Nα-acetylation of the virulence factor EsxA is required for mycobacterial cytosolic translocation and virulence

Javier Aguilera1, Chitra B. Karki2, Lin Li2, Salvador Vazquez-Reyes1, Igor Estevao1, Brian I. Grajeda1, Qi Zhang1,3, Chenoa D. Arico1, Hugues Ouellet1 and Jianjun Sun1*

1Department of Biological Sciences and Border Biomedical Research Center, University of Texas at El Paso, El Paso, TX 79968, USA
2Department of Physics, University of Texas at El Paso, El Paso, TX 79968, USA
3Current address: Zhengzhou University School of Pharmacy, Zhengzhou, P. R. China
*Corresponding: jsun@utep.edu

Running title: Nα-acetylation of EsxA facilitates heterodimer separation

Abstract: The Mycobacterium tuberculosis virulence factor EsxA and its chaperone EsxB are secreted as a heterodimer (EsxA:B) and are crucial for mycobacterial escape from phagosomes and cytosolic translocation. Current findings support the idea that in order for EsxA to interact with host membranes, EsxA must dissociate from EsxB at low pH. However, the molecular mechanism by which the EsxA:B heterodimer separates is not clear. In the present study, using liposome-leakage and cytotoxicity assays, LC-MS/MS–based proteomics, and CCF-4 FRET analysis, we obtained evidence that the Nα-acetylation of the Thr-2 residue on EsxA, a post-translational modification that is present in mycobacteria but absent in Escherichia coli, is required for the EsxA:B separation. Substitutions at Thr-2 that precluded Nα-acetylation inhibited the heterodimer separation and hence prevented EsxA from interacting with the host membrane, resulting in attenuated mycobacterial cytosolic translocation and virulence. Molecular dynamics simulations revealed that at low pH, the Nα-acetylated Thr-2 makes direct and frequent “bind-and-release” contacts with EsxB, which generates a force that pulls EsxB away from EsxA. In summary, our findings provide evidence that the Nα-acetylation at Thr-2 of EsxA facilitates dissociation of the EsxA:B heterodimer required for EsxA membrane permeabilization and mycobacterial cytosolic translocation and virulence.

Introduction

Mycobacterium tuberculosis (Mtb) is the causative agent for tuberculosis (TB), one of the leading infectious diseases in the world with 10 million people falling ill in 2017, and approximately 1.6 million deaths (1, 2). It is believed that after the Mtb-containing aerosolized droplets are inhaled into the lung, Mtb is encountered by alveolar macrophages and internalized into the phagosome, where Mtb manages to survive through arresting phagosome maturation, including inhibition of vATPase-mediated acidification (3-6). Recent compelling evidence support that Mtb penetrates the phagosome and translocates into the cytosol (termed cytosolic translocation), where Mtb replicates and undergoes cell-to-cell spreading (7). The ability of Mtb to arrest phagosome maturation and to translocate from the phagosome to the cytosol has been attributed, at least in part, to the Type VII secretion system, named ESX-1 and the secreted virulence factors EsxA (ESAT-6) and EsxB (CFP-10). The Mtb mutants with either gene deletions or defects in secretion of EsxA and/or EsxB, were not able to translocate into the cytosol and showed significant reduction in host cell lysis and cell-to-cell spreading (7-19).

In our previous studies, we have found that Mtb EsxA exhibits a unique membrane-permeabilizing activity that is not present in the homologous EsxA ortholog from non-pathogenic Mycobacterium smegmatis (Ms) (20). EsxA
undergoes pH-dependent conformational changes, inserts into the membrane and forms a membrane-spanning complex (21). The essential role of EsxA membrane-permeabilizing activity in phagosome rupture and cytosolic translocation is further confirmed by a recent study, in which single-residue mutations at Gln5 (Q5) of EsxA up or down regulated the membrane-permeabilizing activity and consequently up or down regulated mycobacterial cytosolic translocation and virulence in cultured cells and in zebra fish (22).

The genes encoding EsxA and EsxB are located in the same operon within the ESX-1 locus. EsxA and EsxB are co-expressed and co-secreted as a heterodimer (23). Our earlier study has demonstrated that EsxA, but not EsxB, has the membrane-permeabilizing activity, and EsxB is believed to function as a chaperon (20). Current studies support a model that the heterodimer is dissociated at low pH to allow EsxA to penetrate the membranes (24). However, the data regarding to the heterodimer dissociation are conflicting. The native heterodimer extracted from Mtb culture filtrate was found to be dissociated at low pH (24). Surprisingly, however, the studies using the recombinant proteins prepared from E. coli suggest that the heterodimer was not dissociated by acidification. This is evidenced by one of our earlier studies that the heterodimer prepared from E. coli (now termed Ec-heterodimer) was inactive in membrane disruption. In the absence of lipid membranes, EsxA formed aggregates in the acidic solution due to increased solvent-exposed hydrophobicity. In contrast, the heterodimer showed little aggregation at pH 4.0, suggesting that EsxB remains bound to EsxA at low pH and prevents EsxA from forming aggregates, which otherwise would be observed if EsxA was released from EsxB (20). Our data are consistent with an earlier CD analysis showing that the Ec-heterodimer is not dissociated at low pH (25).

We hypothesized that the mycobacteria-produced proteins contain unique features (e.g. post-translational modifications, PTMs) that are required for heterodimer dissociation at low pH. In line with this hypothesis, the native EsxA protein isolated from the culture filtrate of Mtb was displayed as multiple spots in 2D SDS-PAGE, and some of the spots contained a Nα-acetylation at the residue Thr2 (26). Moreover, the heterodimer produced from a Ms strain was found to have a Nα-acetylation on the Thr2 residue of EsxA (27). Interestingly, EsxB preferred to bind the non-acetylated EsxA, but not the acetylated form in a 2-D overlay assay (26). Deletion of the Nα-acetyltransferase in Mycobacterium marinum (Mm) disrupted the homeostasis of EsxA Nα-acetylation and attenuated the virulence (28). Together, these studies suggest that the Nα-acetylation of EsxA plays an important role in mycobacterial virulence through facilitating heterodimer dissociation at low pH.

In the present study, we have obtained the evidence showing that the Nα-acetylation at Thr2 of EsxA is required for EsxA membrane permeabilization, mycobacterial cytosolic translocation and virulence through facilitating heterodimer dissociation.

Results

The Ms-produced Mtb heterodimer, but not Ec-produced heterodimer, disrupted liposomal membrane at low pH. We hypothesized that Ms-heterodimer, but not Ec-heterodimer, dissociates at low pH and permeabilizes the liposomal membrane. The membrane-permeabilizing activity of Ms-heterodimer and Ec-heterodimer was tested with the ANTS/DPX fluorescence de-quenching assay. As expected, Ms-heterodimer permeabilized the membrane at low pH, while Ec-heterodimer was not active (Fig. 1A). NBD–Cl (4-chloro-7-nitrobenzof-urazan) only reacts with free N-terminal α-amino group in non-acetylated proteins and emits fluorescence, but it does not react with Nα-acetylated proteins due to lack of free N-terminal amino group. Thus, we used NBD-Cl to test the states of Nα-acetylation for Ms-heterodimer and Ec-heterodimer. As expected, the Ms-heterodimer exhibited a
significantly lower NBD-Cl fluorescence, compared to Ec-heterodimer, indicating that Ms-heterodimer, but not Ec-heterodimer, is Nα-acetylated (Fig. 1B).

The mutations at Thr2 abolished the membrane-permeabilizing activity of the Ms-heterodimers through blocking separation of EsxA and EsxB.

The Q and A residues have been used to functionally mimic acetylation of amino group of an internal K residue, while R serves as a non-acetylated control (29). Thus, we generated T2A, T2Q and T2R mutations and tested the effects of these mutations on the heterodimer membrane-permeabilizing activity.

Unexpectedly, all of the mutations abolished the Ms-heterodimer’s membrane-permeabilizing activity (Fig. 2A and B). The result suggests that either the mutations blocked the heterodimer separation or abolished the EsxA membrane-permeabilizing activity. To test if the mutations abolished the membrane-permeabilizing activity, we purified the EsxA proteins containing the same mutations from E. coli and applied them to ANTS/DPX dequenching assay. The result showed that the mutations did not affect EsxA membrane-permeabilizing activity (Fig. 2C and D), suggesting that the mutations at Thr2 blocked the heterodimer separation at low pH.

The mutations that block EsxA and EsxB separation do not have Nα-acetylation.

To validate the acetylation state of EsxA wild type and the mutants, we developed a protocol to isolate EsxA and EsxB from the Ms-heterodimer proteins (Fig. 3A). Then the presence of Nα-acetylation on the EsxA proteins was measured by NBD-Cl (Fig. 3B). Consistent with the results in Figure 1B, Ec-EsxA(WT) had a significantly higher fluorescence signal than Ms-EsxA(WT). Similar to Ec-EsxA(WT), the Ms-EsxA mutants (T2A, T2Q, and T2R) emitted significantly higher fluorescence signals than Ms-EsxA(WT), suggesting that the Ms-EsxA mutants were not Nα-acetylated. Next, we applied Ms-EsxA(WT), Ms-EsxA(T2A) and Ec-EsxA(WT) to LC-MS/MS to further confirm the Nα-acetylation states. In addition, Ms-EsxA(T2S), a mutant equivalent to WT, was included in the LC-MS/MS analysis. The results showed that both Ms-EsxA(WT) and Ms-EsxA(T2S) had the first Met residue removed and the second residue (either Thr2 or Ser2) acetylated (Fig. 3C). While Ms-EsxA(T2A) had the first Met residue removed, the second Ala residue was not acetylated. The Ec-EsxA(WT) still has the first Met residue. Interestingly, the LC-MS/MS detected multiple acetylation and oxygenation modifications in the internal sequences of both Ms-EsxA and Ec-EsxA and the roles of these modifications are currently unknown.

EsxB preferred to bind non-acetylated EsxA to inhibit the membrane-permeabilizing activity. An earlier study has shown that EsxB preferred to bind non-acetylated EsxA than acetylated EsxA in a 2D overlay assay (26). Thus, we hypothesize that EsxB will prefer to inhibit the membrane-permeabilizing activity of the non-acetylated Ms-EsxA(T2A) than that of the acetylated Ms-EsxA(WT). First, we tested and confirmed that the proteins Ms-EsxA(WT) and Ms-EsxA(T2A) that were isolated from the heterodimers had similar membrane-permeabilizing activity to Ec-EsxA(WT), which once again confirms that the states of Nα-acetylation do not affect membrane-permeabilizing activity (Fig. 4A and B). Then, Ms-EsxA(WT) and Ms-EsxA(T2B) were incubated with EsxB at a series of EsxB/EsxA molar ratios. As expected, EsxB preferred to inhibit Ms-EsxA(T2A) than Ms-EsxA(WT) in membrane permeabilization, especially at the lower EsxB/EsxA ratios (Fig. 5A, B and C).

The mutations without Nα-acetylation attenuated mycobacterial virulence and inhibited cytosolic translocation.

Here, we investigated the effects of the T2X mutations in mycobacterial pathogenesis. The genes carrying T2X mutations were expressed in the MmΔEsxA:B strain, in which the endogenous
esxB-esxA operon was deleted. We found that the T2X mutations did not affect the expression and secretion of EsxA and EsxB in the Mm strains (Fig. S1). As expected, the Mm strains carrying the non-acetylated mutations T2A, T2Q and T2R had a significantly lower cytotoxicity than the strain carrying the acetylated mutation T2S (Fig. 6A). Mm wild type and MmΔEsxA:B were used as the positive control and negative control, respectively. Furthermore, using the previously established CCF4-FRET assay, we found that the non-acetylated mutations T2A, T2Q and T2R abolished mycobacterial cytosolic translocation, while T2S maintained a similar activity as the wild type (Fig. 6B). The data is consistent to the previous report that deletion of a Nα-acetyltransferase in Mm disrupted the homeostasis of EsxA acetylation and attenuated the virulence (28).

**Molecular dynamic simulation detects frequent “bind-and-release” contacts between the acetylated Thr2(Ac) and EsxB.**

The reported solution structure of EsxA:B heterodimer does not have the Nα-acetylation on Thr2, and the Thr2 residue is distal from the contact interface between EsxA and EsxB. It is not clear how the acetylation at Thr2 affects the heterodimer separation at low pH. Thus, we performed molecular dynamic (MD) simulation on the heterodimers with and without Nα-acetylation of Thr2 at pH 7 and pH 4, respectively (Movie S1 and Fig.7). At pH 7 the non-acetylated Thr2 comes in a close vicinity of EsxB, but it is unable to make a direct contact to EsxB (Fig.7A, C). Compared to non-acetylated Thr2, the acetylated Thr2(Ac) moves further away from EsxB at pH 7 (Fig. 7B, D). Interestingly, at pH 4 the non-acetylated N-terminal loop of EsxA has no direct contact with EsxB (Fig. 7E, G), but the acetylated N-terminal loop is able to make direct contacts with EsxB in a frequent “bind-and-release” mode (Movie S1 and Fig. 7F, H). The electrostatic force was calculated between two sets of residues as demonstrated in Figure S2 using DelPhiForce program. A contact is defined as two residues that contain at least a pair of atoms within 4 Å distance. The magnitude of the force is 43.959 pN and its direction is shown by the orange arrow (Fig. S2). Therefore, when the N-terminus of EsxA is moving away from EsxB, it pulls EsxB away in the direction of the arrow. Hence, the MD simulation data support that the acetylated Thr-2 residue plays a significant role in the dissociation process of the complex.

**Discussion**

EsxA and EsxB are co-expressed and co-secreted as a heterodimer in mycobacteria. The role of ESX-1, EsxA and EsxB in mycobacterial cytosolic translocation and virulence has been confirmed in a series of studies (7, 17-19). Earlier biochemical studies have demonstrated that EsxA has a pH-dependent membrane-permeabilizing activity, while EsxB appears to function as a chaperone for EsxA (20). Current studies support a model that the EsxA:B heterodimer is dissociated at low pH, which allows EsxA to permeabilize the membranes (24). However, the mechanism of the heterodimer separation is not known. For the first time, the present study tested the heterodimers containing EsxA with or without Nα-acetylation and obtained the evidence that the Nα-acetylation at Thr2 of EsxA facilitates the heterodimer separation at low pH, which allows EsxA to permeabilize liposomal membrane in vitro as well as mediate mycobacterial phagosome escape and cytosolic translocation in mycobacteria-infected macrophages.

As discussed above, the essentiality of EsxA and EsxB in mycobacterial pathogenesis has been well documented in a series of reports. Genetic manipulations that either deleted the gene of esxA or esxB, or abolished the secretion of EsxA and EsxB, have attenuated mycobacterial virulence and inhibited the phagosomal rupture, cytosolic translocation and cell-to-cell spreading (7, 10, 17-19). Moreover, the biochemical characterizations have demonstrated that EsxA possesses a unique membrane-permeabilizing activity that is not present in its ortholog in non-pathogenic M. smegmatis (20). Thus, it is reasonable to believe
that during the course of infection, the secreted EsxA exerts its membrane-permeabilizing activity to penetrate the phagosome membranes and facilitate mycobacterial cytosolic translocation. We have reported that the mutations at the Gln 5 residue of EsxA (e.g., Q5V and Q5K) have resulted in up- or down-regulation of EsxA membrane-permeabilizing activity in vitro. Moreover, these mutations up- or down-regulated the mycobacterial virulence and cytosolic translocation accordingly, demonstrating the specific and accurate correlation between EsxA membrane-permeabilizing activity and mycobacterial virulence as well as the ability to penetrate phagosome membrane (22). Once again, the present study provides new evidence that the Nα-acetylation at Thr2 of EsxA is required for mycobacterial virulence and cytosolic translocation through facilitating the heterodimer separation.

Since Thr2 has no any contact with EsxB as shown in the reported solution structure of EsxA:B heterodimer, how Nα-acetylation on Thr2 affect heterodimer separation had become a puzzle. Here, the MD simulation result provides an interesting model, in which the acetylated Thr2(Ac) has frequent “bind-and-release” contacts with EsxB at low pH, generating a dragging force to pull EsxB away from EsxA.

Nα-acetylation is a common modification in eukaryotes and plays important roles in protein-protein interaction, protein activity and stability, and cell growth and cell cycle, etc. (30, 31). While Nα-acetylation in eukaryotes has been well studied, little is known about it in bacteria, including mycobacteria. Over 100 proteins in Mtb have been found to be Nα-acetylated, including EsxA (32), and protein acetylation has been correlated to pathogenesis (33, 34). Currently, three Nα-acetyltransferases in E. coli, RimL, RimL and RimJ, have been identified to acetylate ribosomal proteins S18, L12 and S5, respectively (34-36). A biochemical study has found RimL in Mtb has a relaxed substrate specificity, but little is known about the physiological substrates of the enzyme (37), which warrants further investigation.

A recent study has shown that the recombinant EsxA does not lyse cell membranes, and the lytic activity previously attributed to EsxA is due to residual ASB-14 detergent in the preparation (38). In fact, we had the similar observations that addition of the recombinant EsxA protein to the surface of lung epithelial cell lines WI-26 and A549 did not lyse the cells. Moreover, Conrad et. al. showed that blocking phagosomal acidification by Bafilomycin did not decrease the ESX-1-mediated phagosomal permeabilization, suggesting that acidification is not required for membrane permeabilization (39). Recently, Lienard et. al. employed a collection of Mm ESX-1 transposon mutants, including the mutants that disrupt EsxA secretion, to infect macrophages and showed that the factors independent of EsxAB play a role in cytosolic translocation (40). It is not clear how the discrepancy arises and what is the broken link between the EsxA pH-dependent membrane-permeabilizing activity in liposomal membrane and the ability of mycobacteria to rupture phagosome membrane during infection. Other factors from mycobacteria and host cells, even including properties of target membranes, may be involved in this process, which warrants further investigations.

Materials and Methods

Generation of T2X mutations on the Mtb esxA gene for expression in E. coli, Ms and Mm.

For expression in E. coli: Using the previously reported plasmid pET22b-esxA-His6 as the template (20-22, 41), the mutations T2A, T2Q, T2R, and T2S were introduced into the esxA gene by PCR using the primers listed in Table S1. All of the mutations were confirmed by DNA sequencing. The resulting plasmids were transformed into BL21 (DE3) cells for expression. The cells were grown at 37 °C while shaking at 250 rpm until OD600 reached 0.6-0.8. Protein
expression was induced by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 3-8 h at 37 °C. The cells were harvested and the proteins were purified as previously described (20-22, 41). Typical yield averaged between 30-60 mg/L of culture.

For expression in Ms: The pMyNT plasmid containing the Mtb esxB-esxA operon (27) was used as the template. The mutations T2A, T2Q, T2R and T2S were introduced by overlapping PCR using the primers listed in Table S1. All of the mutations were confirmed by DNA sequencing. The pMyNT plasmids carrying various T2X mutations were electroporated into Ms mc2155 strain (voltage: 2,500 V, capacitance: 25 µF, resistance: 1,000 Ω). The Ms cultures were grown at 37 °C overnight or until OD600 reached 2.0. Protein expression was induced by adding 0.2% (w/v) acetamide for 12-16 h. The cells were harvested. The soluble heterodimer proteins were purified with immobilized metal ion affinity chromatography by passing through a Ni²⁺-column, followed by a sizing exclusion chromatography as previously described (27, 42-45). Typical yield averaged between 20-40 mg/L of culture.

For expression in Mm: The T2X mutations (T2A, T2R, T2Q and T2S) were generated by site-directed mutagenesis (Agilent Quick Change Kit) using the pMH406 plasmid containing esxB-esxA operon as a template. The mutations were confirmed by DNA sequencing. The mutated plasmids were electroporated into MmΔEsxA:B as previously described (44).

Liposome leakage assay

The liposome leakage assay was performed as previously described (20, 21, 41, 46, 47). Briefly, 20 mg of DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) was dried with nitrogen air and left in a vacuum overnight. The samples were rehydrated with 1 ml of the buffer (5 mM HEPES, 50 mM ANTS (8-aminonaphthalene-1,3,6 trisulfonic acid) and 50 mM DPX (p-xylene-bis-pyridinium bromide)). The suspension was subjected to 6x freeze-thaw cycles and extruded via a 0.2 µm membrane filter for 20 times. The liposomes were then desalted to remove excess ANTS and DPX using a Hi-trap desalting column. The desalted liposomes were mixed with 150 mM NaCl, 100 mM NaAc at pH 4.0. Three separate reaction mixtures were generated and individually excited at 350 nm and emissions were recorded at 520 nm in an ISS K2 phase modulation fluorometer. 100 µg of the tested protein was injected into the solution after approximately 30 s of the assay and fluorescence was observed.

Isolation of Mtb EsxA proteins from the Ms-produced Mtb EsxA:B heterodimer

The Mtb EsxA:B heterodimer purified from Ms was incubated in a solution containing 6 M guanidine at 4 °C overnight. The proteins were then passed through a HisTrap column (GE Healthcare). The His-tagged EsxB protein was bound to the column, and the un-tagged EsxA was collected in flow through. The His-tagged EsxB protein was eluted by an imidazole gradient. Both EsxA and EsxB were subjected to an extensive dialysis using a 3,000 MWCO membrane. The samples were then concentrated and passed through gel filtration for a complete buffer exchange.

Detection of Nα-acetylation by NBD-Cl

NBD–Cl (4-chloro-7-nitrobenzof-urazan) only reacts with free N-terminal α-amino group in non-acetylated proteins and emits fluorescence, and it does not react with Nα-acetylated proteins due to lack of free N-terminal amino group (48). Proteins (6 µM) were incubated with 0.5 mM NBD-Cl at room temperature. At different hours of post-incubation, the samples were subjected to fluorescence measurement with excitation at 460 nm and emission at 535 nm.

Sample Preparation for Mass Spectrometry

The concentration of four protein samples, EsxA protein purified from E. coli and the EsxA, EsxA(T2A), and EsxA proteins purified from Ms were determined via bicinchoninic acid assay.
(BCA) according to manufacturer instructions, (Pierce TM BCA Protein Assay kit, cat#23227). Then, 100 µg of each protein sample was used for protein digestion via FASP Protein Digestion (Expedeon, cat#44250), using either trypsin (cat# Sigma, cat#T6567) or pepsin (Sigma, cat# P7012). In summary, 100 µg of each sample was resuspended in 200 µl of 12.48 M urea tris hydrochloride solution (Urea Solution). Then, 10 mg of DL-dithiothreitol (Sigma, Cat#D0632-5G) was added to each sample and placed in a nutating mixer for 45 minutes (min). Samples were then transferred to a 30 kDa filter and centrifuged for 15 min at 14,000 rpm. 200 µl of urea solution was added to each spin filter and centrifuged at 14,000 for 15 min and repeated once more. Then, 100 µl of Iodoacetamide 1X solution (iodoacetamide prepared at a 1:10 ratio in Urea Solution) was added to each spin filter and incubated without mixing for 20 min in the dark. Afterwards, spin filters were centrifuged at 14,000 rpm for 10 min. The spin filters were washed twice by adding 100 µl of Urea Solution and centrifuging at 14,000 rpm for 15 min. Urea was removed from the samples by adding 100 µl of 50 mM ammonium bicarbonate solution to spin filters and centrifuged at 14,000 rpm for 15 min. This step was repeated twice for a total of three times. Spin filters were transferred to new spin filter tubes and 100 µl of digestion solution at 0.02 µg/µl (either with trypsin or pepsin) was added to each sample and incubated at 37°C for 18 hours. 200 µl of 0.1% formic acid water solution was added to each sample and spin filters containing resultant peptides and centrifuged at 14,000 for 10 min. Filtrate containing digested proteins were frozen at -80 °C for two hours and lyophilized for 12 hours. Samples were resuspended with 100 µl of 0.1% formic acid water solution at a final concentration of 1 µg/µl.

**LC-MS/MS and Bioinformatic Method**

Resultant complex peptide mixtures were analyzed via technical duplicates by 2hr-1d-LC-MS/MS with the QE Orbitrap (Thermo Fisher Scientific) along with the Dionex UltiMate 3000 RSLCnano UHPLC system (Thermo Fisher 221 Scientific) using the two digestive protocols (trypsin or pepsin). Samples were loaded in line onto a C18 PicoChip Column (75 µm ID x 15 µm tip packed with 10.5 cm of Reprosil -PUR C18 3µm 120 Å; 25 µm x 50 cm fused-silica tail, New Objective) which was previously equilibrated with Solvent A (95% Water, 5% Acetonitrile, 0.1% Formic Acid) and Solvent B (5% Water, 95% Acetonitrile, 0.1% Formic Acid). Column was conditioned for 10 minutes with a flow rate of 0.5 µL/min with 95% Solvent A, 5% Solvent B. Sample was injected and loaded on column and conditioned for 10 minutes with Solvent A. Elution of peptides was completed by running a linear gradient to 40% Solvent B for 95 minutes, followed by 10 min of 95% Solvent B. Sample was re-equilibrated for 10 minutes with 5% Solvent B. Full scan spectra was collected via Xcalibur (Thermo Fisher Scientific). Two blank injections were run in between samples using a 30 min interval with seesaw washes using 5–80% ACN gradients. QE Orbitrap settings are as follows: Full MS resolution of 70,000, AGC target of 1e6, scan range from 400 to 1600 m/z; MS/MS were run with a resolution of 17,500, AGC target of 2e6, 3 m/z isolation window. The spectra were searched using Proteome Discoverer (PD) 2.1.1.21 (Thermo Fisher Scientific) and filtered via Sequest HT with an estimated false-discovery rate (FDR) of 0.01 against sequences from *M. marinum,* E. coli BL21, human, bovine, human keratin, and porcine trypsin. A 20 ppm precursor, and a 0.02 fragment mass tolerance were used. Cysteine carbamidomethylation, methionine, oxidation and acetylation were set fixed and variable modifications, respectively. The output files were then manually analyzed and collectively used to generate representative data.

**Western Blotting**

*Mycobacterium marinum* (Mm) recombinant strains with respective EssA insertions, were cultured in 7H9 medium and grown to mid-log phase. They were washed with PBS and transferred to Sauton’s medium while normalizing all cultures to OD600 = 0.8. The
bacteria were cultured for two days until harvest. The bacterial cells were collected by centrifugation. The proteins in the culture supernatant were precipitated by trichloroacetic acid (TCA). The bacterial cells were resuspended in 1 ml of PBS containing a cocktail of protease inhibitors (Thermofisher) and sonicated at 30% amplitude for 5 cycles of 30-s pulse and 60-s rest. The culture filtrates and total bacterial lysates were applied to SDS-PAGE and transferred transferring onto PVDF membrane. Western blots were performed to detect EsxA using anti-EsxA antibody (sc-57730, Santa Cruz). As controls, Ag85 (secreted in culture filtrate) and GroEL (only in cell lysate) were also detected by anti-Ag85 (NR-13800, BEI) and anti-GroEl antibodies (NR-13813, BEI), respectively.

**Live/Dead Cytotoxicity Assay**

RAW264.7 cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) with penicillin and streptomycin (100U/mL) at 37°C and 5% CO₂. Raw 264.7 macrophages were plated in a 24-well plate with a density of 5 x10⁵/well for infection on the following day. The Mm strains were prepared with a single cell preparation protocol as previously described (22, 49). RAW264.7 cells were infected with various Mm strains at a multiplicity of infection (MOI) of 10 for 1 hr. The macrophages were washed 3 times with PBS to remove free mycobacteria and incubated for another 3 hrs. The macrophages were stained using Calcein-AM and ethidium homodimer (Life Sciences) for 30 min, enabling visualization under a fluorescence microscope for green cells (live) and red cells (dead). The numbers of dead cells were quantified from dozens of random fields from each sample.

**CCF-4 FRET assay**

Mycobacterial cytosolic translocation was measured by CCF-4 FRET assay as previously described (17, 22, 50). Briefly, RAW264.7 cells were plated in triplicate in a 6-well plate at a density of 2.5 x 10⁶ cells/well. The macrophages were then incubated with CCF4-AM according to the manufacturer’s protocol (Liveblazer B/G loading kit, Life Sciences). Cells were infected with recombinant Mm strains at a MOI of 10 for 2 hrs. Following infection, macrophages were washed 3 times using PBS. DMEM media with 10 % FBS was added to the cells and incubated for approximately 2 days. The samples were then excited at 409 nm and the emissions were measured at 450 nm and 535 nm. The blue/green ratio was calculated as I450/I535.

**Molecular dynamic simulation**

The structure of EsxA:B heterodimer was downloaded from Protein Data Bank with PBD ID 1WA8 (51). DelPhiPka web server (52) was used to obtain the protonation states of ionizable residues at pH 4 and pH 7 and assign the respective states with Visual Molecular Dynamics (VMD) (53). Nα-acetylation of Thr2 was performed on VMD after removal of Met1. The four structures i.e. non-acetylated and acetylated at pH 4 and pH 7 of EsxA:B heterodimer were then solvated in water box with TIP3 (54) water model and ionized with 150 mM NaCl in VMD. The final systems were then simulated with molecular dynamics (MD) simulation program NAMD (55). Each simulation was performed for 20 ns employing force field CHARMM27 (56). The temperature was set as 300 K and the pressure was 1 atm. The snapshots from the simulations were taken to study the behaviors of the N-terminal loop of EsxA with and without Nα-acetylation of Thr2 at pH 4 and pH 7.

**Data Availability Statement:** All data are contained within the manuscript.

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\textbf{Conflict of Interest:}

All authors have read the journal’s policy on disclosure of potential conflicts of interest, and the authors have no conflicts of interest to disclose.
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Figure 1. The EsxA:B heterodimer produced in *M*. *smegmatis* (*Ms*), but not in *E. coli*, permeabilized the liposomes at low pH, implicating the role of N\(^{\alpha}\)-acetylation in heterodimer dissociation. A. The heterodimer EsxA:B purified from *M. smegmatis* (*Ms*) or *E. coli* (*Ec*) were tested in three independent experiments with ANTS/DPX dequenching assay at pH 4.0. B. Equal amount of the heterodimer EsxA:B(*Ms*) or EsxA:B(*Ec*) were incubated with NBD-Cl at room temperature. NBD-Cl is a fluorescent dye that only react with a free N-terminal site without N\(^{\alpha}\)-acetylation was recorded at 535 nm (excitation at 480 nm) and plotted as a function of time.
Figure 2. The mutations at Thr2 of EsxA diminished the membrane-permeabilizing activity of EsxA:B heterodimer. A. The EsxA:B heterodimer proteins (wild type and the mutants carrying mutations T2A, T2Q and T2R) were purified from *M. smegmatis* (*Ms*). The membrane-permeabilizing activity of the purified heterodimer proteins was tested by ANTS/DPX fluorescence dequenching assay. The representative ANTS/DPX fluorescent dequenching curves were shown. B. The average end-point fluorescence intensities from at least three independent experiments were calculated. Results represent the average of 3 replicates and error bars represent S.D. C. The EsxA proteins (WT and T2A, T2Q and T2R mutants) were purified from *E. coli* (*Ec*). The membrane-permeabilizing activity of the *Ec*-EsxA proteins purified from *Ec* was tested by ANTS/PDX assay. The representative curves were shown. D. The average end-point fluorescence intensities from at least 3 independent experiments were calculated, with error bars denoting S.D.
Figure 3. Detection of Nα-acetylation of EsxA by NBD-Cl and mass spectrometry. A. The EsxA:B heterodimer purified from M. smegmatis (Ms) was separated by 6 M guanidine, after which EsxA and EsxB were purified separately via nickel affinity as described in Experimental Procedures. B. The indicated EsxA proteins, purified from Ms, were incubated with NBD-Cl. At the indicated times, the fluorescence intensity of NBD-Cl was measured. C. The indicated EsxA proteins, purified from Ms or E. Coli (Ec) as indicated were analyzed via LC/MS/MS to identify the post-translational modifications. The residues with acetylation were labeled as “A” and the residues with oxidation were labeled as “O”.
Figure 4. The $\text{N}^\alpha$-acetylation of EsxA did not affect the membrane-permeabilizing activity of EsxA. **A.** The indicated $Ms$-EsxA proteins isolated from the *M* smegmatis (*Ms*)-heterodimer were tested for membrane-permeabilizing activity using ANTS/DPX assay. The *Ec*-EsxA (WT) protein, purified from *E. Coli* (*Ec*) was used as a control. The representative curves from at least three independent experiments were shown. **B.** The average end-point fluorescence intensity from at least three independent experiments was calculated and shown. ($p < 0.05$).
Figure 5. EsxB preferentially inhibited non-acetylated EsxA(T2A) over its Nα-acetylated counterpart. The Ms-produced EsxA(WT) and EsxA(T2A) proteins were incubated with various concentrations of EsxB as the indicated molar ratios. The mixtures were tested in triplicate by ANTS/DPX assay for membrane-permeabilizing activity. The representative curves were shown in A and B, respectively. The relative inhibition from at least three independent experiments for a total n = 9 was summarized in C. For EsxA:B 0.5:1.0 ratio, p = 0.0015 and for EsxA:B 1:1 ratio p = 0.0012. Error bars represent S.D.
Figure 6. The non-\(N^\alpha\)-acetylate EsxA diminished mycobacterial virulence and cytosolic translocation in macrophages.  

A. RAW263.4 cells were infected with the indicated \(Mm\) strains at MOI of 10. The cytotoxicity was measured by using the Live/Dead assay. Dead cells were counted in random fields (***: \(n = 22, p < 0.0001\)).

B. Mycobacterial cytosolic translocation was monitored by using CCF4-AM as a FRET reporter. The Blue/Green ratio was measured by comparing emissions at 450nm and 530nm with excitation at 409 nm. The data were calculated from at least three independent experiments (***: \(n = 3, p < 0.0001\)).
Figure 7. Molecular dynamic simulation detects the acetylated Thr2(Ac) interacts with EsxB in a “bind-and-release” mode. The structures of *Mtb* EsxA:B heterodimers with/out N$\alpha$-acetylation were analyzed by molecular dynamic simulation. The figures were generated from snapshots of 20 ns MD simulations at pH 7 and pH 4. EsxA, EsxB and the Thr2 residue are shown in cyan, pink and red, respectively. The structures of EsxA:B heterodimer with non-acetylated Thr2 at pH 7 and pH 4 are shown in A (pH 7, top view), C (pH 7, side view), E (pH 4, top view) and G (pH 4, side view), respectively. The structures of EsxA:B heterodimer with acetylated Thr2(Ac) are shown in B (pH 7, top view), D (pH 7, side view), F (pH 4, top view) and H (pH 4, side view), respectively.
"N^\text{O-}acetylation of the virulence factor EsxA is required for mycobacterial cytosolic translocation and virulence"

Javier Aguilera, Chitra B Karki, Lin Li, Salvador Vazquez Reyes, Igor Estevao, Brian I Grajeda, Qi Zhang, Chenoa D. Arico, Hugues Ouellet and Jianjun Sun

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