is also examined to determine its exact role as a member of the GNAT superfamily as well as to understand why a tyrosine phosphatase, MPtpB would want to interact with an acetyltransferase such as Eis.

MATERIALS AND METHODS

Cell lines. RAW 264.7 (ATCC TIB-71) and HEK 293T were cultured in DMEM medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 μM L-glutamine, 50 μM 2-mercapto-ethanol, 100 μg/ml streptomycin-penicillin, and 50 μg/ml gentamicin (Sigma, St. Louis, MO) at 37 °C under 5% CO_2.

Antibodies and reagents. For Western blot analysis, the following antibodies were purchased: mouse anti-FLAG mAb conjugated to horseradish peroxidase (HRP) (Sigma) and anti-phosphotyrosine mAb conjugated to HRP (4G10, Upstate Biotechnology Inc., Waltham, MA). Rat monoclonal anti-MPtpB antibody was generated by immunizing rats with intact recombinant MPtpB.

Bacterial strains, growth and preparation. *Mycobacterium smegmatis* (mc^2155) was cultured in Luria-Broth (LB) supplemented with 0.05% Tween-80. *M. smegmatis* transformed with various modified pMV762 plasmids were grown to mid-logarithmic phase growth (OD_{600} = 0.8-1.0) in LB supplemented with 50 μg/mL Hygromycin B (A.G. Scientific, San Diego, CA) and 0.05% Tween-80. Cells were centrifuged and resuspsended in lysis buffer containing protease inhibitors and phenylmethylsulfonyl fluoride (PMSF). Cells were lysed using a sonicator. The resulting lysate was centrifuged to pellet any non-lysed cells and cellular debris. For immunoprecipitations, FLAG

agarose beads (Sigma) were added to the lysates and rotated overnight at 4˚C. The beads were then washed four times in lysis buffer.

Plasmids. The cDNA encoding full-length MPtpB (Rv0153c) and Eis (Rv2416c) were PCR amplified from *M. tuberculosis* H37Rv genomic DNA using KOD high fidelity polymerase (Novagen, Madison, WI). For the pMV762 plasmid, MPtpB had a BamHI site included in the forward primer (5'-GAGAGGATCCGATGGCTGTCCGTGAACT-GCCGGGCGCG-3') and a 3X FLAG site and a HindIII site was included in the reverse primer (5'-GAGAGAATTCGGGTAACCCTATGCAGTCGTCGAGGAATTGCTA-TTA-3'). The MPtpB PCR product was digested with BamHI and HindIII restriction enzymes and then ligated into the BamHI and HindIII sites of the pMV762 plasmid. MPtpA had a NcoI site included in the forward primer (5'-GAGACCATGGGGGTGT-CTGATCCGCTGCACGTCACATTCGTTTGT-3') and a 3X FLAG site and a PstI site was included in the reverse primer (5'-GAGACTGCAGGGGTAACCCTATGCAGTC-GTCGAGGAATTGCTATTA-3'). The MPtpA PCR product was digested with NcoI and PstI restriction enzymes and then ligated into the NcoI and PstI sites of the pMV762 plasmid. For the pAS4 plasmid, MPtpB had a glycine linker and an EcoRI site included in the forward primer (5'-GAGAGAATTCGGAGGAGGAGGAGGAATGGCTGTCCG-TGAACTGCCGGGCGCG-3') and a PstI site was included in the reverse primer (5'-GAGACTGCAGTTATTATCCGAGCAGCACCCCGCGCATCCG-3'). The MPtpB PCR product was digested with EcoRI and PstI restriction enzymes and then ligated into the EcoRI and PstI sites of the pAS4 plasmid.

Site-directed mutagenesis. MPtpB was mutagenized to create either a substrate trapping mutant (D82A, GAC→GCA), or a catalytically inactive mutant (R166M, CGC→ATG).

Pervanadate treatment of RAW 264.7 cells and immunoprecipitation/Western blot assay. RAW264.7 cells ($1x10^7$ cells/sample) were treated for 30 min at 37˚C with 0.1 mM pervanadate (2 µl of a fresh solution containing 50 mM sodium metavanadate ($NaVO_3$) and 50 mM H_2O_2 was added to 1 ml of media). After stimulation, cells were immediately washed in ice-cold PBS to stop the reaction. Next, cells were washed twice in ice-cold PBS and lysed in 0.5 ml of lysis buffer (50 mM MOPS (pH 7.2), 150 mM NaCl, 0.0025% NaN_3, 10 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4, and 1% Nonidet P-40). A protease inhibitor mixture (Calbiochem, San Diego, CA) and PMSF were added to the lysis buffer just before use. Cell lysates were incubated for 30 min on ice and then centrifuged at 13,000 x g for 15 min at 4˚C. Cleared lysates were transferred to a new tube and incubated with *M. smegmatis* lysates expressing either MPtpB WT or MPtpB D82A substrate trapping mutant on FLAG agarose beads for various time points at 37˚C. Beads were washed four times in ice-cold lysis buffer followed by boiling in SDS-PAGE Sample Reducing Buffer. The proteins were separated by a 10% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked overnight at 4˚C in TBST with 3% Blot Qualified BSA (Promega, Madison, WI), and washed two times with TBST before the addition of the primary antibody, 4G10. Membranes were incubated for 1 hr at room temperature with the primary antibody diluted into TBST with 1% Blot Qualified BSA, and washed six times with TBST. Proteins of interest were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and subsequent exposure to autoradiographic film (Eastman Kodak Co).

Operons. MPtpB and Eis or fbpA (Rv3804c) were placed in an operon with MPtpB upstream of Eis. For MPtpB, a BamHI site was included in the forward primer

(5'-GAGAGGATCCGATGGCTGTCCGTGAACGGCCGGGCGCG-3') and a 3X FLAG and SpeI site were included in the reverse primer (5'-GAGAACTAGTGGGT-AACCCTATGCAGTCGTCGAGGAATTGCTATTA-3'). For Eis, a Shine-Delgarno sequence (ribosomal binding site) and SpeI site were included in the forward primer (5'-GAGAACTAGTGTGGCCGCGGCATATGCCACAGTCG-3') and a myc tag and ClaI site were included in the reverse primer (5'-GAGAATCGATTTATTACAGATCCT-CTTCTGAGATGAGTTTTTGTTCGAACTCGAACGCGGTCTGGACGGGAAC-3'). To determine the region of MPtpB that contains the export signal peptides, multiple deletions of MPtpB were created for this operon model. These MPtpB deletions are: Δ1-30 amino acids (AA); Δ31-60 AA; Δ61-90 AA; Δ91-120 AA; Δ121-150 AA; Δ151-180C AA; Δ181-210C AA; Δ211-240C AA; Δ241-276C AA and 117-218 AA, consisting only of the catalytic region.

Intracellular survival assay. RAW 264.7 macrophages were plated at a cell density of 5 x 10^5 cells/mL warm antibiotic free DMEM media in 12 well plates and allowed to adhere overnight at 37˚C. The following day, macrophages are washed three times with warm antibiotic free DMEM media and 400 µl media added back to each well. *M. smegmatis* expressing MPtpB:pMV762, MPtpB D82A:pMV762, MPtpB R166M:pMV762, MPtpA:pMV762 and pMV762 alone were centrifuged at top speed for 5 min and resuspended in appropriate volume of warm antibiotic free DMEM media and added at an MOI 10:1 (mycobacteria:macrophage) per well. Plate was rocked back and forth to mix and incubated at 37˚C for 4 hrs. Following incubation any *M. smegmatis* that was not phagocytosed were washed away three times in warm antibiotic free DMEM media. To lyse any extracellular mycobacteria not phagocytosed, 1 ml of warm DMEM plus antibiotics was added per well and incubated at 37˚C for 2 hrs. After 2 hrs, wash

three times and replace with warm DMEM plus antibiotics and incubate at 37˚C. Lyse macrophages at Day 0, 1, and 2 by adding 400 µl 1% Triton X-100 per well for 15 min at 37˚C. Pipet and scrape lysed macrophages and plate various dilutions onto LB plates containing 0.05% Tween 80 and incubate plates at 37˚C for 2-3 days. Count colonies and chart accordingly.

Yeast two hybrid assay. MPtpB was cloned into pAS4 containing an EcoRI site with an alanine linker in the forward primer and a PstI site in the reverse primer. MPtpB:pAS4 was transformed into the yeast strain p69A and selected on synthetic complete (SC) lacking Ura plates following the protocol in the Clontech Matchmaker manual. A mouse lung infected with *M. tuberculosis* library was transformed into MPtpB:pAS4 expressing yeast cells using the scaled-up protocol suggested in the Clontech Matchmaker manual. The cells were plated on SC lacking Ura, Leu, Ade and His + X-α-gal (20µg/ml, Glycosynth) plates and incubated at 30˚C. Colonies that grew and turned blue were transferred to 96-well plates containing SC lacking Leu media in order to drop out the bait. The colonies were replica plated to another 96-well plate containing SC lacking Leu media. Following growth of this plate, colonies were replica plated to SC lacking Leu + 5-fluoroorotic acid (FOA, 1 mg/ml) plates. Surviving colonies that lost the bait plasmid were replica plated to another 96-well plate containing SC lacking Leu media and then replica plated to SC lacking Leu + FOA plates to confirm the bait was lost. Those colonies that did not grow were transferred from the frozen glycerol stock plate to a new plate and mated with control plasmid pLAM (-Trp) in yeast strain p69α to eliminate false positives. These transformants were replica plated to SC lacking Trp and Leu plates and then to SC lacking Trp, Leu and Ade plates. Clones that did not grow were transferred from the frozen glycerol stock plate to SC lacking Leu media and allowed to

mate with MPtpB:pAS4 in yeast strain p69α. To verify positive interactions, the transformants were replica plated to SC lacking Ura and Leu plates and then to SC lacking Ura, Leu and Ade plates. Any colony that grew was lysed and sequenced using ABI Prism Big Dye Terminator reaction mix and an ABI Prism Sequencer 3700.

RESULTS

Mycobacterial PTPases are capable of being secreted from non-pathogenic strains. Both MPtpA and MPtpB were cloned into an episomal mycobacterial vector pMV762 and transformed into *M. smegmatis*. *M. smegmatis* transformants were grown to mid-log phase growth and culture supernatants collected to determine if these PTPases are capable of being secreted from non-pathogenic mycobacteria. To our surprise, both MPtpA and MPtpB were secreted from *M. smegmatis* indicating that non-pathogenic mycobacteria strains have the same export machinery as the pathogenic strains in secreting these PTPases (Figure 1). As seen in Figure 2, the growth curve for these transformed mycobacteria were the same as non-transformed mycobacteria indicating that the PTPases alone did not interfere with nor enhance the growth of *M. smegmatis*.

Role of mycobacterial PTPases in uptake and bactericidal killing. To determine if these secreted PTPases are capable of enhancing uptake or sufficient to reduce bactericidal killing in macrophages, an intracellular survival assay was performed. RAW 264.7 macrophages were infected with a MOI 10:1 (mycobacteria:macrophage). After washing and adding antibiotics to kill any extracellular mycobacteria that were not internalized, macrophages were lysed on Days 0, 1 and 2 and various lysate dilutions were plated in triplicate. The uptake of mycobacteria on Day 0 were counted and plotted in Figure 3. Not only were MPtpA or MPtpB not capable of enhancing uptake, but they al-

so were not sufficient to interfere with bactericidal killing by the macrophages (data not shown) indicating that other virulent factors are involved in keeping the mycobacteria alive in the host phagosome.

Tyrosine phosphatase activity of MPtpB. Previous studies have shown that MPtpB is capable of dephosphorylating tyrosine residues on MBP (22). To determine which substrates MPtpB might dephosphorylate in host macrophages, MPtpB WT and MPtpB D82A (substrate trapping mutant) was immunoprecipitated from the culture supernatant using FLAG agarose beads from transformed *M. smegmatis* and these beads were incubated with pervanadate-treated RAW 264.7 macrophage lysates for various time points. To detect which host proteins are becoming dephosphorylated, the beads were immunoblotted using an anti-phosphotyrosine antibody. Figure 4 shows a representative blot where it appears that MPtpB WT was not able to dephosphorylate any of the substrates that it might interact with.

Monoclonal antibody production of MPtpB. A monoclonal antibody was created by cloning MPtpB into pETcoco. The resulting plasmid was used to transform *E. coli* and the expressed His-fusion protein was purified using a Ni-NTA matrix. The purified protein was then injected into rats. The lymph node was harvested and hybridomas were created and tested for MPtpB interaction. One particular clone (3E6-2) was able to immunoprecipitate, immunoblot and stain native protein by flow cytometry (Figure 5 and data not shown).

Yeast two hybrid. To determine the substrates for MPtpB in the host, a yeast two hybrid screen was performed on a *M. tuberculosis* infected mouse lung library. MPtpB was cloned into the yeast vector pAS4 and the resulting plasmid was cotransformed with either library. Positive interactions were selected on SC lacking ura, Leu,

Ade, His + X-α-gal plates. Clones underwent rigorous testing to verify positive interactions before they were lysed and sequenced. The proteins MPtpB interacted with from the mouse infected lung library are shown in Table 1.

Determining the secretory signal region of MPtpB. Since MPtpB does not have a known secretory signal peptide, truncated mutants of MPtpB were created in the operon plasmid (Figure 6, 7). Each truncated mutant was created to be thirty amino acids shorter than the previous mutant for a total of nine mutants. Another mutant was created that contained only the PTP catalytic domain of MPtpB. To determine where the signal sequence of MPtpB is located, the mycobacterial mutants were grown to mid-log phase growth and the culture supernatant was harvested. The supernatants were immunoprecipitated with either FLAG agarose beads or the monoclonal MPtpB antibody bound to magnetic beads. The beads were then immunoblotted with either the FLAG or monoclonal MPtpB antibody to see which of the mutants affected secretion. As seen in Figure 8, deleting the region between amino acids 151-210 of MPtpB causes a slight decrease in the amount of MPtpB protein in the culture supernatant. This region of MPtpB consists of part of the catalytic domain and the amino portion leading up to the lid.

DISCUSSION

Mycobacterial species are well suited to survive the hostile environment of macrophage phagosomes by using several methods that are not seen in other bacteria. There is still a poor understanding of the mechanisms of interaction between mycobacteria and host cell proteins and the consequent changes that occur in the signaling pathways such as downregulation of TLRs, MAPKs and JAK/STATs. What is known is that the entry and survival of intracellular pathogens in host cells require a complex dialogue of signal-

ing events between the host cells and the pathogenic bacteria. Such dialogue requires mycobacterial species to secrete virulence factors into the host cell to reprogram the host signaling network to allow mycobacteria to propagate. Two such potential virulence factors are MPtpB and Eis.

MPtpB has been shown to dephosphorylate tyrosine but not serine/threonine residues (22). Since MPtpB with no known signal peptide is secreted from *M. tuberculosis* and there are no known tyrosine kinases in the mycobacteria, then MPtpB is hypothesized to dephosphorylate host proteins that are involved in various cell signaling pathways essential for the intracellular killing of mycobacteria. In this work, MPtpB was shown to be secreted from the non-pathogenic mycobacteria transformed with an episomal plasmid containing MPtpB indicating that the non-pathogenic strain, *M. smegmatis* has the same secretory machinery as the pathogenic strain *M. tuberculosis*. Also, MPtpB did not alter *M. smegmatis* ability to grow. With this knowledge, *M. smegmatis* expressing MPtpB were used for intracellular killing assays on macrophages to determine if MPtpB is sufficient for survival. Unfortunately, MPtpB is neither able to enhance the uptake of non-pathogenic *M. smegmatis* nor is MPtpB capable of increasing the survival rate of *M. smegmatis* in macrophages demonstrating that MPtpB alone is not sufficient for mycobacteria to survive inside the host.

Since MPtpB alone is not sufficient for mycobacterial survival, a yeast two hybrid screen was performed on a genomic *M. tuberculosis* library to determine what other mycobacterial proteins MPtpB might interact with to enhance survival. This library screen revealed that MPtpB interacted with enhanced intracellular survival (Eis) gene. Previous reports demonstrated that Eis, which also does not contain no know signal peptide, is secreted from the pathogenic strains and that Eis enhanced the survival of non-pathogenic

M. smegmatis in a macrophage-like cell line, U-937 (51). Besides being able to enhance survival in macrophages, Eis has no other known function. To delineate the region where Eis interacts with MPtpB, truncated mutants of MPtpB were made and inserted into an operon containing Eis. From the results obtained, it seemed that Eis might be interacting at the carboxyl terminus of MPtpB. This region consisted of a majority of the lid that protects the active site. Another benefit of these MPtpB truncation mutants was the ability to determine where MPtpBs signal peptide is located. Again, it looked as if MPtpBs signal peptide might be located somewhere between the catalytic site and the lid.

Another yeast two hybrid assay was performed on a mouse lung infected with a *M. tuberculosis* library to determine MPtpB targets in the host. Unfortunately, the targets that were identified could not be easily tied to the function of MPtpB. Likewise, a substrate trapping mutant of MPtpB was used to try and trap potential targets in the host macrophage, but even then MPtpB did not seem to be trapping any putative targets.

Analysis of the sequence of Eis revealed it to have a putative acetyltransferase site. Eis was found to closely resemble the GNAT superfamily. This superfamily has been shown to be involved in acetylating histones, allowing bacteria to be resistant to antibiotics and converting serotonin to melatonin in the involvement of the light-dark cycle. Comparing other known GNAT members to Eis revealed a conserved, key tyrosine residue (Y126) found in the catalytic region, or Motif B. In this study, Eis was shown to acetylate free histones, whereas mutating this key tyrosine residue to a phenylalanine ablated free histone acetylation. Eis, though, was not able to acetylate histones found in a nucleosomal complex. In summary, Eis has been demonstrated to be an acetyltransferase with the key tyrosine residue being involved in this activity, but that histones were not the main target of Eis. Several attempts were made to try and determine host substrates in

the cytoplasm and the nucleus of macrophages with which Eis might acetylate by probing with a pan anti-acetyl lysine antibody, but unfortunately there was no clear evidence of any such substrates. This negative result could be in part due to the poor performance of the antibody, and the possibility that Eis might need to be activated in order to acetylate its substrates.

Why then does a tyrosine phosphatase such as MPtpB interact with an acetyltransferase such as Eis? It might be possible for MPtpB to influence the activity of Eis. Surprisingly, Eis was shown to become tyrosine phosphorylated, albeit not as pronounced as its level of expression in the 293 cell line. When examining various tyrosine mutants of Eis that were capable of becoming phosphorylated, it was seen that the key catalytic tyrosine residue (Y126) was the tyrosine involved in phosphorylation, but several other attempts did not reveal this particular tyrosine(s) to be involved in phosphorylation. Several attempts have been made to determine if MPtpB could dephosphorylate tyrosine phosphorylated Eis, but all attempts have failed. Ongoing efforts are continuing to find the optimal pH and buffer conditions for MPtpB to possibly dephosphorylate Eis.

One theory as to why MPtpB and Eis interact with one another is when Eis is secreted from mycobacteria into the host macrophage, it is active and capable of acetylating its targets in order for the mycobacterium to survive. Tyrosine kinases from the host target and phosphorylate Eis making it inactive. Since MPtpB and Eis interact with one another, MPtpB dephosphorylates Eis allowing Eis to become active again so that it can acetylate its targets allowing the mycobacterium to survive.

REFERENCES

1. **Ainsa, J. A., E. Perez, V. Pelicic, F. X. Berthet, B. Gicquel, and C. Martin.** 1997. Aminoglycoside 2'-N-acetyltransferase genes are universally present in mycobacteria: characterization of the aac(2')-Ic gene from Mycobacterium tuberculosis and the aac(2')-Id gene from Mycobacterium smegmatis. Mol Microbiol **24:**431-41.

2. **Aliprantis, A. O., R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski, and A. Zychlinsky.** 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science **285:**736-9.

3. **Balcewicz-Sablinska, M. K., J. Keane, H. Kornfeld, and H. G. Remold.** 1998. Pathogenic Mycobacterium tuberculosis evades apoptosis of host macrophages by release of TNF-R2, resulting in inactivation of TNF-alpha. J Immunol **161:**2636-41.

4. **Barford, D., A. K. Das, and M. P. Egloff.** 1998. The structure and mechanism of protein phosphatases: insights into catalysis and regulation. Annu Rev Biophys Biomol Struct **27:**133-64.

5. **Black, D. S., and J. B. Bliska.** 1997. Identification of p130Cas as a substrate of Yersinia YopH (Yop51), a bacterial protein tyrosine phosphatase that translocates into mammalian cells and targets focal adhesions. Embo J **16:**2730-44.

6. **Brazil, D. P., J. Park, and B. A. Hemmings.** 2002. PKB binding proteins. Getting in on the Akt. Cell **111:**293-303.

7. **Brownell, J. E., and C. D. Allis.** 1995. An activity gel assay detects a single, catalytically active histone acetyltransferase subunit in Tetrahymena macronuclei. Proc Natl Acad Sci U S A **92:**6364-8.

8. **Cappelli, G., P. Volpe, A. Sanduzzi, A. Sacchi, V. Colizzi, and F. Mariani.** 2001. Human macrophage gamma interferon decreases gene expression but not replication of Mycobacterium tuberculosis: analysis of the host-pathogen reciprocal influence on transcription in a comparison of strains H37Rv and CMT97. Infect Immun **69:**7262-70.

9. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell.** 1998. De-

ciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature **393:**537-44.

10. **Dahl, J. L., J. Wei, J. W. Moulder, S. Laal, and R. L. Friedman.** 2001. Subcellular localization of the Iitracellular survival-enhancing Eis protein of Mycobacterium tuberculosis. Infect Immun **69:**4295-302.

11. **Deretic, V., and R. A. Fratti.** 1999. Mycobacterium tuberculosis phagosome. Mol Microbiol **31:**1603-9.

12. **Ernst, J. D.** 1998. Macrophage receptors for Mycobacterium tuberculosis. Infect Immun **66:**1277-81.

13. **Ferrari, G., H. Langen, M. Naito, and J. Pieters.** 1999. A coat protein on phagosomes involved in the intracellular survival of mycobacteria. Cell **97:**435-47.

14. **Fu, Y., and J. E. Galan.** 1999. A salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. Nature **401:**293-7.

15. **Grundner, C., H. L. Ng, and T. Alber.** 2005. Mycobacterium tuberculosis protein tyrosine phosphatase PtpB structure reveals a diverged fold and a buried active site. Structure (Camb) **13:**1625-34.

16. **Hegde, S. S., F. Javid-Majd, and J. S. Blanchard.** 2001. Overexpression and mechanistic analysis of chromosomally encoded aminoglycoside 2'-N-acetyltransferase (AAC(2')-Ic) from Mycobacterium tuberculosis. J Biol Chem **276:**45876-81.

17. **Hickman, A. B., M. A. Namboodiri, D. C. Klein, and F. Dyda.** 1999. The structural basis of ordered substrate binding by serotonin N-acetyltransferase: enzyme complex at 1.8 A resolution with a bisubstrate analog. Cell **97:**361-9.

18. **Hussain, S., B. S. Zwilling, and W. P. Lafuse.** 1999. Mycobacterium avium infection of mouse macrophages inhibits IFN-gamma Janus kinase-STAT signaling and gene induction by down-regulation of the IFN-gamma receptor. J Immunol **163:**2041-8.

19. **Keane, J., H. G. Remold, and H. Kornfeld.** 2000. Virulent Mycobacterium tuberculosis strains evade apoptosis of infected alveolar macrophages. J Immunol **164:**2016-20.

20. **Kennelly, P. J., and M. Potts.** 1996. Fancy meeting you here! A fresh look at "prokaryotic" protein phosphorylation. J Bacteriol **178:**4759-64.

21. **Klein, D. C., S. L. Coon, P. H. Roseboom, J. L. Weller, M. Bernard, J. A. Gastel, M. Zatz, P. M. Iuvone, I. R. Rodriguez, V. Begay, J. Falcon, G. M. Cahill, V. M. Cassone, and R. Baler.** 1997. The melatonin rhythm-generating

enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. Recent Prog Horm Res **52:**307-57; discussion 357-8.

22. **Koul, A., A. Choidas, M. Treder, A. K. Tyagi, K. Drlica, Y. Singh, and A. Ullrich.** 2000. Cloning and characterization of secretory tyrosine phosphatases of Mycobacterium tuberculosis. J Bacteriol **182:**5425-32.

23. **Koul, A., T. Herget, B. Klebl, and A. Ullrich.** 2004. Interplay between mycobacteria and host signalling pathways. Nat Rev Microbiol **2:**189-202.

24. **Llano-Sotelo, B., E. F. Azucena, Jr., L. P. Kotra, S. Mobashery, and C. S. Chow.** 2002. Aminoglycosides modified by resistance enzymes display diminished binding to the bacterial ribosomal aminoacyl-tRNA site. Chem Biol **9:**455-63.

25. **Lopez, M., L. M. Sly, Y. Luu, D. Young, H. Cooper, and N. E. Reiner.** 2003. The 19-kDa Mycobacterium tuberculosis protein induces macrophage apoptosis through Toll-like receptor-2. J Immunol **170:**2409-16.

26. **Maiti, D., A. Bhattacharyya, and J. Basu.** 2001. Lipoarabinomannan from Mycobacterium tuberculosis promotes macrophage survival by phosphorylating Bad through a phosphatidylinositol 3-kinase/Akt pathway. J Biol Chem **276:**329-33.

27. **Malik, Z. A., S. S. Iyer, and D. J. Kusner.** 2001. Mycobacterium tuberculosis phagosomes exhibit altered calmodulin-dependent signal transduction: contribution to inhibition of phagosome-lysosome fusion and intracellular survival in human macrophages. J Immunol **166:**3392-401.

28. **Malik, Z. A., C. R. Thompson, S. Hashimi, B. Porter, S. S. Iyer, and D. J. Kusner.** 2003. Cutting edge: Mycobacterium tuberculosis blocks Ca2+ signaling and phagosome maturation in human macrophages via specific inhibition of sphingosine kinase. J Immunol **170:**2811-5.

29. **Mariotti, S., R. Teloni, E. Iona, L. Fattorini, F. Giannoni, G. Romagnoli, G. Orefici, and R. Nisini.** 2002. Mycobacterium tuberculosis subverts the differentiation of human monocytes into dendritic cells. Eur J Immunol **32:**3050-8.

30. **Noss, E. H., R. K. Pai, T. J. Sellati, J. D. Radolf, J. Belisle, D. T. Golenbock, W. H. Boom, and C. V. Harding.** 2001. Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of Mycobacterium tuberculosis. J Immunol **167:**910-8.

31. **Pai, R. K., M. Convery, T. A. Hamilton, W. H. Boom, and C. V. Harding.** 2003. Inhibition of IFN-gamma-induced class II transactivator expression by a 19-kDa lipoprotein from Mycobacterium tuberculosis: a potential mechanism for immune evasion. J Immunol **171:**175-84.

32. **Pancholi, P., A. Mirza, N. Bhardwaj, and R. M. Steinman.** 1993. Sequestration from immune CD4+ T cells of mycobacteria growing in human macrophages. Science **260:**984-6.

33. **Persson, C., N. Carballeira, H. Wolf-Watz, and M. Fallman.** 1997. The PTPase YopH inhibits uptake of Yersinia, tyrosine phosphorylation of p130Cas and FAK, and the associated accumulation of these proteins in peripheral focal adhesions. Embo J **16:**2307-18.

34. **Peters, C., and A. Mayer.** 1998. Ca2+/calmodulin signals the completion of docking and triggers a late step of vacuole fusion. Nature **396:**575-80.

35. **Redpath, S., P. Ghazal, and N. R. Gascoigne.** 2001. Hijacking and exploitation of IL-10 by intracellular pathogens. Trends Microbiol **9:**86-92.

36. **Rich, R. C., and H. Schulman.** 1998. Substrate-directed function of calmodulin in autophosphorylation of Ca2+/calmodulin-dependent protein kinase II. J Biol Chem **273:**28424-9.

37. **Roach, S. K., and J. S. Schorey.** 2002. Differential regulation of the mitogen-activated protein kinases by pathogenic and nonpathogenic mycobacteria. Infect Immun **70:**3040-52.

38. **Roberts, E. A., A. Clark, S. McBeth, and R. L. Friedman.** 2004. Molecular characterization of the eis promoter of Mycobacterium tuberculosis. J Bacteriol **186:**5410-7.

39. **Rojas, M., L. F. Garcia, J. Nigou, G. Puzo, and M. Olivier.** 2000. Mannosylated lipoarabinomannan antagonizes Mycobacterium tuberculosis-induced macrophage apoptosis by altering Ca+2-dependent cell signaling. J Infect Dis **182:**240-51.

40. **Salmeen, A., J. N. Andersen, M. P. Myers, T. C. Meng, J. A. Hinks, N. K. Tonks, and D. Barford.** 2003. Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. Nature **423:**769-73.

41. **Shtrichman, R., and C. E. Samuel.** 2001. The role of gamma interferon in antimicrobial immunity. Curr Opin Microbiol **4:**251-9.

42. **Singh, R., A. Singh, and A. K. Tyagi.** 2005. Deciphering the genes involved in pathogenesis of Mycobacterium tuberculosis. Tuberculosis (Edinb) **85:**325-35.

43. **Spiegel, S., and S. Milstien.** 2002. Sphingosine 1-phosphate, a key cell signaling molecule. J Biol Chem **277:**25851-4.

44. **Stenger, S., K. R. Niazi, and R. L. Modlin.** 1998. Down-regulation of CD1 on antigen-presenting cells by infection with Mycobacterium tuberculosis. J Immunol **161:**3582-8.

45. **Sterner, D. E., and S. L. Berger.** 2000. Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev **64:**435-59.

46. **Stone, R. L., and J. E. Dixon.** 1994. Protein-tyrosine phosphatases. J Biol Chem **269:**31323-6.

47. **Sturgill-Koszycki, S., P. H. Schlesinger, P. Chakraborty, P. L. Haddix, H. L. Collins, A. K. Fok, R. D. Allen, S. L. Gluck, J. Heuser, and D. G. Russell.** 1994. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science **263:**678-81.

48. **Szalai, G., R. Krishnamurthy, and G. Hajnoczky.** 1999. Apoptosis driven by IP(3)-linked mitochondrial calcium signals. Embo J **18:**6349-61.

49. **Underhill, D. M., A. Ozinsky, K. D. Smith, and A. Aderem.** 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proc Natl Acad Sci U S A **96:**14459-63.

50. **van Montfort, R. L., M. Congreve, D. Tisi, R. Carr, and H. Jhoti.** 2003. Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. Nature **423:**773-7.

51. **Wei, J., J. L. Dahl, J. W. Moulder, E. A. Roberts, P. O'Gaora, D. B. Young, and R. L. Friedman.** 2000. Identification of a Mycobacterium tuberculosis gene that enhances mycobacterial survival in macrophages. J Bacteriol **182:**377-84.

52. **Wojciechowski, W., J. DeSanctis, E. Skamene, and D. Radzioch.** 1999. Attenuation of MHC class II expression in macrophages infected with Mycobacterium bovis bacillus Calmette-Guerin involves class II transactivator and depends on the Nramp1 gene. J Immunol **163:**2688-96.

53. **Yarden, Y., and A. Ullrich.** 1988. Growth factor receptor tyrosine kinases. Annu Rev Biochem **57:**443-78.

Table 1. Mouse lung infected with *M. tuberculosis* library screen results.

Name	Description
Fzd2	Frizzled homolog 2
SftpC	Surfactant associate protein C
Scgb1a1	Secretoglobin, family 1A, member 1
Itm2b	Integral membrane protein 2B

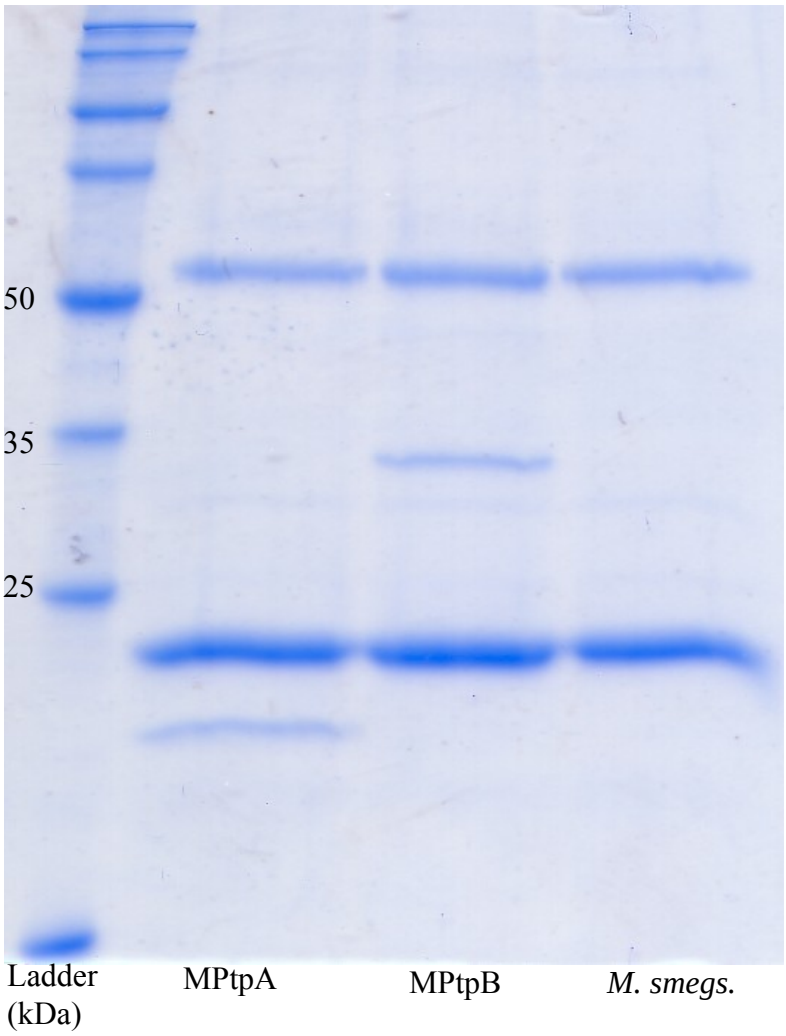

Figure 1. Secretion of MPtpA and MPtpB in *M. smegmatis*. *M. smegmatis* was grown to mid-log phase growth (OD_{600} = 0.8 – 1.0) and culture filtrate proteins were incubated with FLAG agarose beads and loaded on a 10% SDS-PAGE and stained with Coomassie blue. Lane 1, ladder; lane 2, MPtpA (22 kDa); lane 3, MPtpB (35 kDa); lane 4, *M. smegs.*

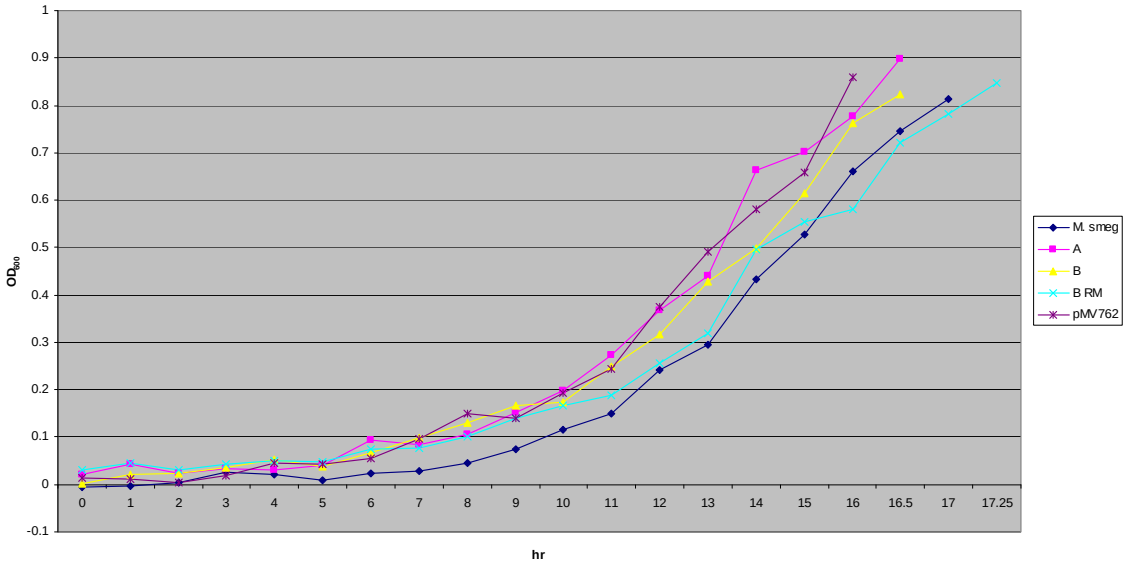

Figure 2. Growth curve of *M. smegmatis* expressing MPtpA, MPtpB, vector control (pMV762), and *M. smegmatis* only. The OD_{600} was checked hourly until mid-log phase growth to determine if MPtpA or MPtpB might affect the growth of *M. smegmatis*. M. smeg, *M. smegmatis*; A, MPtpA; B, MPtpB; B RM, MPtpB R166M.

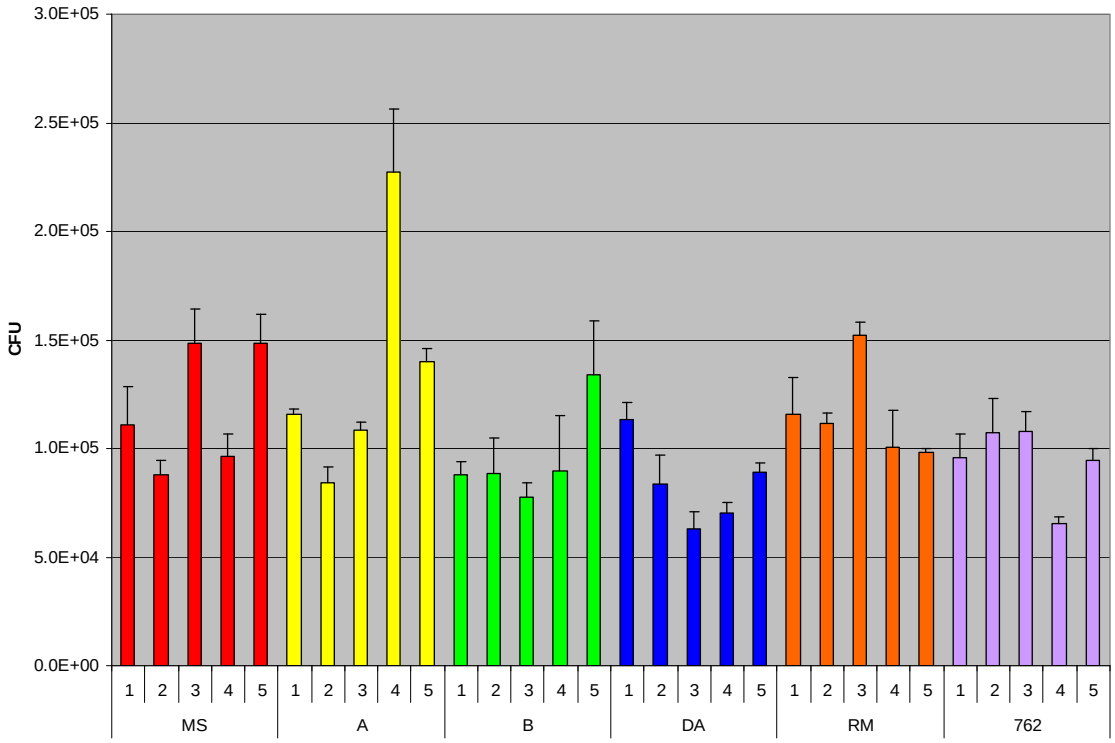

Figure 3. *M. smegmatis* transformants infecting macrophages to determine uptake. Five different mycobacterial colonies per construct were grown to mid-log phase growth and used to infect RAW 264.7 macrophages at an MOI 10:1 (mycobacteria:macrophages). On day 0 post-infection, macrophages were lysed and the number of intracellular mycobacteria was assessed by plating on LB/0.05% Tween-80 plates. The experiment was carried out in triplicate and the data depicts the mean of all three values ±SE. MS, *M. smegmatis*; A, MPtpA; B, MPtpB; DA, MPtpB D82A; RM, MPtpB R166M; 762, pMV762.

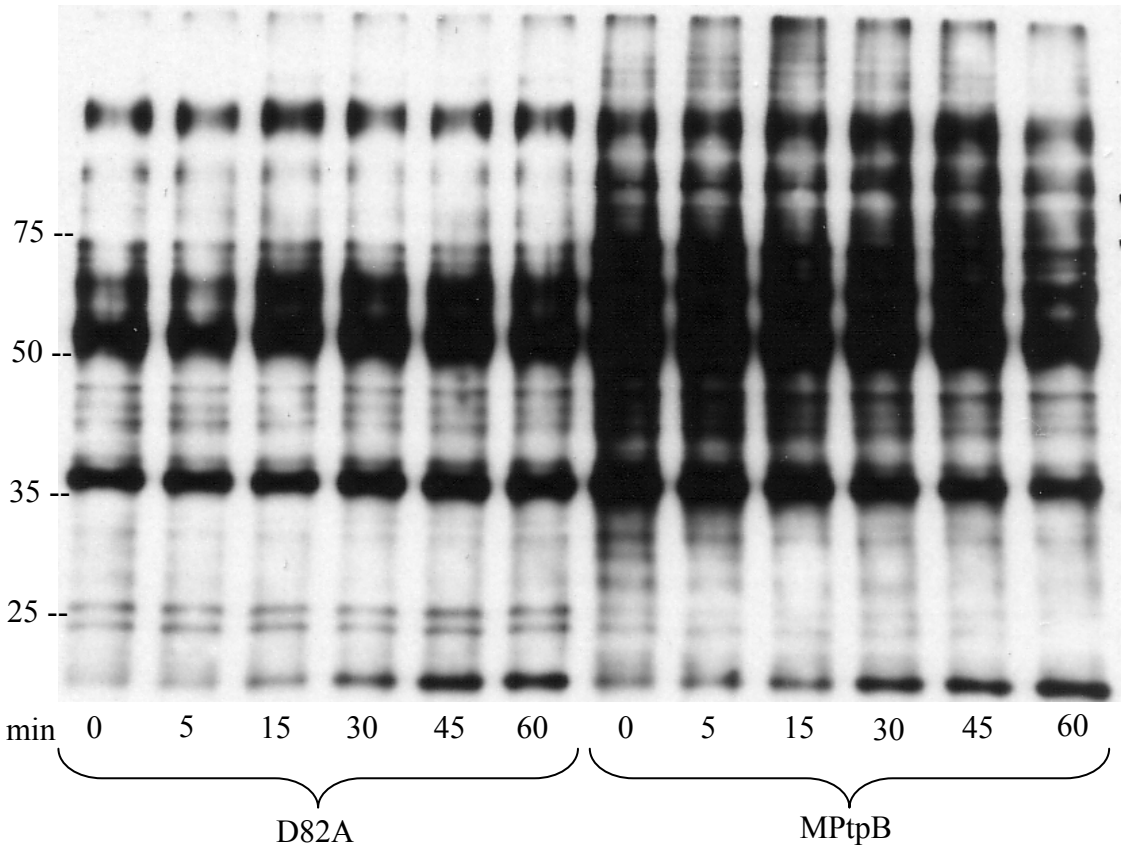

Figure 4. Immunoprecipitation of pervanadate treated RAW264.7 macrophage lysates incubated with either MPtpB WT or its substrate trapping mutant (D82A) from *M. smegmatis*. MPtpB and its substrate trapping mutant were harvested from *M. smegmatis* culture supernatant with FLAG agarose beads. These beads were then incubated with pervanadate treated macrophages and then immunoblotted on a 10% SDS-PAGE. Blot was probed with anti-phosphotyrosine (4G10) antibody conjugated to HRP and developed using enhanced chemiluminescence reagents.

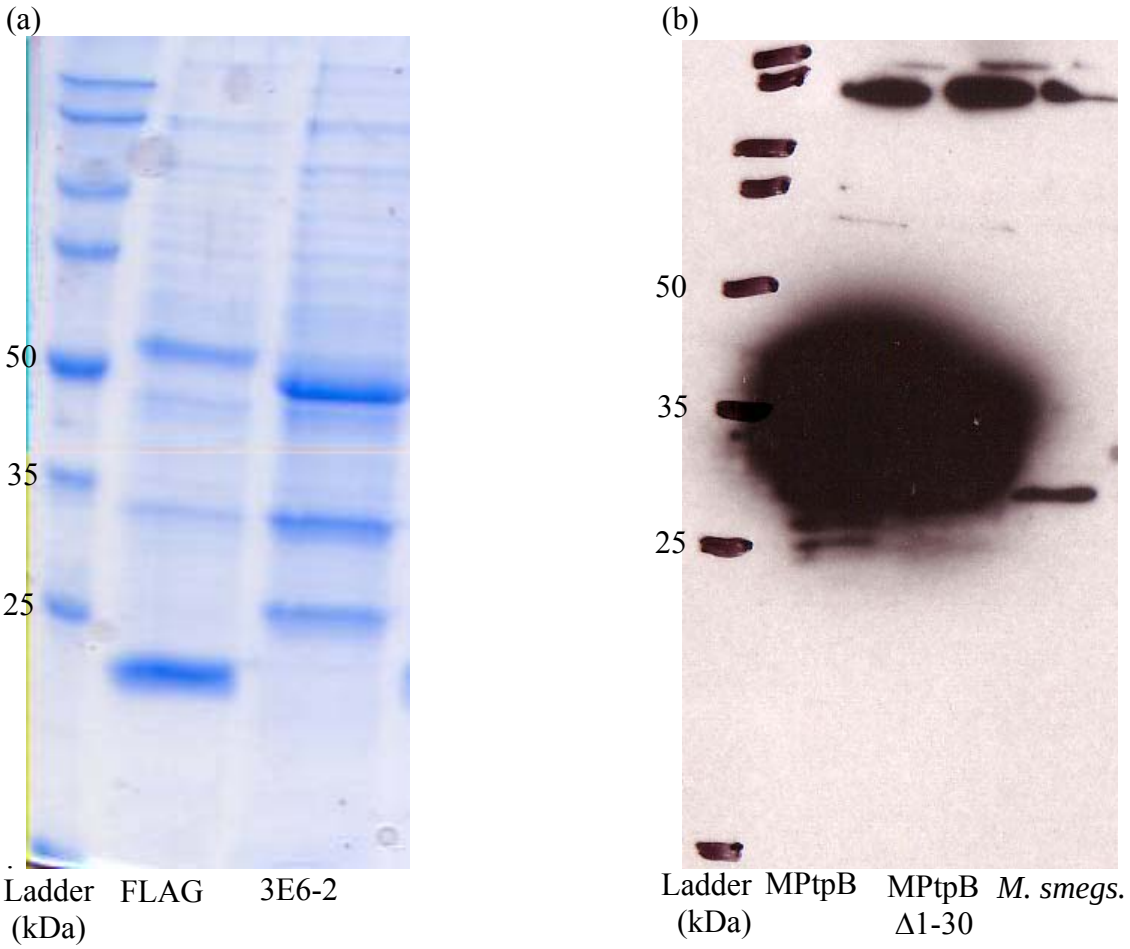

Figure 5. Immunoprecipitation and immunoblot of monoclonal antibody raised against MPtpB. Monoclonal rat anti-MPtpB (3E6-2) is capable of immunoprecipitating MPtpB (a) and blotting MPtpB (b). *M. smegmatis* expressing MPtpB was grown to mid-log phase growth and harvested. (a) Whole cell lysates were incubated with either FLAG agarose beads or anti-MPtpB 3E6-2:Protein G and loaded on a 10% SDS-PAGE and stained with Coomassie blue. (b) Whole cell lysates were loaded on a 10% SDS-PAGE and probed with anti-MPtpB 3E6-2.

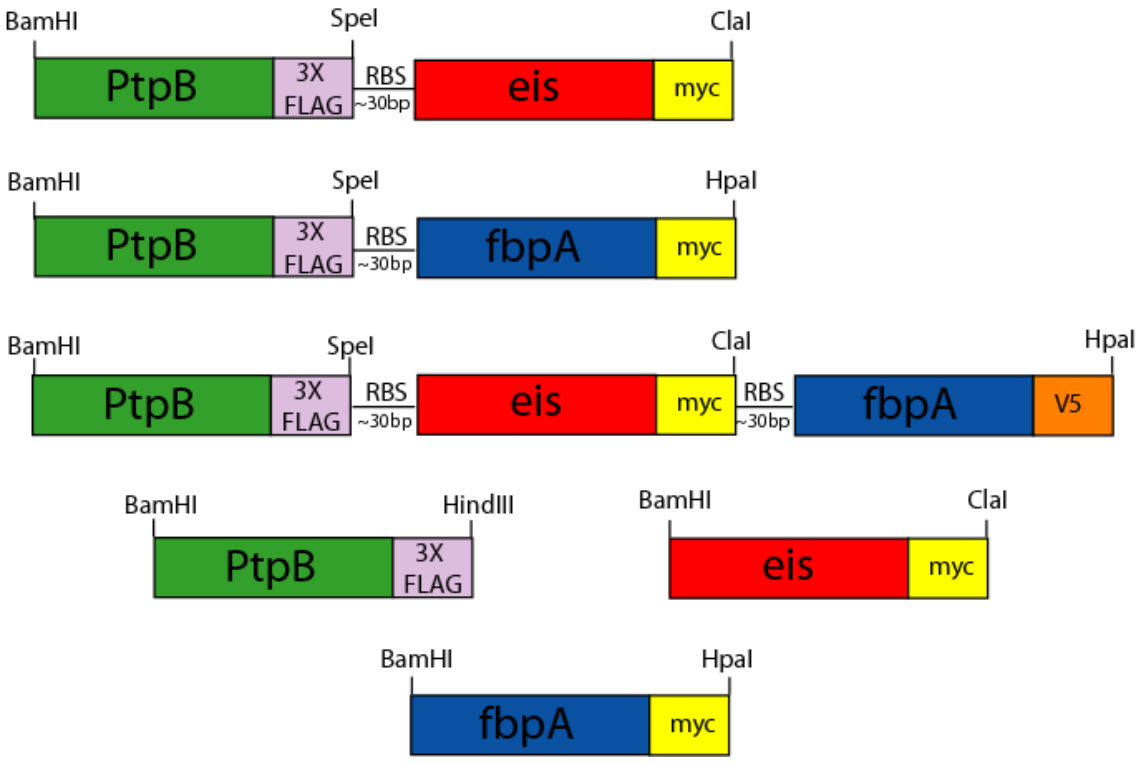

Figure 6. Diagram of operons built for determining the region of interaction between MPtpB with Eis or fbpA. MPtpB containing a 3X FLAG was cloned upstream of Eis or fbpA that was myc-tagged. A ribosomal binding site (RBS) was placed upstream of Eis or fbpA. This operon was then cloned into the mycobacterial episomal pMV762 plasmid.

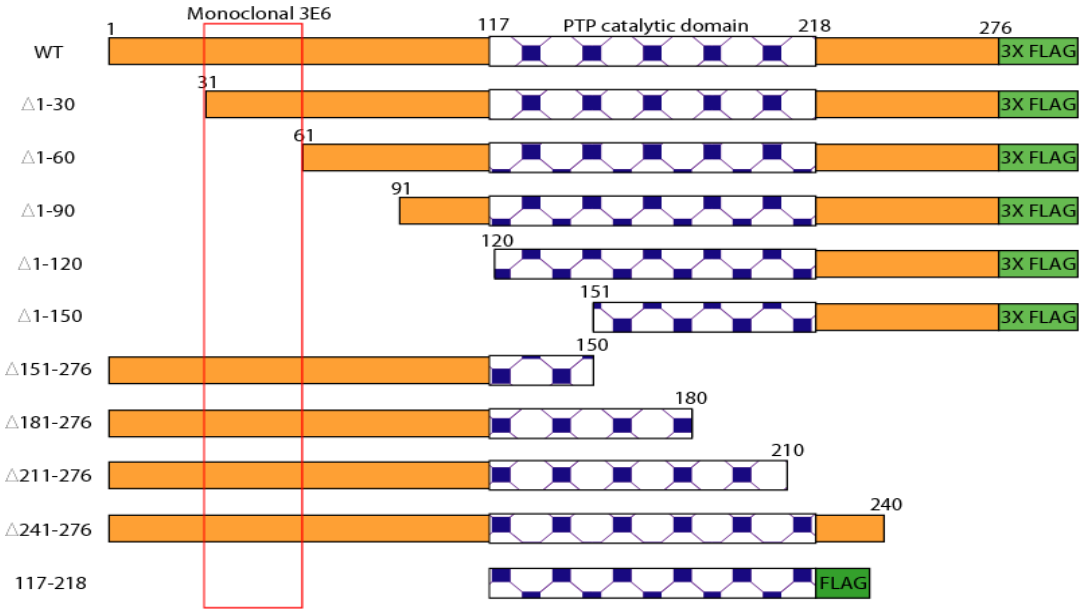

Figure 7. Truncation mutants of MPtpB. Schematic diagram of the various truncated mutants of MPtpB inserted into the operon. The red box denotes the region of MPtpB where the rat anti-MPtpB (3E6-2) monoclonal antibody recognizes.

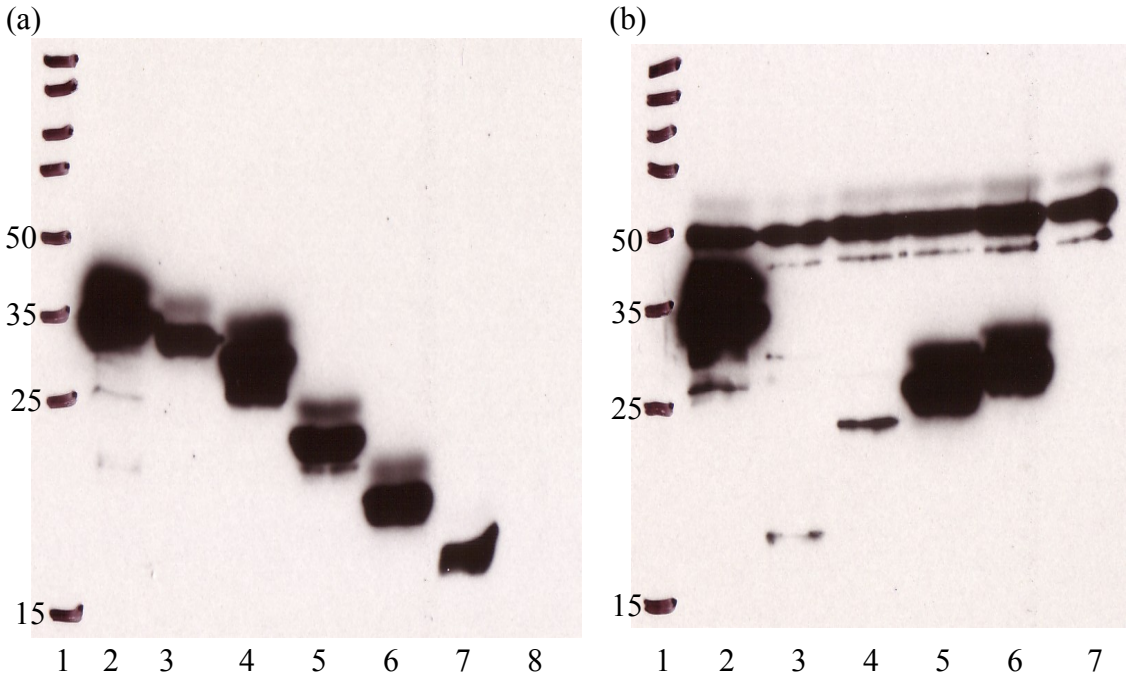

Figure 8. MPtpB secretion. The culture supernatant from *M. smegmatis* expressing the MPtpB:Eis operon was harvested at mid-log phase growth and immunoprecipitated with either FLAG agarose beads (a) or rat anti-MPtpB (3E6-2) bound to magnetic beads (b) and probed with either anti-FLAG-HRP (A) or rat anti-MPtpB (3E6-2). For (a), lane 1, ladder (kDa); lane 2, MPtpB:Eis; lane 3, MPtpB Δ1-30:Eis; lane 4, MPtpB Δ1-60:Eis; lane 5, MPtpB Δ1-90:Eis; lane 6, MPtpB Δ1-120:Eis; lane 7, MPtpB Δ1-150:Eis; lane 8, Eis. For (b), lane 1, ladder (kDa); lane 2, MPtpB:Eis; lane 3, MPtpB Δ151-276:Eis; lane 4, MPtpB Δ181-276:Eis; lane 5, MPtpB Δ211-276:Eis; lane 6, MPtpB Δ241-276:Eis; lane 7, Eis.

CHARACTERIZATION OF AN ACETYLTRANSFERASE AND ITS INTERACTION WITH A TYROSINE PHOSPHATASE IN *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

Mycobacterium tuberculosis has been shown to manipulate various signal transduction pathways in the host macrophage in order for intracellular survival. *M. tuberculosis* protein tyrosine phosphatase MPtpB is thought to be secreted into the host thereby interrupting unknown signal pathways. To better understand what virulence factors are needed with MPtpB, a yeast two hybrid analysis of a *M. tuberculosis* H37Rv library was analyzed and these results revealed an interaction with enhanced intracellular survival (Eis) protein. Previous results demonstrated that Eis is capable of enhancing survival in primary human monocytes infected with Eis overexpressed in *Mycobacterium smegmatis*. Eis recently was shown to modulate the secretion of IL-10 and TNF-α. A BLAST search revealed a putative acetyltransferase domain in the amino terminus of Eis. In this present work, we show that Eis is an acetyltransferase by it ability to acetylate free histones. Eis also appears to inhibit the phosphatase activity of MPtpB indicating that Eis plays some sort of regulatory role in the activity of MPtpB inside mycobacteria.

INTRODUCTION

Mycobacterium tuberculosis is the main causative agent of tuberculosis (TB) and is a major cause of death in the world. According to the Centers for Disease and Control (CDC) and the World Health Organization (WHO), TB kills about two million people annually, and approximately one-third of the world's population (or 1.7 billion people) are asymptomatically infected with *M. tuberculosis*. *M. tuberculosis* deaths are exacerbated with the spread of AIDS and the development of resistance against most of the antibiotics used in the treatment of tuberculosis. Currently, there is insufficient knowledge

in the understanding of how *M. tuberculosis* enters the host cell, circumvents host defenses and spreads into neighboring cells.

In order for pathogenic mycobacteria to survive inside macrophages, mycobacteria must be able to inhibit several host-cell processes, whereas non-pathogenic species are not capable. Host processes inhibited by pathogenic mycobacteria includes the fusion of phagosomes with lysosomes, antigen presentation, apoptosis and the stimulation of bactericidal responses due to the activation of pathways involving mitogen-activated protein kinases (MAPKs), Interferon-γ (IFN-γ) and calcium (Ca^{+2}) signaling (8, 10, 11).

For bacterial pathogens to evade these host immune responses, many bacteria secrete virulence factors into the host causing modification of host proteins allowing for the bacteria to survive. Tyrosine phosphorylation is extremely important in eukaryotic cells. One example is reversible phosphorylation of tyrosine residues which has been shown to represent a key mechanism for the transduction of signals that regulate cell growth, differentiation, mobility, metabolism, and survival (17). The level of phosphorylation on tyrosine residues required for normal cell function is maintained by the opposing actions of tyrosine kinases (15). For some bacteria, protein phosphorylation has been shown to play an important role in sensing extracellular signals by a pathogen and coordinating intracellular events (7). Protein tyrosine phosphatases (PTPases) have been identified as virulence factors in a number of bacterial species and are essential for their development and pathogenesis (7). For example, these pathogenic bacteria have tyrosine phosphatases that act as virulence factors: *Yersinia pseudotuberculosis* contains YopH, *Salmonella enterica* serovar Typhimurium contains SptP, and enterophatogenic *Escherichia coli* (1, 5, 12).

The genome of *M. tuberculosis* codes for two PTPases, MPtpA (Rv2234) and MPtpB (Rv0153c), but no known tyrosine kinases have been found (2). Both MPtpA and

MPtpB exhibit protein tyrosine phosphatase activity but not serine/threonine phosphatase activity upon myelin basic protein (9). MPtpA is classified as a low-molecular weight (LMW) phosphatase, and the MPtpB sequence falls into the conventional PTP or dual-specificity phosphatases (DSP) class. Whereas MPtpA was found in both pathogenic and non-pathogenic species, MPtpB was found only in the slow-growing pathogenic species (9). When the *mptpB* gene was deleted from *M. tuberculosis*, the mutant strain caused accelerated mycobacterial cell death in the lung and spleen of guinea pigs (14). In addition, wild-type and mutant *mptpB* strains were equally able to survive in resting macrophages, but the ability of *mptpB* mutants to survive in macrophages activated with IFN-γ was highly impaired. The crystal structure of MPtpB revealed two unusual elaborations: a disordered, 31-residue acidic loop, and a flexible, two-helix lid that covers the active site, which are specific to mycobacterial orthologs (6).

In this present study, we report the interaction of MPtpB with the mycobacterial protein, "enhanced intracellular survival" or Eis (Rv2416c). Eis is present only in the slow-growing pathogenic species and Eis from *M. tuberculosis* was shown to significantly enhance intracellular survival of *M. smegmatis* containing *eis* in the human macrophage cell line U937 (3, 16). We also demonstrate Eis' capabilities as an acetyltransferase since a BLAST search revealed Eis to contain a putative acetyltransferase domain belonging to the GCN5-related N-acetyltransferases (GNAT) superfamily (4). Eis also appears to regulate the tyrosine phosphatase activity of MPtpB by decreasing the activity of MPtpB inside mycobacteria.

MATERIALS AND METHODS

Cell lines. HEK 293T were cultured in DMEM medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 μM L-glutamine, 50 μM 2-mercapto-ethanol, 100 μg/ml streptomycin-penicillin, and 50 μg/ml gentamicin (Sigma, St. Louis, MO) at 37°C under 5% CO_2.

Antibodies and reagents. For Western blot analysis, the following antibodies were purchased: mouse anti-FLAG mAb conjugated to horseradish peroxidase (HRP) (Sigma), anti-phosphotyrosine mAb conjugated to HRP (4G10, Upstate Biotechnology Inc., Waltham, MA), mouse anti-c-myc (9E10, Roche, Indianapolis, IN) and mouse anti-c-myc conjugated to HRP (9E10, Roche). Rat monoclonal anti-MPtpB antibody was generated by immunizing rats with intact recombinant MPtpB.

Bacterial strains, growth and preparation. *Mycobacterium smegmatis* (mc^2155) was cultured in Luria-Broth (LB) supplemented with 0.05% Tween-80. *M. smegmatis* transformed with various modified pMV762 plasmids were grown to mid-logarithmic phase growth (OD_{600} = 0.8-1.0) in LB supplemented with 50 μg/mL Hygromycin B (A.G. Scientific, San Diego, CA) and 0.05% Tween-80. Cells were centrifuged, washed in PBS and resuspsended in various lysis buffers containing protease inhibitors and phenylmethylsulfonyl fluoride (PMSF). Cells were either lysed using a sonicator or a French press. The resulting lysates were centrifuged to pellet any non-lysed cells and cellular debris. For immunoprecipitations, either FLAG agarose beads (Sigma), α-c-myc or α-MPtpB were added to the lysates and rotated for 2 hrs at 4˚C (FLAG beads were rotated overnight). After 2 hrs, Protein G, blocked in 5% BSA, was added and allowed to rotate overnight at 4˚C. The beads were then washed four times in the appropriate lysis buffer.

Plasmids. The cDNA encoding full-length MPtpB (Rv0153c) and Eis (Rv2416c) were PCR amplified from *M. tuberculosis* H37Rv genomic DNA using KOD high fidelity polymerase (Novagen, Madison, WI). For Eis, an EcoRV site was included in the forward primer (5'-GAGAGATATCGCCGCCATGACTGTGACCCTGTGTAGCCCGACCG-3') and a FLAG tag and NotI site were included in the reverse primer (5'-GAGAGCGGCCGCTTATTACTTGTCATCGTCGTCCTTGTAGTCGAACTCGAACGCGGTCTGGACGGGAACA-3'). The Eis PCR product was digested with EcoRV and NotI restriction enzymes and then ligated into the EcoRV and NotI sites of the pIRESpuro2 vector (Clontech, Mountain View, CA). For the pMV762 plasmid, MPtpB had a BamHI site included in the forward primer (5'-GAGAGGATCCGATGGCTGTCCGTGAACTGCCGGGCGCG-3') and a HindIII site was included in the reverse primer (5'-GAGAAAGCTTTTATTATCCGAGCAGCACCCCGCGCATCCG-3'). For Eis in the pMV762 plasmid, a BamHI site was included in the forward primer (5'-GAGAGGATCCGATGACTGTGACCCTGTGTAGCCCGACCG-3') and a FLAG tag and a HindIII site were included in the reverse primer (5'-GAGAAAGCTTTTATTACTTGTCATCGTCGTCCTTGTAGTCGAACTCGAACGCGGTCTGGACGGGAAC-3'). For AAC(2')-IC (Rv0262c) in the pMV762 plasmid, a BamHI site was include in the forward primer (5'-GAGAGGATCCGATGCACACCCAGGTACACACGGCCCG-3') and a FLAG tag and a HindIII site were included in the reverse primer (5'-GAGAAAGCTTTTATTACTTGTCATCGTCGTCCTTGTAGTCCCAGACGTCGCCCGCGCGCCAAT-3'). The MPtpB, Eis and AAC PCR product were digested with BamHI and HindIII restriction enzymes and then ligated into the BamHI and HindIII sites of the pMV762 vector.

Site-directed mutagenesis. Eis was mutagenized to create either a catalytically inactive mutant (Y126F, TAC→GCA), or various other tyrosine residues thought to be involved in tyrosine phosphorylation (Y113F, TAT→TTT; Y179F, TAC→GAC).

Transient transfection. Eis-FLAG:pIRESpuro2, Eis Y113F-FLAG:pIRESpuro2, Eis Y126F-FLAG:pIRESpuro2, Eis Y179F-FLAG:pIRESpuro2 or empty pIRESpuro2 plasmids were transfected into 293T cells using Lipofectamine from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions.

Pervanadate treatment of transient transfected cells and immunoprecipitation/Western blot assay. 293T transiently transfected cells ($1x10^7$ cells/sample) were treated for 30 min at 37˚C with 0.1 mM pervanadate (2 µl of a fresh solution containing 50 mM sodium metavanadate ($NaVO_3$) and 50 mM H_2O_2 was added to 1 ml of media). After stimulation, cells were immediately washed in ice-cold PBS to stop the reaction. Next, cells were washed twice in ice-cold PBS and lysed in 0.5 ml of lysis buffer (50 mM MOPS (pH 7.2), 150 mM NaCl, 0.0025% NaN_3, 10 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4, and 1% Nonidet P-40). A protease inhibitor mixture (Calbiochem, San Diego, CA) and PMSF were added to the lysis buffer just before use. Cell lysates were incubated for 30 min on ice and then centrifuged at 13,000 x g for 15 min at 4˚C. Cleared lysates were transferred to a new tube and 50 µl FLAG agarose was added and allowed to rotate overnight at 4˚C. Beads were washed four times in ice-cold lysis buffer followed by boiling in SDS-PAGE Sample Reducing Buffer. The proteins were separated by a 10% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked overnight at 4˚C in TBST with 5% nonfat milk (or TBST with 3% Blot Qualified BSA (Promega, Madison, WI) for 4G10 blotting), and washed two times with TBST before the addition of the primary antibody. Membranes

were incubated for 1 hr at room temperature with the primary antibody diluted into TBST with 1% nonfat milk (or TBST with 1% Blot Qualified BSA), and washed six times with TBST. For primary antibodies not directly conjugated to HRP, secondary goat $F(ab')_2$ anti-rat Ig's conjugated to HRP (Biosource International) were incubated with the membranes for 1 hr at room temperature and then washed six times with TBST. Proteins of interest were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and subsequent exposure to autoradiographic film (Eastman Kodak Co).

Operons. MPtpB and Eis were placed in an operon with MPtpB upstream of Eis. For MPtpB, a BamHI site was included in the forward primer (5'-GAGAGGATCCGATGGCTGTCCGTGAACTGCCGGGCGCG-3') and a 3X FLAG and SpeI site were included in the reverse primer (5'-GAGAACTAGTGGGTAACCCTATGCAGTCGTCGAGGAATTGCTATTA-3"). For Eis, a Shine-Delgarno sequence (ribosomal binding site) and SpeI site were included in the forward primer (5'-GAGAACTAGTGTGGCCGCGGCATATGCCACAGTCG-3') and a myc tag and ClaI site were included in the reverse primer (5'-GAGAATCGATTTATTACAGATCCTCTTCTGAGATGAGTTTTTGTTCGAACTCGAACGCGGTCTGGACGGGAAC-3'). To determine the region of MPtpB that is responsible for interacting with Eis, multiple deletions of MPtpB were created for this operon model. These MPtpB deletions are: Δ1-30 amino acids (AA); Δ1-60 AA; Δ1-90 AA; Δ1-120 AA; Δ1-150 AA; Δ1-180C AA; Δ181-276 AA; Δ211-276 AA; Δ241-276 AA and Δ150-210.

HAT assay on free histones. Add 20 μg Type IIA calf thymus histones (Sigma), Eis bound to FLAG beads from *M. smegmatis*, 37 μl 1X HAT buffer (50 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate), 3 μl ^{14}C-

acetyl CoA (0.05 mCi/ml, 53 mCi/mmol). For positive control, add 10 µl p300 (0.5 µg/µl) (Upstate, Temecula, CA), and for a negative control, substitute Eis with 10 µl water. Incubate at room temperature for 1 hr. Separate on a 15% SDS-PAGE gel. Soak the gel in Amplify (Amersham, Piscataway, NJ) for 30 min. Dry the gel onto a piece of filter paper at 80˚C for 45 min. Place dried gel in a film cassette with autoradiographic film and expose after 5-20 days.

HAT assay on nucleosomes. To form the histone octamer, dissolve core histones in 4 ml of unfolding buffer (6 M Guanidinium chloride, 20 mM Tris-HCl (pH 7.5), 5 mM DTT) at a concentration of 2 mg/ml. Incubate at room temperature for 2 hrs. Dialyze at 4˚C for 6 hrs, then overnight, then 6 hrs in three changes of refolding buffer (2 M NaCl in TE (pH 7.5)). Concentrate to 1 ml final volume. Centrifuge for 15 min at 3,500 g to remove precipitated protein. Next, prepare a whole cell extract of HeLa cells. Phenol chloroform extract, ethanol precipitate and resuspend DNA in TE buffer. Use this DNA as template PCR with the forward primer (5'-GGGAAGCTTGCTAGCAATC-TGCCAGTGGATATTTGGACC-3') and the reverse primer (5'-CCCAAGCTTGTC-GACTACAAAAAGAGTGTTTCAAA-3') to create the 186 bp α-satellite DNA to wrap around the histone octamer. Mix 10 µg of histone octamer with 10 µg of satellite DNA in 6 M urea, 2 M NaCl and 1 mg/ml BSA (100 µl final). Dialyze against the following sequence of buffers at 4˚C (2 hrs each): 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM DTT, with 2 M, 0.85 M, 0.65 M, and 0.2 M NaCl, successively. To confirm reconstitution run a gel shift assay. Perform HAT assay as described above by substituting the nucleosomal complex in place of the free histones.

Yeast two hybrid assay. MPtpB was cloned into pAS4 containing an EcoRI site with an alanine linker in the forward primer and a PstI site in the reverse primer.

MPtpB:pAS4 was transformed into the yeast strain p69A and selected on synthetic complete (SC) lacking Ura plates following the protocol in the Clontech Matchmaker manual. A *M. tuberculosis* genomic library was transformed into MPtpB:pAS4 expressing yeast cells using the scaled-up protocol suggested in the Clontech Matchmaker manual. The cells were plated on SC lacking Ura, Leu, Ade and His + X-α-gal (20μg/ml, Glycosynth, England, UK) plates and incubated at 30˚C. Colonies that grew and turned blue were transferred to 96-well plates containing SC lacking Leu media in order to drop out the bait. The colonies were replica plated to another 96-well plate containing SC lacking Leu media. Following growth of this plate, colonies were replica plated to SC lacking Leu + 5-fluoroorotic acid (FOA, 1 mg/ml) plates. Surviving colonies that lost the bait plasmid were replica plated to another 96-well plate containing SC lacking Leu media and then replica plated to SC lacking Leu + FOA plates to confirm the bait was lost. Those colonies that did not grow were transferred from the frozen glycerol stock plate to a new plate and mated with control plasmid pLAM (-Trp) in yeast strain p69α to eliminate false positives. These transformants were replica plated to SC lacking Trp and Leu plates and then to SC lacking Trp, Leu and Ade plates. Clones that did not grow were transferred from the frozen glycerol stock plate to SC lacking Leu media and allowed to mate with MPtpB:pAS4 in yeast strain p69α. To verify positive interactions, the transformants were replica plated to SC lacking Ura and Leu plates and then to SC lacking Ura, Leu and Ade plates. Any colony that grew was lysed and sequenced using ABI Prism Big Dye Terminator reaction mix and an ABI Prism Sequencer 3700.

Phosphatase activity using pNPP. *M. smegmatis* expressing either MPtpB:pMV762, MptpB:Eis:pMV762 or control vector pMV762 were grown to mid-log phase and lysed in enzyme dilution buffer (50 mM Bis-Tris, 2 mM EDTA, 5 mM DTT,

20% glycerol, 0.1% Triton X-100, pH 6.3). The reaction at 25˚C was initiated by adding 20 µl of the protein in enzyme dilution buffer to 180 µl of assay buffer (50 mM Bis-Tris, 2 mM EDTA, 5 mM DTT, pH 6.3) containing various amounts of pNPP (16.7-0.17 mM) in a 96-well plate and mixed vigorously for 1 min. The reaction was terminated 3 min later with 20 µl of 10N NaOH and read at 405 nm on a plate reader (Versamax from Molecular Devices).

RESULTS

Interaction of MPtpB with Eis and fbpA. To determine the substrates for MPtpB in mycobacteria, a yeast two hybrid screen was performed on a *M. tuberculosis* H37Rv genomic library. The proteins MPtpB interacted with from the *M. tuberculosis* genomic library are listed in Figure 1a. To verify the interaction between MPtpB and Eis, an operon was designed for *M. smegmatis* where MPtpB and Eis were cloned into the pMV762 plasmid and transformed into *M. smegmatis*. Mycobacterial cultures were grown to mid-logarithmic phase growth, harvested, immunoprecipitated with either α-MPtpB or α-c-myc and immunoblotted. As seen in Figure 1b MPtpB was capable of interacting with both Eis and fbpA.

Identifying the region of MPtpB which interacts with Eis. To better understand the region(s) of interaction between MPtpB and Eis, truncated mutants of MPtpB were created in the operon plasmid (Figure 1c). Each truncated mutant was designed to be thirty amino acids shorter than the previous mutant for a total of eight mutants. The mycobacterial mutants were grown to mid-log phase growth, harvested and lysed. The mycobacterial lysates were immunoprecipitated with either α-MPtpB or α-c-myc bound to Protein G beads and immunoblotted. When MPtpB was truncated from either the ami-

no or carboxyl terminus, Eis was still able to interact with MPtpB, albeit more strongly in the truncated mutants than in wild type (Figure 1d-g). Figure 1h shows levels of MPtpB and Eis protein expression in the mycobacterial lysates. If a portion of the catalytic domain of MPtpB was deleted (Δ150-210), the interaction between MPtpB and Eis was lost (Figure 1i; let's hope this is it).

Eis acetylates free histones. Since various prediction programs indicated that Eis was a putative acetyltransferase, a free histone assay was performed. Eis consists of an acetyltransferase domain at the amino terminus between amino acids 50-132 (Figure 2a). When Eis was aligned with other GNAT family members, a key tyrosine residue (Y126) was conserved which if mutated, ablates acetyltransferase activity. So, to confirm acetyltransferase activity, Eis WT, catalytically inactive Eis Y126F mutant and a negative control acetyltransferase, aminoglycoside 2'-N-acetyltransferase (AAC(2')-IC), was cloned into the pMV762 plasmid. These proteins were immunoprecipitated from mid-log phase growth *M. smegmatis* with FLAG agarose beads and then incubated with free histones and ^{14}C-Acetyl CoA. As seen in Figure 2b, Eis WT is capable of acetylating free histones, as compared to the positive control, p300, whereas the catalytically inactive Eis Y126F mutant and the negative control AAC(2')-IC were not capable of acetylating free histones (Figure 2c).

Since Eis is able to acetylate free histones, we next wanted to determine if Eis could acetylate a nucleosomal complex. Eis WT and the catalytically inactive Eis Y126F mutant were examined and Figure 2d shows that Eis was not able to acetylate a nucleosomal complex as compared to the positive control p300. In summary, Eis is an acetyltransferase but histones are not its primary target.

Tyrosine phosphorylation of Eis. To better understand the interaction between the tyrosine phosphatase MPtpB and the acetyltransferase Eis, we hypothesized that Eis could be modified in the host macrophage by becoming tyrosine phosphorylated causing Eis to become inactive. Since MPtpB interacts with Eis, we hypothesized that MPtpB could possibly play a role in dephosphorylating Eis thereby allowing Eis to become active again. Eis WT and various tyrosine mutants of Eis were cloned into pIRESpuro2 and transiently transfected into 293 cells. The transfected cells were treated with pervanadate and lysed and the lysates were immunoprecipitated with FLAG agarose beads to pull down phosphorylated Eis and its various tyrosine single and double mutants and immunoblotted with the anti-phosphotyrosine antibody, 4G10. As seen in Figure 3, Eis is capable of tyrosine phosphorylation whereas the catalytically inactive tyrosine (Y126) and another downstream tyrosine (Y179) have decreased phosphorylation when singly mutated but when double mutated, tyrosine phosphorylation of Eis is ablated. Even though Eis is tyrosine phosphorylated, MPtpB does not seem capable of dephosphorylating Eis.

Phosphatase activity of MPtpB with Eis. We next wanted to see if Eis was either interfering or enhancing the PTPase activity of MPtpB so we performed a pNPP assay. Again, *M. smegmatis* expressing either MPtpB alone or in the operon with Eis in the pMV762 plasmid were grown to mid-log phase growth and harvested. The whole cell lysates were then used with various concentrations of pNPP. As seen in Figure 4, MPtpB alone has phosphatase activity, but when coexpressed with Eis, the activity of MPtpB is reduced.

DISCUSSION

Mycobacterial species are well suited to survive the hostile environment of macrophage phagosomes by using several methods that are not seen in other bacteria. Yet there is still a poor understanding of the mechanisms of interaction between mycobacteria and host cell proteins and the consequent changes that occur in the signaling pathways such as downregulation of TLRs, MAPKs and JAK/STATs. What is known is that the entry and survival of intracellular pathogens in host cells requires a complex dialogue of signaling events between the host cells and the pathogenic bacteria. Such dialogue requires mycobacterial species to secrete virulence factors into the host cell to reprogram the host signaling network to allow mycobacteria to propagate. Two such potential virulence factors are MPtpB and Eis.

To determine what other proteins MPtpB interacts with in *M. tuberculosis*, a yeast two hybrid screen was performed on a genomic *M. tuberculosis* library. This library screen revealed that MPtpB interacted with enhanced intracellular survival (Eis) gene. Previous reports demonstrated that Eis enhanced the survival of non-pathogenic *M. smegmatis* in a macrophage-like cell line, U-937 (16). Recently, Eis has been shown to modulate secretion of IL-10 and TNF-α in primary human monocytes in response both to infection with *M. tuberculosis* and to stimulation with recombinant Eis protein (13). To delineate the region where Eis interacts with MPtpB, truncated mutants of MPtpB were designed and inserted into an operon containing Eis. From the results obtained, it appears that Eis might be interacting inside the catalytic region of MPtpB.

Analysis of the sequence of Eis revealed it to have a putative acetyltransferase domain. Eis was found to closely resemble the GCN5-related N-acetyltransferases (GNAT) superfamily. This superfamily has been shown to be involved in acetylating his-

tones, allowing bacteria to be resistant to antibiotics and converting serotonin to melatonin in the involvement of the light-dark cycle (4). Comparing other known GNAT members to Eis revealed a conserved, key tyrosine residue (Y126) found in the catalytic region, or Motif B. In this study, Eis was shown to acetylate free histones, whereas mutating this key tyrosine residue to a phenylalanine ablated free histone acetylation. Eis, though, was not able to acetylate histones found in a nucleosomal complex. In summary, Eis has been demonstrated in this study to be an acetyltransferase with the key tyrosine residue being involved in this activity, but that histones were not the main target of Eis.

Why then does the tyrosine phosphatase MPtpB interact with the acetyltransferase Eis? It might be possible for MPtpB to influence the activity of Eis. Eis was discovered to become tyrosine phosphorylated, albeit not as pronounced as its level of expression in the 293T cell line. When examining various tyrosine mutants of Eis that were capable of becoming phosphorylated, it was seen that the key catalytic tyrosine residue (Y126) and another tyrosine downstream (Y179) were the tyrosines involved in phosphorylation, as seen by the double mutant. Although we could not show that MPtpB was involved in dephosphorylating tyrosine phosphorylated Eis it is possible that MPtpB might regulate the function of Eis by dephosphorylating the tyrosines of Eis. On the other hand, Eis was capable of inhibiting the PTPase activity of MPtpB as seen by the pNPP results. Eis could play a role in keeping MPtpB in check while residing inside the mycobacterium.

REFERENCES

1. **Black, D. S., and J. B. Bliska.** 1997. Identification of p130Cas as a substrate of Yersinia YopH (Yop51), a bacterial protein tyrosine phosphatase that translocates into mammalian cells and targets focal adhesions. Embo J **16:**2730-44.

2. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell.** 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature **393:**537-44.

3. **Dahl, J. L., J. Wei, J. W. Moulder, S. Laal, and R. L. Friedman.** 2001. Subcellular localization of the Iitracellular survival-enhancing Eis protein of Mycobacterium tuberculosis. Infect Immun **69:**4295-302.

4. **Dyda, F., D. C. Klein, and A. B. Hickman.** 2000. GCN5-related N-acetyltransferases: a structural overview. Annu Rev Biophys Biomol Struct **29:**81-103.

5. **Fu, Y., and J. E. Galan.** 1999. A salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. Nature **401:**293-7.

6. **Grundner, C., H. L. Ng, and T. Alber.** 2005. Mycobacterium tuberculosis protein tyrosine phosphatase PtpB structure reveals a diverged fold and a buried active site. Structure **13:**1625-34.

7. **Kennelly, P. J., and M. Potts.** 1996. Fancy meeting you here! A fresh look at "prokaryotic" protein phosphorylation. J Bacteriol **178:**4759-64.

8. **Knutson, K. L., Z. Hmama, P. Herrera-Velit, R. Rochford, and N. E. Reiner.** 1998. Lipoarabinomannan of Mycobacterium tuberculosis promotes protein tyrosine dephosphorylation and inhibition of mitogen-activated protein kinase in human mononuclear phagocytes. Role of the Src homology 2 containing tyrosine phosphatase 1. J Biol Chem **273:**645-52.

9. **Koul, A., A. Choidas, M. Treder, A. K. Tyagi, K. Drlica, Y. Singh, and A. Ullrich.** 2000. Cloning and characterization of secretory tyrosine phosphatases of Mycobacterium tuberculosis. J Bacteriol **182:**5425-32.

10. **Maiti, D., A. Bhattacharyya, and J. Basu.** 2001. Lipoarabinomannan from Mycobacterium tuberculosis promotes macrophage survival by phosphorylating Bad through a phosphatidylinositol 3-kinase/Akt pathway. J Biol Chem **276:**329-33.

11. **Perskvist, N., L. Zheng, and O. Stendahl.** 2000. Activation of human neutrophils by Mycobacterium tuberculosis H37Ra involves phospholipase C gamma 2, Shc adapter protein, and p38 mitogen-activated protein kinase. J Immunol **164:**959-65.

12. **Persson, C., N. Carballeira, H. Wolf-Watz, and M. Fallman.** 1997. The PTPase YopH inhibits uptake of Yersinia, tyrosine phosphorylation of p130Cas and FAK, and the associated accumulation of these proteins in peripheral focal adhesions. Embo J **16:**2307-18.

13. **Samuel, L. P., C. H. Song, J. Wei, E. A. Roberts, J. L. Dahl, C. E. Barry, 3rd, E. K. Jo, and R. L. Friedman.** 2007. Expression, production and release of the Eis protein by Mycobacterium tuberculosis during infection of macrophages and its effect on cytokine secretion. Microbiology **153:**529-40.

14. **Singh, R., V. Rao, H. Shakila, R. Gupta, A. Khera, N. Dhar, A. Singh, A. Koul, Y. Singh, M. Naseema, P. R. Narayanan, C. N. Paramasivan, V. D. Ramanathan, and A. K. Tyagi.** 2003. Disruption of mptpB impairs the ability of Mycobacterium tuberculosis to survive in guinea pigs. Mol Microbiol **50:**751-62.

15. **Stone, R. L., and J. E. Dixon.** 1994. Protein-tyrosine phosphatases. J Biol Chem **269:**31323-6.

16. **Wei, J., J. L. Dahl, J. W. Moulder, E. A. Roberts, P. O'Gaora, D. B. Young, and R. L. Friedman.** 2000. Identification of a Mycobacterium tuberculosis gene that enhances mycobacterial survival in macrophages. J Bacteriol **182:**377-84.

17. **Yarden, Y., and A. Ullrich.** 1988. Growth factor receptor tyrosine kinases. Annu Rev Biochem **57:**443-78.

Figure 1

(a)

Gene Name	Accession #
accD5 Propyonyl-CoA carboxylase β chain 5	Rv3280
Eis "Enhanced intracellular survival"	Rv2416c
fbpA Antigen 85A	Rv3804c
PE3	Rv0159c

(b)

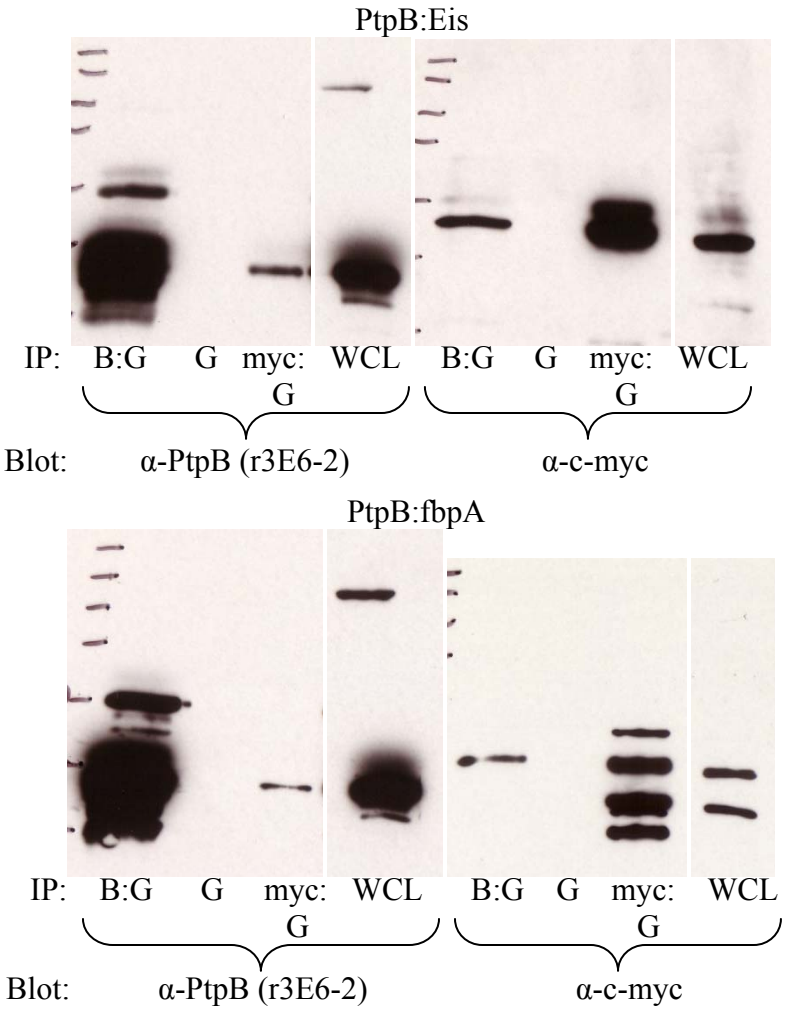
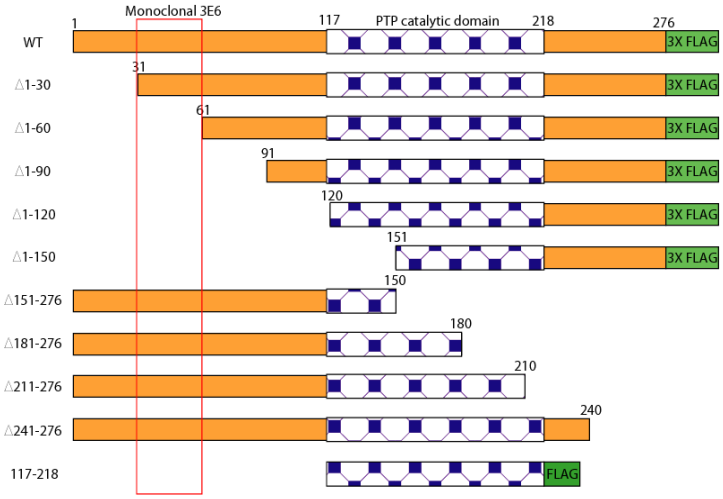


Figure 1 continued

(c)



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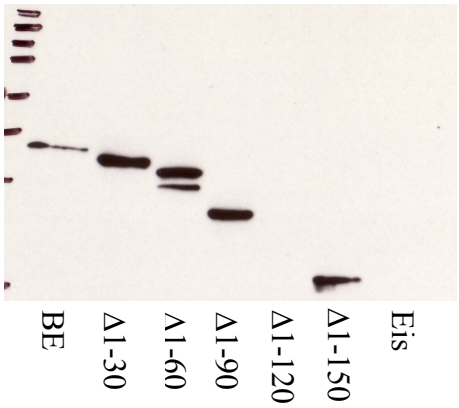
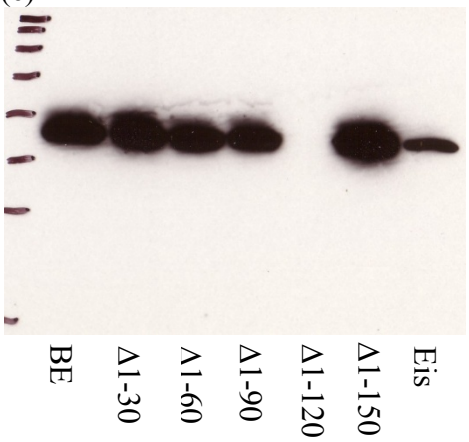


IP: myc:G
Blot: α-PtpB

(e)



B:G
α-c-myc

Figure 1 continued

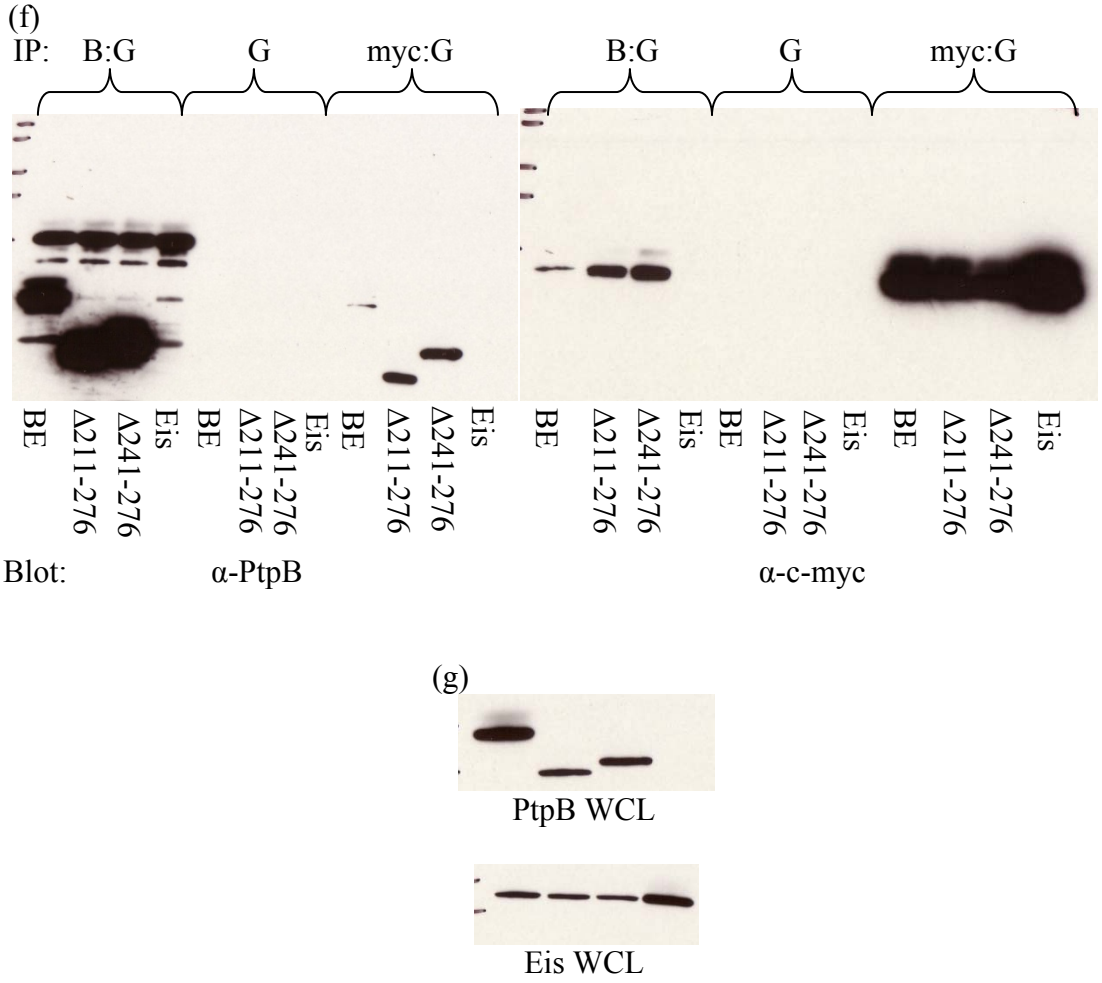
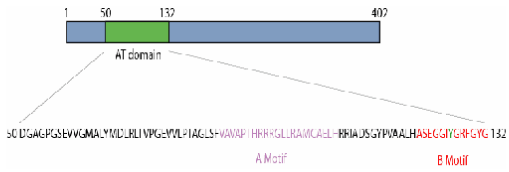


Figure 1. Interaction of MPtpB with Eis and fbpA. (a) Yeast two hybrid results of MPtpB interactions in a *M. tuberculosis* H37Rv genomic library. (b) *M. smegmatis* expressing the operons were grown to mid-log phase growth. Whole cell lysates (WCL) were immunoprecipitated with either rat α-MPtpB (3E6-2):Protein G or α-c-myc:Protein G and loaded on a 10% SDS-PAGE and probed with either α-c-myc or α-MPtpB. (c) Schematic diagram of the various truncated mutants of MPtpB inserted into the operon. The red box denotes the region of MPtpB where the rat anti-MPtpB (3E6-2) monoclonal antibody recognizes. (d,e) The amino terminus (d,e) and carboxyl terminus (f) truncated mutants of MPtpB were immunoprecipitated with the above antibodies in (b) and blotted to determine the region of MPtpB that interacts with Eis. (g) Immunoblots showing levels of protein expression of MPtpB and Eis in *M. smegmatis* WCL.

Figure 2

(a)



N-acetyltransferase domain consists of 4 motifs
C: involved in substrate recognition
D: probably involved in substrate recognition
A: acetyl-CoA contacts
B: catalytic region

Least conserved

Most conserved

M. tuberculosis DGAGPGSEVVGMALYMDLRLTVPGEVVLPTAGLSFVAVAPTHRRRGLLRA
M. bovis DGAGPGSEVVGMALYMDLRLAVPGEVVLPTAGLSFVAVAPTHRRRGLLRA
M. ulcerans VVGMALFIDLQLTVPGGNVLSTAGMSIVAVAPTHRRRGLLRA

A Motif

M. tuberculosis MCAELHRRIADSGYPVAALHASEGGIYGRFGYG
M. bovis MCAELHRRIADSGYPVAALHASEGGIYGRFGYG
M. ulcerans MCGELHRRMADSGYAVAALHASEGGIYGRFGYG

B Motif

(b)

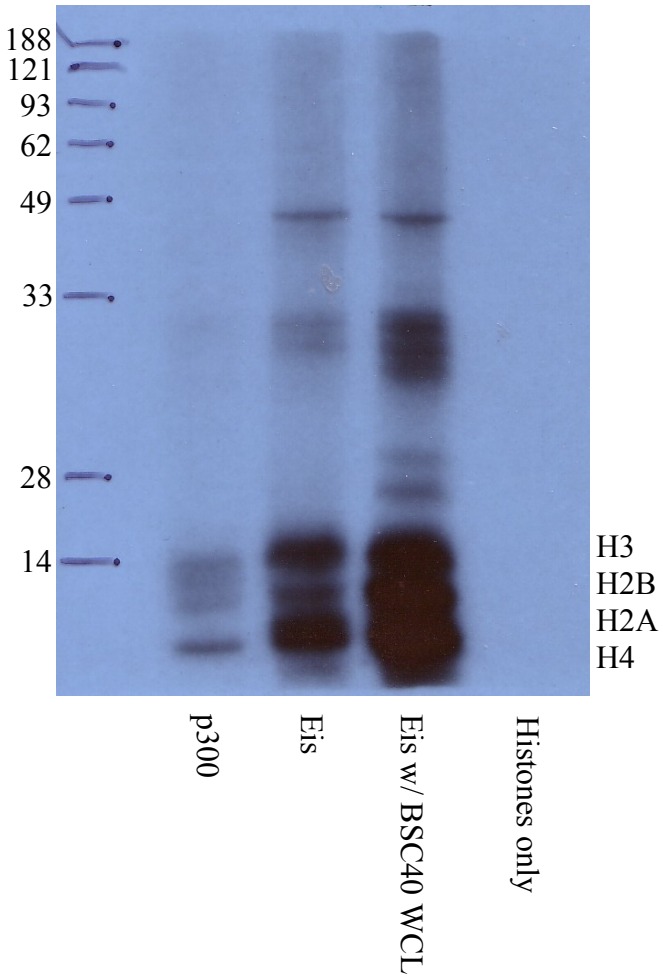


Figure 2 continued

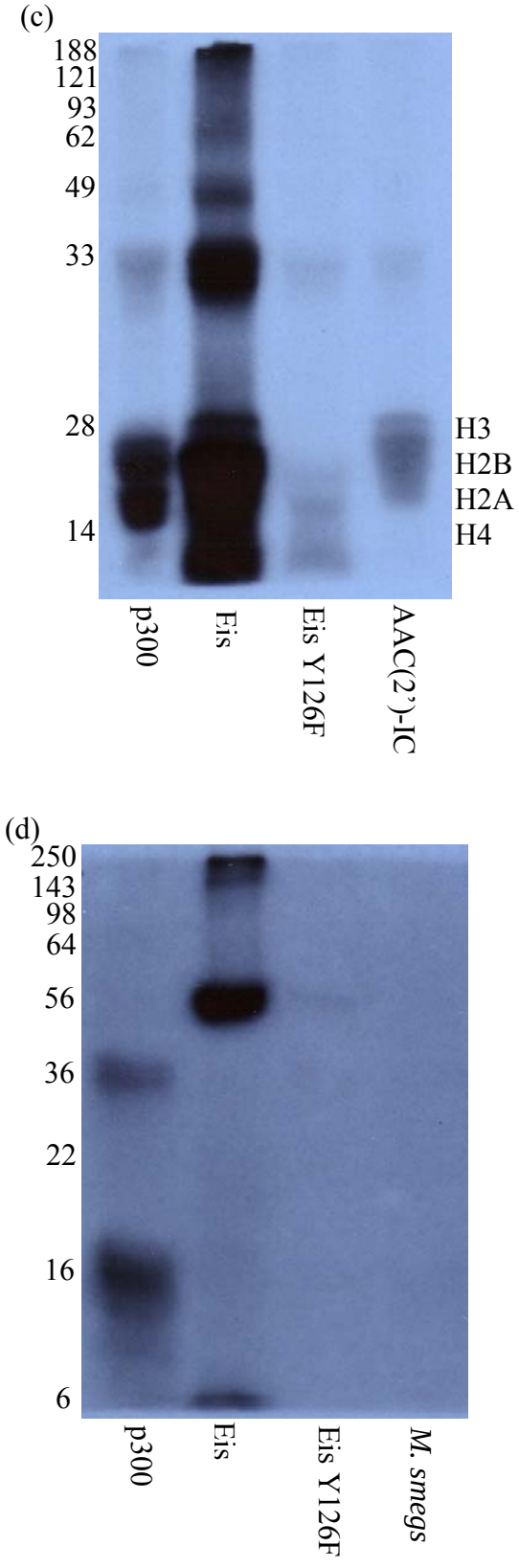


Figure 2 continued

(e)

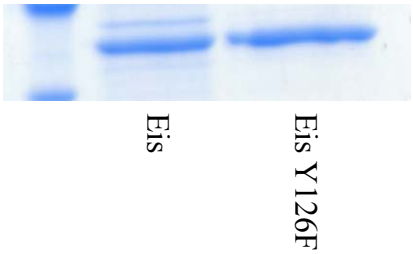


Figure 2. Acetyltransferase activity of Eis. (a) Schematic diagram of the putative acetyltransferase site of Eis and an alignment of the catalytic region with other pathogenic mycobacteria species. (b,c) In the HAT assay on free histones, *M. smegmatis* whole cell lysates expressing either Eis, catalytically inactive Eis Y126F mutant, AAC(2')-IC or *M. smegmatis* alone were immunoprecipitated with FLAG agarose beads. The beads were then incubated with free histones and ^{14}C-Acetyl CoA and loaded on a 15% SDS-PAGE. Histone H3 = 17 kDa; histone H2B = 15 kDa; histone H2A = 13.5 kDa; histone H4 = 10 kDa. (d) in the HAT assay on nucleosomes *M. smegmatis* expressing either Eis or catalytically inactive Eis Y126F mutant were harvested and immunoprecipitated as described in (b,c) and incubated with the nucleosomal complex and ^{14}C-Acetyl CoA and loaded on a 15% SDS-PAGE. (e) Coomassie of immunoprecipitated Eis and Eis Y126F showing equal loading for the above assays.

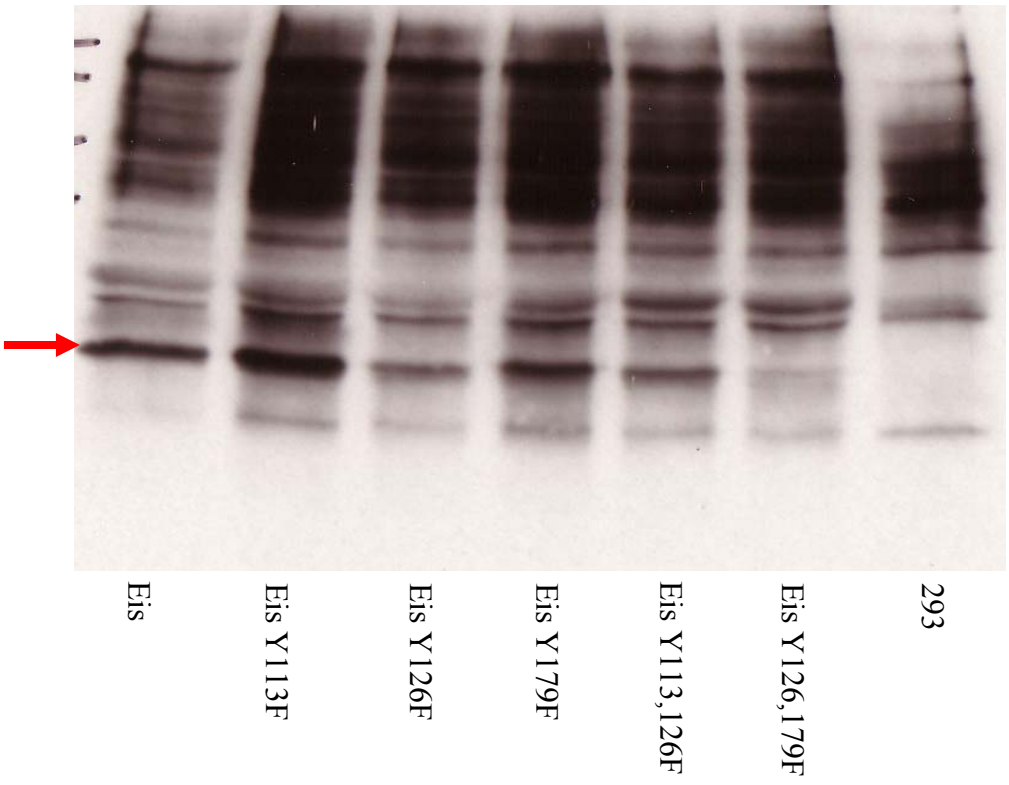
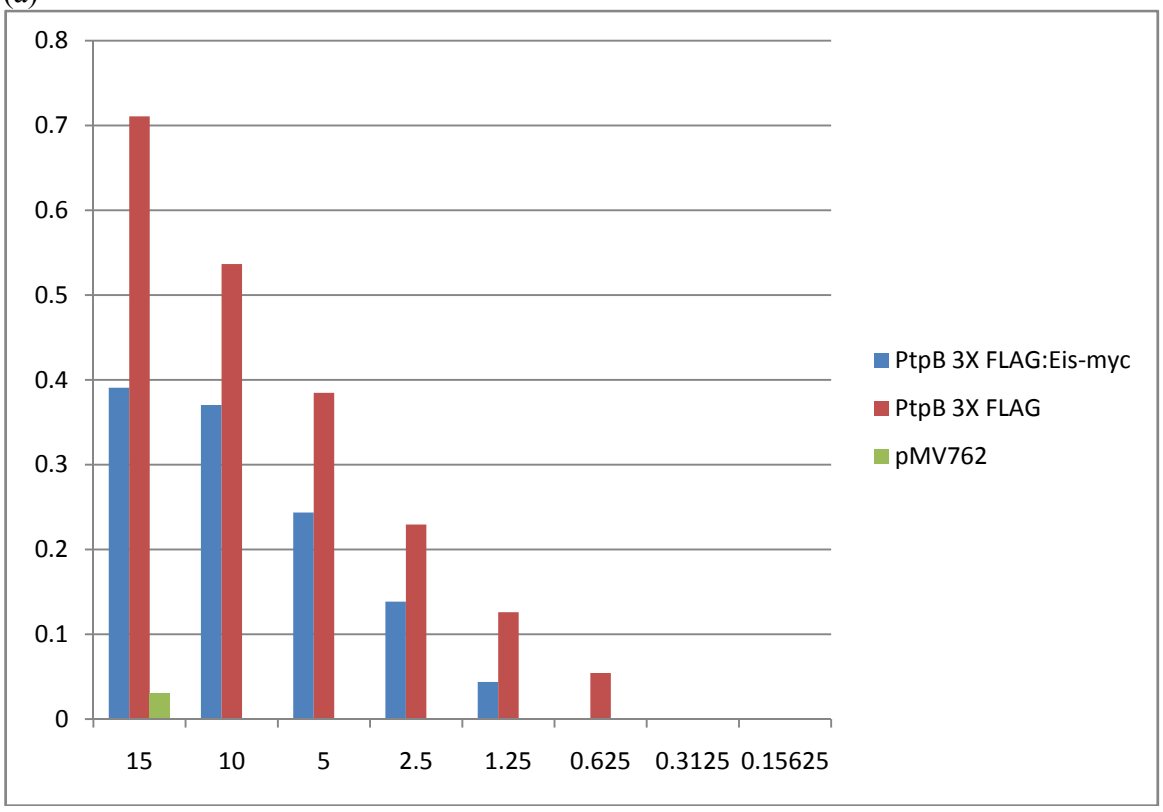


Figure 3. Tyrosine phosphorylation of Eis. Eis (46 kDa) and its tyrosine mutants were transiently transfected into 293 cells and pervanadate treated. Eis and the mutants were immunoprecipitated with FLAG agarose and loaded on a 10% SDS-PAGE and probed with anti-phosphotyrosine (4G10) HRP-conjugated antibody.

(a)



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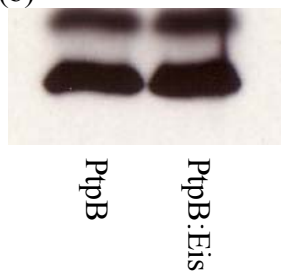

Figure 4. PTPase activity of MPtpB with Eis. (a) *M. smegmatis* expressing MPtpB alone or in the operon with Eis were grown to mid-log phase growth and harvested in enzyme dilution buffer. Various concentrations of pNPP was added to equal protein concentrations of MPtpB and the reaction was stopped three minutes later with NaOH. (b) Equal concentration of MPtpB and MPtpB expressed in the operon.

CONCLUSIONS

How the pathogenicity of *M. tuberculosis* can enable it to survive and replicate inside host macrophages remains largely a mystery. For *M. tuberculosis* to enter the macrophage and survive requires the mycobacterium to secrete virulence factors that are thought to be involved in the reprogramming of the host cell signaling network. One such virulence factor is MPtpB, a tyrosine phosphatase. Several bacterial tyrosine phosphatases such as YopH from *Yersinia pseudotuberculosis* and SptP from *Salmonella enteric* serovar Typhimurium have been shown to modify signal transduction pathways of the host for the benefit of the pathogen (1, 4, 6). When the *mptpB* gene from *M. tuberculosis* was disrupted, the ability of the mutant strain to survive in activated macrophages and guinea pigs was greatly impaired (8). Since MPtpB is important for survivability, I set out to determine what MPtpB might be targeting inside the host macrophage and also to determine what other mycobacterial virulence factors MPtpB interacts with.

In the first chapter, I wanted to use the fast-growing non-pathogenic species, *M. smegmatis* to help expedite all the experiments. First, I had to verify that MPtpB could still be secreted from the non-pathogenic strain. As seen in Figure 1, MPtpB is capable of being secreted from *M. smegmatis* indicating that the non-pathogenic strains have the same conserved secretion pathways as the pathogenic strains. These *M. smegmatis* expressing MPtpB did not have an altered growth pattern as compared to wild type *M. smegmatis* (Figure 2). When the *M. smegmatis* expressing MPtpB were used to deter-

mine intracellular survival inside the host macrophage there was no significant increase in their survival (Figure 3) indicating that the macrophages were clearing the non-pathogenic strains too quickly for MPtpB to have time to affect the host cell signaling pathways. Since MPtpB does not contain a secretory signal peptide, truncated mutants of MPtpB were created (Figure 7) to determine the domain that was involved in secretion. As seen in Figure 8, deleting the region between amino acids 151-210 of MPtpB causes a decrease in the amount of MPtpB protein in the culture supernatant. To determine the host macrophage substrates of MPtpB, a yeast two hybrid screen was performed on a mouse lung infected with *M. tuberculosis* library. As seen in Table 1, MPtpB was found to interact with various macrophage substrates, but after further analysis none of the substrates seemed plausible for tyrosine dephosphorylation by MPtpB.

The next chapter demonstrates the interaction between MPtpB and Eis. A yeast two hybrid screen was performed on a *M. tuberculosis* genomic library to determine what other virulence factors MPtpB interacts with. As seen in Figure 1a, MPtpB interacts with Eis. Eis was shown to enhance *M. smegmatis* intracellular survival in the human macrophage cell line U-937 (9). Recently, Eis was shown to modulate the secretion of IL-10 and TNF-α by primary human monocytes in response both to infection with *M. tuberculosis* and to stimulation with recombinant Eis protein (7). In this study, an operon was designed that included MPtpB and Eis and expressed in *M. smegmatis* to verify the interaction. In Figure 1b, MPtpB was capable of interacting with Eis by either immunoprecipitating MPtpB or Eis and then immunoblotting with either MPtpB or Eis. To determine the region of MPtpB involved in the interaction with Eis, the truncated mutants of MPtpB were used (Figure 1c). As seen in Figures 1d-g, the truncated mutants of MPtpB did not lose it ability to interact with Eis. It is thought that the interaction between MPtpB and

Eis must lie within the catalytic region of MPtpB and further studies need to be completed to verify the catalytic region deletion of amino acids 150-210. BLAST analysis revealed that Eis is an acetyltransferase belonging to the GCN5-related family of N-acetyltransferases. A schematic of the acetyltransferase domain of Eis is shown in Figure 2a. To verify that Eis is indeed an acetyltransferase, a free histone assay was performed. In Figures 2b-c, Eis is capable of acetylating free histones whereas the catalytically inactive mutant of Eis (Y126F) was not capable of acetylation. Histones were not the primary target of Eis as seen in Figure 2d when a nucleosomal assay was performed on histones found in a nucleosomal complex. Surprisingly, Eis has the ability to become tyrosine phosphorylated. The tyrosines were mapped to determine which tyrosine was involved in phosphorylation. Figure 3 revealed that the catalytically inactive mutant (Y126F) and another tyrosine residue further downstream (Y179F) were involved in tyrosine phosphorylation as seen by the double mutant. Unfortunately, MPtpB, a tyrosine phosphatase was not capable of dephosphorylating Eis. To determine if Eis had any effect on MPtpB phosphatase activity, a pNPP assay was performed. As seen in Figure 4, Eis downregulates the phosphatase activity of MPtpB. It is plausible that Eis is suppressing the activity of MPtpB when inside the mycobacterium.

GENERAL LIST OF REFERENCES

1. **Black, D. S., and J. B. Bliska.** 1997. Identification of p130Cas as a substrate of Yersinia YopH (Yop51), a bacterial protein tyrosine phosphatase that translocates into mammalian cells and targets focal adhesions. Embo J **16:**2730-44.

2. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell.** 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature **393:**537-44.

3. **Dahl, J. L., J. Wei, J. W. Moulder, S. Laal, and R. L. Friedman.** 2001. Subcellular localization of the Iitracellular survival-enhancing Eis protein of Mycobacterium tuberculosis. Infect Immun **69:**4295-302.

4. **Fu, Y., and J. E. Galan.** 1999. A salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. Nature **401:**293-7.

5. **Koul, A., A. Choidas, M. Treder, A. K. Tyagi, K. Drlica, Y. Singh, and A. Ullrich.** 2000. Cloning and characterization of secretory tyrosine phosphatases of Mycobacterium tuberculosis. J Bacteriol **182:**5425-32.

6. **Persson, C., N. Carballeira, H. Wolf-Watz, and M. Fallman.** 1997. The PTPase YopH inhibits uptake of Yersinia, tyrosine phosphorylation of p130Cas and FAK, and the associated accumulation of these proteins in peripheral focal adhesions. Embo J **16:**2307-18.

7. **Samuel, L. P., C. H. Song, J. Wei, E. A. Roberts, J. L. Dahl, C. E. Barry, 3rd, E. K. Jo, and R. L. Friedman.** 2007. Expression, production and release of the Eis protein by Mycobacterium tuberculosis during infection of macrophages and its effect on cytokine secretion. Microbiology **153:**529-40.

8. **Singh, R., V. Rao, H. Shakila, R. Gupta, A. Khera, N. Dhar, A. Singh, A. Koul, Y. Singh, M. Naseema, P. R. Narayanan, C. N. Paramasivan, V. D. Ramanathan, and A. K. Tyagi.** 2003. Disruption of mptpB impairs the ability of Mycobacterium tuberculosis to survive in guinea pigs. Mol Microbiol **50:**751-62.

9. **Wei, J., J. L. Dahl, J. W. Moulder, E. A. Roberts, P. O'Gaora, D. B. Young, and R. L. Friedman.** 2000. Identification of a Mycobacterium tuberculosis gene that enhances mycobacterial survival in macrophages. J Bacteriol **182:**377-84.