Mycobacterium tuberculosis ESAT-6 exhibits a unique membrane-interacting activity that is not found in its ortholog from non-pathogenic Mycobacterium smegmatis

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Running title: Differential membrane interactions of ESAT-6 proteins from M. tuberculosis and M. smegmatis

Keywords: Mycobacterium tuberculosis, ESAT-6, membrane interaction

Background: Mycobacterium tuberculosis ESAT-6 (MtbESAT-6) is required for phagosomal rupture and bacterial cytosolic translocation.

Results: MtbESAT-6 underwent a pH-dependent conformational change and induced leakage of membrane vesicles, while its ortholog from non-pathogenic Mycobacterium smegmatis, MsESAT-6, did not.

Conclusion: MtbESAT-6 possesses a unique membrane-interacting activity that is not found in MsESAT-6.

Significance: This study links membrane-interacting activity of ESAT-6 to virulence of M. tuberculosis.

Abstract

Mycobacterium tuberculosis ESAT-6 (MtbESAT-6) reportedly shows membrane/cell-lysis activity, and recently its biological roles in pathogenesis have been implicated in rupture of the phagosomes for bacterial cytosolic translocation. However, molecular mechanism of MtbESAT-6-mediated membrane interaction, particularly in relation with its biological functions in pathogenesis, is poorly understood. In this study, we investigated the pH-dependent membrane interaction of MtbESAT-6, MtbCFP-10, and the MtbESAT-6/CFP-10 heterodimer, by using liposomal model membranes that mimic phagosomal compartments. MtbESAT-6, but neither MtbCFP-10 nor the heterodimer, interacted with the liposomal membranes at acidic conditions, which was evidenced by release of K⁺ ions from the liposomes. Most importantly, the orthologous ESAT-6 from non-pathogenic Mycobacterium smegmatis (MsESAT-6) was essentially inactive in release of K⁺. The differential membrane interactions between MtbESAT-6 and MsESAT-6 were further confirmed in an independent membrane leakage assay using the dye/quencher pair, 8-aminonaphthalene-1,3,6 trisulfonic acid (ANTS)/p-xylene-bis-pyridinium bromide (DPX). Finally, using intrinsic and extrinsic fluorescence approaches, we probed the pH-dependent conformational changes of MtbESAT-6 and MsESAT-6. At acidic pH conditions, MtbESAT-6 underwent a significant conformational change, which was featured by an increased solvent-exposed hydrophobicity, while MsESAT-6 showed little conformational change in response to acidification. In conclusion, we have demonstrated that MtbESAT-6 possesses a unique membrane-interacting activity that is not found in MsESAT-6 and established the utility of rigorous biochemical approaches in dissecting the virulence of M. tuberculosis.

Introduction

Mycobacterium tuberculosis, a tremendously successful pathogen, infects one-third of the world population and kills 2-3 million people each year (1). The cellular and molecular mechanisms of its pathogenesis have begun to be elucidated, but are still not fully understood. There is a widely accepted concept that after being internalized into the host macrophages, M. tuberculosis inhibits phagosome maturation and remains inside the phagosomes (2-4). This concept has recently been extended by a series of studies suggesting that at the later stage of infection, the pathogen is able to translocate from the phagolysosomal compartments into the cytosol of host cells, which...
may be a privileged niche (5-7). The ability of the pathogen to arrest phagosome maturation and to translocate from the phagosome to the cytosol have been attributed, at least in part, to a Type VII secretion system, named ESX-1 (5-9). Comparative genomic studies have revealed that the esx-1 locus is present in the genome of M. tuberculosis, but is absent in the genome of the attenuated vaccine strain Mycobacterium bovis Bacille Calmette-Guérin (BCG) (10). Deletion of esx-1 from M. tuberculosis resulted in attenuation of virulence (11-13), while transfer of esx-1 into BCG partially restored virulence (14, 15). The esx-1 locus comprises 11 genes that encode the ESX-1 secretion system and two secreted proteins: 6-kDa early-secreted antigenic target (MtbESAT-6, also called EsxA or Rv3875) and 10-kDa culture filtrate protein (MtbCFP-10, also called EsxB or Rv3874). ESAT-6 and CFP-10 are secreted by ESX-1 in a co-dependent manner (16). Mutants of M. tuberculosis with either gene deletions or defects in secretion of MtbESAT-6 and/or MtbCFP-10, are not translocated into the cytosol and showed reduced host cell lysis and cell-to-cell spreading (5-7, 11, 13). Biochemical analysis has showed that MtbESAT-6 and MtbCFP-10 form a 1:1 heterodimeric complex (Fig. 1A) (17, 18), and MtbESAT-6 exhibits membrane-lysis activity (11, 19). However, the molecular mechanism of MtbESAT-6’s membrane interaction has not been defined.

While ESX-1 is essential for virulence of M. tuberculosis, it is not unique to pathogenic mycobacteria. Mycobacterium smegmatis, the fast-growing, non-pathogenic mycobacterial species, also contains a highly conserved and functional ESX-1 system that is essential for DNA conjugation (20). M. smegmatis ESAT-6 (MsESAT-6) and CFP-10 (MsCFP-10) share ~70% amino acid identity with MtbESAT-6 and MtbCFP-10, respectively. Moreover, the M. smegmatis ESX-1 system is capable of secreting exogenously-expressed MtbESAT-6 and MtbCFP-10, suggesting that it is functionally related to that of M. tuberculosis (21). Importantly, phagosomal-localized M. smegmatis has been found to secrete MsESAT-6 into the phagosomes, but fails to translocate into the cytosol (7, 21). Therefore, we hypothesize that MtbESAT-6 and MsESAT-6 have different membrane-interacting activities, which contributes to the distinct virulence phenotypes of M. tuberculosis and M. smegmatis.

In this study, we characterized the pH-dependent membrane interactions and conformational changes of MtbESAT-6 in comparison with MsESAT-6. We showed that MtbESAT-6 induced leakage of membrane vesicles and underwent a significant conformational change upon acidification, while MsESAT-6 was essentially inactive in membrane interaction and showed no conformational changes in response to acidification.

Materials and Methods

Genes and Plasmids - The genes encoding MtbESAT-6 and MtbCFP-10 were amplified by PCR from the genomic DNA extract of M. tuberculosis (H37Rv). The genes encoding MsESAT-6 and MsCFP-10 were amplified by PCR from the genomic DNA extract of M. smegmatis. The amplified genes of MtbESAT-6 and MsESAT-6 were cloned into pET22b vector at NdeI/XhoI sites to produce C-terminally His-tagged proteins. The amplified genes of MtbCFP-10 and MsCFP-10 were cloned into pGEX4T-1 vector at BamHI/EcoRI sites to produce N-terminally GST-tagged proteins. All of the constructs were confirmed by DNA sequencing.

Purification of MtbESAT-6 and MsESAT-6 from bacterial inclusion bodies – Either pET22b-MtbESAT-6 or pET22b-MsESAT-6 was transformed into Escherichia coli BL21(DE3) cells, and the cells were grown at 37 °C in 2.5-L of ECPM1 medium containing 100 µg/ml carbenicillin in a BioFlo 115 bioreactor. At OD$_{600}$ =1.0, protein expression was induced with 1 mM IPTG for 5 hrs at 37 °C. The cells were harvested by centrifugation and lysed by sonication in the lysis buffer (20 mM TrisHCl, 100 mM NaCl, 0.5 mM EDTA and 100 µM  PMSF, pH 7.3). Inclusion bodies were collected by centrifuging at 15,200 x g for 40 mins at 4°C. The supernatant was loaded onto a Ni$^{2+}$-charged
sepharose column that had been pre-equilibrated with the pre-equilibration buffer (20 mM TrisHCl, 8 M urea, and 20% glycerol, pH 7.3). The bound proteins were refolded on the column by gradually removing urea with a linear gradient (0-88%) of the refolding buffer (20 mM TrisHCl, 500 mM NaCl, 20% glycerol, pH 7.3) on an AKTA FPLC (GE healthcare) at flow rate of 0.4 ml/min for 2.5 hrs. The refolded proteins were then eluted from the column with a linear gradient (10-100%) of the refolding buffer supplemented with 250 mM imidazole at flow rate 5 ml/min. The eluted proteins were further clarified through gel filtration chromatography using a Superdex-75 column in the gel filtration buffer (20 mM TrisHCl, 100 mM NaCl, pH 7.3). The resulting purified proteins normally reach greater than 90% purity.

**Purification of MtbCFP-10 and MsCFP-10**

Either pGEX4T-MtbCFP-10 or pGEX4T-MsCFP-10 was transformed into *E. coli* BL21(DE3) cells, and the cells were grown at 37°C in 4-L of LB medium containing 100 µg/ml carbenicillin. At OD$_{600}$ = 0.8, protein expression was induced by addition of IPTG (1 mM) for 3 hrs. The cells were harvested and resuspended in 1 x PBS (3 ml/gram of cell weight) containing lysozyme (0.2 mg/ml), MgCl$_2$ (1 mM), and PMSF (1 mM). The cells were lysed by sonication and the cell lysate was centrifuged at 32,000 x g for 1 hr at 4°C. The soluble fraction was loaded onto a glutathione sepharose 4B column that was pre-equilibrated with 1x PBS. The column was then washed with 5 column volumes of 1x PBS. The bound proteins were eluted with a linear gradient of the elution buffer (50 mM TrisHCl, 10 mM reduced glutathione, pH 8.0). The eluted proteins were further purified by gel filtration chromatography using a Superdex-75 column that was pre-equilibrated in the gel filtration buffer. For thrombin digestion, the purified GST-CFP-10 proteins were incubated with thrombin (1 unit/mg of protein) at RT for 14 hrs. The thrombin-treated samples were then passed through a glutathione sepharose 4B column. The GST and GST-tagged CFP-10 were bound to the column, while the cleaved CFP-10 was collected in the flow through fractions. The resulting cleaved CFP-10 is at least 90% pure.

**Purification of ESAT-6/CFP-10 heterodimers**

The purified His-tagged ESAT-6 proteins were incubated with excess amount of the purified CFP-10 proteins (The molar ratio of CFP-10: ESAT-6 is at least 3:1) in the binding buffer (20 mM TrisHCl, 100 mM NaCl, pH 7.3) at RT for 2 hrs. The mixture was loaded into a Ni$^{2+}$-column to allow the His-tagged protein complexes to bind. The column was washed three times with the binding buffer, and the bound proteins were eluted with a linear gradient of the elution buffer (20 mM TrisHCl, 100 mM NaCl, 500 mM imidazole, pH 7.3). The eluted proteins were applied to gel filtration using the Superdex 75 column, and the fractions of the heterodimer was then collected, concentrated to at least 1 mg/ml and stored at -80°C.

**Native gel shift assay**

- 1 µM of MtbCFP-10 was incubated with MtbESAT-6 at various concentrations (0.5µM, 1 µM, 1.5 µM, and 2 µM, respectively) at RT for 2 hrs. The samples were applied to native gel electrophoresis, followed by Coomassie brilliant blue staining.

**Liposome Preparation**

Liposomes were prepared as previously described (22, 23). Briefly, 1, 2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) (20 mg/ml in chloroform) was dried under nitrogen gas to form a lipid film, followed by vacuum overnight to remove residual solvent. The resulting dry lipid film was rehydrated by six freeze-thaw cycles in 10 mM HEPES (pH 7.4) and 150 mM KCl, and then extruded by passage through a 200-nm pore size polycarbonate filter (Nucleo-pore) in a mini-extruder (Avanti Polar Lipids). Subsequently, the liposome solution was applied to a G-25 desalting column equilibrated with 10 mM HEPES (pH 7.4) and 150 mM NaCl for buffer exchange. The resulting liposome solution has K$^+$ ion inside the liposomes and Na$^+$ outside. Similarly, the liposomes containing the dye/quencher pair, 8-aminonapthalene-1,3,6 trisulfonic acid (ANTS)/p-xylene-bis-pyridinium bromide (DPX), were prepared by rehydrating the dry lipid film in 50 mM ANTS, 50 mM DPX, 5 mM HEPES (pH 7.3), followed by extrusion through a 200-nm filter and by desalting in a G-25 column in 5 mM HEPES, 150 mM NaCl (pH 7.3).
The K⁺ release assay was performed as previously described (22, 23). Briefly, 200 µl of the K⁺-doped liposomes were added into 5 ml of 10 mM buffers at various pH (NaAc, pH 4.5–5.0; Bistris, pH 5.5–6.5; HEPES, pH 7.0–7.5; Tris-Cl, pH 8–9) containing 150 mM NaCl. The purified proteins were injected into the solution with continuous stirring (final concentration 3 µM). Release of K⁺ from liposomes was monitored using a K⁺-selective electrode (Orion Research) connected to a microcomputer pH/mV/ Temp meter 6171. The signal was transmitted by a data transmitter (DATAQ) and displayed with DATAQ software.

The time-lapse intensity measurement of ANTS/DPX dequenching – The ANTS fluorescence dequenching was measured in an ISS-K2 multiphase frequency and modulation fluorometer with excitation at 380 nm and emission at 520 nm. Briefly, 100 µl of the liposomes containing ANTS/DPX after desalting was diluted into 1.3 ml of 50 mM NaAc, 150 mM NaCl (pH 5.0) with continuous stirring. After the base line was stabilized, 100 µl of purified MtbESAT-6 or MsESAT-6 (total 100 µg) was injected into the cuvette, and the fluorescence signal was monitored in real-time. Crossed polarizers on excitation and emission beams, and a 395-nm long-path filter were used to reduce the background scatter.

Intrinsic tryptophan fluorescence – 10 µM of the purified proteins (MtbESAT-6, MsESAT-6, or MtbCFP-10) were incubated either in a neutral pH buffer (20 mM TrisHCl, 100 mM NaCl, pH 7.0) or an acidic buffer (20 mM NaAc, 100 mM NaCl, pH 5.0) for 30 mins. The intrinsic tryptophan fluorescence was measured in the ISS-K2 fluorometer with excitation at 295 nm and emission at 310–450 nm. A 305 nm long-path filter was applied in the emission channel to reduce the background. The emission spectra of the proteins were calibrated with the emission spectra of the same pH buffers without proteins in VINCI software.

ANS fluorescence – 5 µM of MtbESAT-6 or MsESAT-6 were incubated with 100 µM of 8-anilino-1-naphthalenesulfonate (ANS) in either pH 7.0 or pH 5.0 buffers for 30 mins at dark. The ANS emission spectra of the samples were measured in the ISS-K2 fluorometer with excitation at 380 nm and emission at 400–600 nm. A 395 nm long-path filter was placed in the emission path to reduce the backgrounds. The emission spectra of the samples were calibrated with the same buffers (pH 7 or pH 5) without proteins in VINCI software.

Results
The recombinant MtbESAT-6 and MtbCFP-10 were purified into homogeneity and reconstituted into 1:1 heterodimer. Since the previous study has shown that a C-terminal His-tag does not affect MtbESAT-6 secretion and function (24), we engineered a His-tag into the C-terminus of MtbESAT-6. The C-terminally His-tagged MtbESAT-6 was over-expressed as insoluble aggregates in E. coli inclusion bodies, comprising nearly 1/3 of the total protein. In previous reports (17, 18, 25), purification of the recombinant MtbESAT-6 protein was accomplished by extracting the protein aggregates from inclusion bodies with 8 M urea or 6 M guanidine, followed by an overnight dialysis in a refolding buffer. A C-terminal truncation of the purified MtbESAT-6 after overnight dialysis has been observed. To minimize protein degradation and increase the purification efficiency, we developed an “on-column” refolding and purification protocol that allowed us refold and purify the protein in a single programmed operation using FPLC. The purified MtbESAT-6 protein is soluble in aqueous solution and migrates as a single band in SDS-PAGE with at least 90% purity (Fig 1B). The identity and integrity of the purified protein were confirmed by western blots using either an anti-His-tag antibody, which detects the presence of the C-terminal His-tag, or an anti-ESAT-6 antibody, which detects the N-terminal epitope on MtbESAT-6 (data not shown).

The GST-tagged MtbCFP-10 was expressed as soluble protein in E. coli and purified through affinity chromatography. After thrombin treatment, the cleaved MtbCFP-10 was purified as a soluble protein with above 90% purity (Figure 1B). The purified MtbESAT-6 and MtbCFP-10 bound to each other in a dose-dependent manner, and formed a heterocomplex that migrated as a distinct band as indicated in the native gel electrophoresis (Figure 1C). To purify the heterocomplex, excess
amount of MtbCFP-10 (no His-tag) was incubated with His-tagged MtbESAT-6 on a Ni²⁺-column, which was followed by washing off the free MtbCFP-10 and elution of the His-tagged heterocomplex. The resulting heterocomplex was confirmed as a 1:1 heterodimer in the gel filtration chromatography that was calibrated with molecular weight markers (Figure 1D) and in SDS-PAGE where the bands of MtbESAT-6 and MtbCFP-10 showed similar density (Figure 1B).

**MtbESAT-6, but neither MtbCFP-10 nor the heterodimer, induced release of K⁺ from the liposomes at low pH conditions.** While MtbESAT-6 membrane/cell-lysis activity has been implicated in virulence of *M. tuberculosis*, characterization of MtbESAT-6 for pH-dependent membrane interaction using a model membrane system is lacking. Here, we applied the purified MtbESAT-6, MtbCFP-10, and the heterodimer, to the well-established K⁺ release assay, in which the release of K⁺ ions from the liposomes was used as a sensitive indicator for membrane disruption. In the measurements with a pH titration (pH 4.0–9.0), MtbESAT-6 induced a significant release of K⁺ ions from the liposomes at acidic conditions (pH 4.0–5.0) (Figure 2A, B). In contrast, MtbCFP-10 induced a limited release of K⁺ from the liposomes, suggesting that MtbCFP-10 is less active in membrane interaction. Interestingly, the heterodimer induced little release of K⁺ from the liposomes, indicating that the heterodimer is not dissociated at low pH conditions, and MtbESAT-6, when bound to MtbCFP-10, is incapable of interacting with the membranes. This result was further confirmed in the aggregation assay (Figure 2C). It is predicted that, in the absence of liposomes, MtbESAT-6 will form aggregates in the acidic solution due to increased solvent-exposed hydrophobicity resulting from the low pH-induced conformational changes. As predicted, MtbESAT-6 formed aggregates at pH 4.0, but not pH 7.0. In contrast, MtbCFP-10 alone and the heterodimer showed little aggregation at both pH 4.0 and pH 7.0. This data suggests that MtbCFP-10 remains bound to MtbESAT-6 at low pH and prevents MtbESAT-6 from forming aggregates, which otherwise would be observed if MtbESAT-6 was released from MtbCFP-10.

**Compared to MtbESAT-6, MsESAT-6 induced little release of K⁺ from the liposomes.** MsESAT-6 shares high sequence homology with MtbESAT-6, including most of the conserved hydrophobic residues (Figure 3A). To test if MsESAT-6 has a differential membrane-interacting activity relative to MtbESAT-6, we purified the recombinant proteins of MsESAT-6, MsCFP-10 and the MsESAT-6/CFP-10 heterodimer using the same approaches as we purified the Mtb-proteins (Figure 3B). Subsequently, we applied these proteins to the K⁺ release assay. In the measurements with a pH titration (pH 4–9), MsESAT-6 was most active in reducing release of K⁺ at pH 4–5 (data not shown). But even at low pH, the level of K⁺ release induced by MsESAT-6 was significantly lower than that induced by equal amount of MtbESAT-6. Not surprisingly, MsCFP-10 and the MsESAT-6/CFP-10 heterodimer induced little release of K⁺ as their Mtb-counterparts (Figure 3C).

**MtbESAT-6 induced a stronger fluorescence dequenching than MsESAT-6 from the liposomes containing the dye/quencher pair, ANTS/DPX.** We sought to further confirm the differential membrane interaction between MtbESAT-6 and MsESAT-6 using a membrane leakage assay other than K⁺ release. ANTS/DPX, the anion/cation fluorophore/quencher pair, is widely used for membrane leakage (26, 27). ANTS fluorescence is quenched by DPX inside the liposomes, and it is dequenched upon release into the medium. As expected, addition of Triton X-100 caused rapid membrane lysis and induced a sharp increase of ANTS fluorescence, while the buffer without protein did not induce fluorescence dequenching. Consistent with the results obtained in the K⁺ release assay, MtbESAT-6 induced dequenching of ANTS fluorescence, at a level that was significantly higher than that induced by MsESAT-6 (Figure 4A and B).

**MtbESAT-6, but not MsESAT-6, underwent significant conformational change upon acidification.** Transition of an aqueous-soluble protein into a membrane-interacting protein is always accompanied by significant conformational changes. Since all of the tryptophan (Trp) residues (3 in total) are conserved between MtbESAT-6
and MsESAT-6 (Figure 3A), we measured the intrinsic Trp fluorescence of the proteins to probe the acidification-dependent conformational changes (Figure 5). The emission spectra of MtbESAT-6 showed that there was a dramatic shift (~12 nm) of the peak maximum from pH 7 to pH 5 (Figure 5A), suggesting a significant conformational change occurred upon acidification. In contrast, MsESAT-6 showed little difference in the emission spectra between pH 7 and pH 5 (Figure 5B), which was similar to MtbCFP-10 (Figure 5C). This data is consistent with the results obtained from the K⁺ release assay (Figure 3), the ANTS/DPX dequenching assay (Figure 4), and the aggregation assay (Figure 2C). It demonstrated that acidification induced a significant conformational change on MtbESAT-6, but not MsESAT-6.

The acidification-induced conformational change is associated with an increased solvent-exposed hydrophobicity. To further confirm that the pH-dependent membrane interaction is due to an increased solvent-exposed hydrophobicity, an extrinsic fluorescence dye, 8-anilino-1-naphthalenesulfonate (ANS), was incubated with the proteins either in a neutral or an acidic solution (Figure 6). ANS is hardly fluorescent in aqueous environment, but becomes highly fluorescent when it binds to hydrophobic sites of proteins. As expected, MtbESAT-6 showed a significant increase of ANS fluorescence at pH 5, compared to pH 7, suggesting that acidification induced conformational changes on MtbESAT-6, which increased solvent-exposed hydrophobic residues. Compared to MtbESAT-6, MsESAT-6 showed little ANS fluorescence at both neutral and acidic conditions, suggesting that MsESAT-6 has less solvent-exposed hydrophobic residues at neutral pH, and its conformation is less responsive to acidification. Again, this data is consistent with the results obtained from the K⁺ release assay, the ANTS/DPX dequenching assay, the aggregation assay, and the Trp fluorescence measurements.

Discussion

MtbESAT-6 has been extensively investigated as an important virulence factor of M. tuberculosis. However, the molecular mechanism of the MtbESAT-6-mediated membrane interaction has not been defined. In this study, we provide evidence that upon acidification MtbESAT-6 undergoes a significant conformational change and induces leakage of membrane vesicles, while MsESAT-6, the orthologous ESAT-6 from non-pathogenic M. smegmatis, showed essentially no membrane interaction. This finding establishes a strong link between the membrane-interacting activity of ESAT-6 proteins and the virulence of mycobacterial species, and suggests that MtbESAT-6 contributes to the pathogenesis of M. tuberculosis through acidification-triggered membrane interaction within the phagosomes.

MsESAT-6 shares over 70% sequence identity with MtbESAT-6, including most of the hydrophobic residues (Figure 3A). Like MtbESAT-6, the recombinant MsESAT-6 is expressed as insoluble aggregates in the inclusion bodies of E. coli, and the refolded MsESAT-6 is capable of binding to MsCFP-10 to form a 1:1 heterodimer, which was confirmed by gel filtration (data not shown) and SDS-PAGE (Figure 3B). These observations indicate that MsESAT-6 has similar biochemical properties to MtbESAT-6. In fact, other biochemical and biophysical analyses have also suggested that members of WXG protein family (EsxA and EsxB) share conserved structural features and solution properties (28). Given these similarities, it is therefore surprising that MtbESAT-6 and MsESAT-6 exhibit significant differences in membrane interaction and conformational changes in response to acidification. This strongly suggests that, regardless of the general similarity, the capacity to interact with the membranes in response to acidification is a hallmark of the ESAT-6 molecules associated with virulence. These findings are consistent with the membrane interaction studies of MmESAT-6, the reportedly membrane-interacting molecule from the pathogenic M. marinum (29). It would be interesting to look into the membrane interaction of other orthologous ESAT-6 proteins from both pathogenic and non-pathogenic species in relation to virulence. More importantly, identification of the key regions/residues that are responsible for the differential membrane interaction of ESAT-6 proteins is of great interest for understanding of the molecular mechanism of MtbESAT-6-mediated membrane interaction.

Recent microscopic studies of the M. tuberculosis mutants defective in MtbESAT-6
secretion suggest that MtbESAT-6 is required for bacterial cytosolic translocation (6, 7). To date, however, there is scant evidence that MtbESAT-6 mediates this pathogenic process by directly interacting with the host membranes. We do not exclude the possibility that other factors (either from pathogen or host) are also involved in the ESAT-6-mediated pathogenesis, by directly or indirectly interacting with the membranes. In addition to MtbCFP-10, other bacterial factors have been found to associate MtbESAT-6 and/or the heterodimer (16, 28, 30, 31). For example, EspA has been found to be co-secreted with the MtbESAT-6/CFP-10 heterodimer (16), perhaps by forming a complex with the heterodimer (28). Thus, the membrane specificity of MtbESAT-6 and the effects of EspA as well as unknown factors on ESAT-6-mediated membrane interaction remain to be addressed in future.

There are currently conflicting data in the literature as to whether or not the MtbESAT-6/CFP-10 heterodimer dissociates at acidic pH. In our study, the recombinant heterodimer was inactive in release of K⁺ and showed no aggregation at acidic pH, suggesting that MtbESAT-6 was not dissociated from MtbCFP-10, even under acidic conditions. Consistent with our results, an earlier circular dichroism study showed that the complex formed by MtbESAT-6 and MtbCFP-10 was too stable to dissociate at low pH (25). In contrast, a published study using the native MtbESAT-6 extracted from the culture filtrate of M. tuberculosis suggested that MtbESAT-6 was dissociated from MtbCFP-10 at low pH (19). One possible explanation for this discrepancy is that the native proteins possess unique properties that are absent in the recombinant proteins. These might include post-translational modifications (PTMs), which allows the two proteins dissociate at low pH. Several PTMs have been found on mycobacterium-expressed MtbESAT-6, including a N-terminal acetylation on threonine 2 (Thr2). Moreover, MtbCFP-10 discriminated the non-acetylated and acetylated MtbESAT-6 by differential binding (32), suggesting that acetylation of Thr2 may be a factor that affects the interaction of MtbESAT-6 and MtbCFP-10 in response to pH changes. However, a later study showed that replacement of Thr2 with a histidine did not affect ESAT-6 secretion and bacterial growth in SCID mice, indicating that the acetylation of Thr2 does not have a major effect upon ESAT-6 function (24). In addition to PTMs, other factors that show specific association with the heterodimer may also affect MtbESAT-6 and MtbCFP-10 interaction in vivo. For instance, MtbCFP-10 has been shown to interact with several other proteins, including ClpC1, an AAA-ATPase chaperone involved in protein translocation and quality control, and ClpC1 associates with the proteolytic component ClpP2 (33). It has recently been shown that ClpP1 and ClpP2 form a single proteolytic complex and function together in protein degradation, and both are required for bacterial viability in vitro and during infection (34, 35). Thus, further studies of the roles of PTMs and other interacting factors in the heterodimer dissociation will facilitate our understanding of the MtbESAT-6-mediated pathogenesis.

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**Figure legend**

**Figure 1.** Purification of MtbESAT-6, MtbCFP-10 and MtbESAT-6/CFP-10 heterodimer. A. The solution structure of MtbESAT-6/CFP-10 heterodimer. The structural coordinate (1WA8) was downloaded from PDB website and the structure was displayed in swiss-pdb-viewer. B. The recombinant proteins of MtbCFP-10, MtbESAT-6 and MtbESAT-6/CFP-10 heterodimer were purified as described in Material and Method and applied to SDS-PAGE, followed by Coomassie blue staining. C. MtbESAT-6 and MtbCFP-10 were incubated at the indicated molar ratio at pH 7.0 and then applied to native gel electrophoresis, followed by Coomassie blue staining. *: The hetero complex of MtbESAT-6/CFP-10. D. The purified MtbESAT-6/CFP-10 heterocomplex was applied to gel filtration chromatography using a Superdex-75 column. The positions of the gel filtration calibration markers were indicated in the elution profile.

**Figure 2.** MtbESAT-6, but neither MtbCFP-10 nor the heterodimer, induced release of K⁺ ion from liposomes at low pH. A. 3 µM of the purified proteins (MtbESAT-6, MtbCFP-10, or the heterodimer) were mixed with the K⁺-containing liposomes in the solutions at various pH conditions (pH 4 – 9). The release of K⁺ from the liposomes was measured in real-time by a K⁺-selective probe. The representative recordings of K⁺ release at pH 4.0 are shown. B. The release of K⁺ at 60 seconds of post-mixing in the pH titration was quantified from at least three independent measurements. C. 3 µM of the purified proteins (MtbESAT-6, MtbCFP-10, or the heterodimer) were incubated in either pH 4 or pH 7 buffers without liposomes for 1 hr. The formation of protein aggregates in the solution was measured at OD₃₄₀ in a plate reader.

**Figure 3.** At low pH, MtbESAT-6 induced a stronger release of K⁺ from the liposomes than MsESAT-6. A. Sequence alignment of MtbESAT-6 and MsESAT-6. B. The recombinant proteins of MsESAT-6, MsCFP-10 and MsESAT-6/CFP-10 heterodimer were purified and examined by SDS-PAGE, followed by Coomassie staining. C. 3 µM of the indicated proteins were injected into the liposome solutions at pH 5.0. The release of K⁺ ions from the liposomes at 60 seconds of post-injection was measured and quantified from at least three independent experiments.

**Figure 4.** At low pH, MtbESAT-6 induced a stronger leakage of the membrane vesicles containing ANTS/DPX than MsESAT-6. The liposomes containing the dye/quencher pair, ANTS/DPX, were diluted into a pH 5 buffer with continuous stirring. Once the fluorescence base line was stabilized, either MtbESAT-6 or MsESAT-6 was injected into the solution (final concentration 10 µM). The dequenching of ANTS fluorescence due to leakage of the membrane vesicles was recorded at emission 520 nm with excitation at 380 nm. Triton X-100 (final concentration 0.06%) and the buffer without protein were tested as positive control and negative control, respectively. The representative recordings of ANTS dequenching are shown in A. The fluorescence intensity at 200 seconds of post-injection was quantified from three independent measurements and shown in B. Note: the fluorescence intensity (y₁ axis) induced by Triton X-100 is marked on the right of the graph.

**Figure 5.** Acidification triggered a significant conformational change on MtbESAT-6, but not on MsESAT-6. 10 µM of MtbESAT-6 (A), MsESAT-6 (B), or MtbCFP-10 (C) were incubated either at pH 7 or at pH 5 for 30 mins. The samples were excited at 295 nm and the emission spectra of Trp fluorescence of the samples were recorded at 310 – 450 nm. The emission spectra of the samples were calibrated with the emission spectra of the same buffers (pH 7 or pH 5) without proteins. The representative data from at least three independent experiments are shown in the figure.

**Figure 6.** MtbESAT-6, but not MsESAT-6, exhibited enhanced ANS fluorescence upon acidification. 5 µM of MtbESAT-6 or MsESAT-6 were incubated with 100 µM of ANS at either pH 7 or pH 5 buffers for 30 mins. The samples were excited at 380 nm and the emission fluorescence intensity of ANS was recorded at 400 – 600 nm. The emission spectra of the samples were calibrated with the same buffers (pH
7 or pH 5) without proteins. The representative results are shown in A. The peak emission intensity at 468 nm was quantified from at least three independent experiments and shown in B.
Figure 2
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Mycobacterium tuberculosis ESAT-6 exhibits a unique membrane-interacting activity that is not found in its ortholog from non-pathogenic Mycobacterium smegmatis
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