Evidence for Pore Formation in Host Cell Membranes by ESX-1-Secreted ESAT-6 and Its Role in \textit{Mycobacterium marinum} Escape from the Vacuole

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The ESX-1 secretion system plays a critical role in the virulence of \textit{M. tuberculosis} and \textit{M. marinum}, but the precise molecular and cellular mechanisms are not clearly defined. Virulent \textit{M. marinum} is able to escape from the \textit{Mycobacterium}-containing vacuole (MCV) into the host cell cytosol, polymerize actin, and spread from cell to cell. In this study, we have examined nine \textit{M. marinum} ESX-1 mutants and the wild type by using fluorescence and electron microscopy detecting MCV membranes and actin polymerization. We conclude that ESX-1 plays an essential role in \textit{M. marinum} escape from the MCV. We also show that the ESX-1 mutants acquire the ability to polymerize actin after being artificially delivered into the macrophage cytosol by hypotonic shock treatment, indicating that ESX-1 is not directly involved in initiation of actin polymerization. We provide evidence that \textit{M. marinum} induces membrane pores ~4.5 nm in diameter, and this activity correlates with ESAT-6 secretion. Importantly, purified ESAT-6, but not the other ESX-1-secreted proteins, is able to cause dose-dependent pore formation in host cell membranes. These results suggest that ESAT-6 secreted by \textit{M. marinum} ESX-1 could play a direct role in producing pores in MCV membranes, facilitating \textit{M. marinum} escape from the vacuole and cell-to-cell spread. Our study provides new insight into the mechanism by which ESX-1 secretion and ESAT-6 enhance the virulence of mycobacterial infection.

\textit{Mycobacterium tuberculosis} infects one-third of the world’s population and kills 2 to 3 million people each year (13). The molecular and cellular mechanisms governing the pathogenesis of \textit{M. tuberculosis} are beginning to be elucidated but are not fully understood. \textit{Mycobacterium marinum} is a close relative of \textit{M. tuberculosis}. \textit{M. marinum} causes a tuberculosis-like disease in fish with symptoms similar to those of human tuberculosis and has been used as a surrogate model for studying the pathogenesis of \textit{M. tuberculosis} (7, 17, 20, 46, 47).

Previous studies have identified and partially characterized a specialized protein secretion system, ESX-1, in \textit{M. tuberculosis} (14, 23, 24, 33, 44) and \textit{M. marinum} (17, 50). This secretion system has recently been named the type VII secretion system (1). ESX-1 is encoded by genes of RD1 (region of difference 1) (17, 50). This secretion system could secrete a pore-forming protein into the MCV to compromise the integrity of the vacuole membrane and facilitate the escape of \textit{M. marinum} into the host cell cytosol. In this study, we show that ESX-1 plays a critical role in the escape of \textit{M. marinum} from the MCV. We provide evidence that ESX-1 secretion and secreted ESAT-6 play a critical role in causing pore formation in host cell membranes. These results suggest that ESAT-6 secretion by ESX-1 may cause membrane pore formation in the MCV, facilitating \textit{M. marinum} escape from the vacuole and spreading.

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Bacteria and media. *M. marinum* strain M was cultured and maintained as described previously (16). The *M. marinum* ESX-1 mutants were produced as described previously (17). The mb3777::tn mutant was recently isolated from an *M. marinum* transposon mutant library (16). The mb3881::tn mutant and its complementation were described previously (50).

**Generation of *M. marinum* Δesat-6 mutant.** Δesat-6 mutant *M. marinum* was generated by allelic exchange. The right flanking fragment was digested with EcoRI and HindIII. The left and right flanking fragments and a kanamycin resistance cassette (the right flanking fragment was cut with EcoRI and HindIII. The left and right flanking fragments and a kanamycin resistance cassette (`kan") were ligated into pBluescript. The entire sequence containing the flanking sequences and `kan" was cut from the pBluescript clone and ligated into pLYG304.zeo (19) to generate the esat-6 knockout plasmid.

The plasmid was electroporated into wild-type (WT) *M. marinum*, and homologous recombinants were selected as described previously (19). Confirmation of the Δesat-6 mutation was carried out by PCR with two primer pairs. One pair of primers anneals to a sequence upstream from the flanking sequence (5'-GGATTCAGCCTCCGGTGGCCCTGGAG3' and another that anneals to a sequence downstream from the flanking sequence (5'-GGATTACGCCACCGGTGCCGTGG3' and another that anneals to a sequence downstream from the flanking sequence (5'-GGATTACCGCGCTCCGGGCTGTGG3' and another that anneals to a sequence downstream from the flanking sequence (5'-GGATTACGCCACCGGTGCCGTGG3'). These were used to confirm recombinants within the flanking sequence, in which a primer that anneals to a sequence downstream from the flanking sequence (5'-GGATTACCGCGCTCCGGGCTGTGG3') and another that anneals to a sequence in *kan" (5'-CACCTCCTCCTGAGCCGACCTGACC3') were used.

**Complementation of *M. marinum* Δesat-6 mutant.** To complement the Δesat-6 mutant with both the *esat-6* and *clf-10* genes together, a fragment containing both genes was amplified by PCR from the *M. marinum* genome with primers 5'-CACAGATGAAAGCAGATGCCGTCCCTGC3' and 5'-GGCCGGATCTTATGATGATGATGATGACGACATCCCGTCCG3'. The reverse primer contains a six-His tag fused to the C terminus of *ekur*. The primer pair recombinates within the flanking sequence, in which a primer that anneals to a sequence downstream from the flanking sequence (5'-GGATTACCGCGCTCCGGGCTGTGG3') and another that anneals to a sequence in *kan" (5'-CACCTCCTCCTGAGCCGACCTGACC3') were used.

Macrophages. J774A1 (ATCC TIB67) or Raw264.7 (ATCC TIB-71) murine macrophage-like cells were cultured and maintained as described previously (16). Bone marrow-derived macrophages (BMDMs) were obtained from C57BL/6 mice as previously described (38). Cells were harvested 8 to 10 days after plating and allowed to adhere to fibronectin-coated coverslips (Becton Dickinson) for infecting with *M. marinum* *Δesat-6* mutant.

**Dil labeling of MCV membranes.** BMDMs on glass coverslips were infected with *M. marinum* at a multiplicity of infection (MOI) of 2 for 2 h, followed by three washes with phosphate-buffered saline (PBS) and 1 h of incubation with 200 μg/ml amikacin to kill the extracellular bacteria. At the end of the antibiotic incubation, the cells were washed twice with PBS and incubated at 32°C in 5% CO₂ for 72 h. CM-Dil (Molecular Probes) was added to the cells at a 2 μM final concentration and incubated for 1 h. The cells were then washed twice with PBS to remove excess Dil, incubated for 1 h in the cell culture medium, and fixed with 4% formaldehyde. The fixed cells were washed three times with PBS, mounted on a glass slide with Prolong Antifade (Molecular Probes), and imaged.

**Fluorescent labeling of F-actin.** BMDMs on glass coverslips were infected at an MOI of 2 as described above. At 48 to 72 h post infection, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, stained with Alexa Fluor phalloidin (Molecular Probes), and imaged. To artificially deliver the ESX-1 mutant bacteria into macrophage cytosol, the cells at 48 h postinfection were treated for 5 min with a hypotonic solution (4 parts phenol red-free Dulbecco modified Eagle medium [DMEM] to 1 part H₂O₂), incubated in cell culture medium at 32°C in 5% CO₂ for an additional 24 h, and then processed for actin staining. The procedures for the hypotonic shock treatment were similar to those described by Okada and Reichstein (31).

**Detection of pore formation in red blood cell membranes.** Induction of pore formation in red blood cell membranes by *M. marinum* was detected by hemolysis assay as previously described (15, 17). Briefly, *M. marinum* grown in TH medium to mid-log phase was washed twice with PBS. A volume of 1.3 ml of *M. marinum* suspension (containing 2.5 × 10⁷ bacteria) was mixed with 400 μl of sheep red blood cells (sRBC, Quad Five) (containing 1 × 10⁷ cells) in a microcentrifuge tube and centrifuged at 8,000 × g for 2 min. The tubes were incubated at 32°C for designated periods of time. The pellets were then resuspended and centrifuged. The amount of the *mda₉₀* of the supernatants was measured. To examine the role of energy-dependent secretion in membrane pore formation, *M. marinum WT* bacteria were pretreated for 15 min with 20 μM carbonyl cyanide m-chlorphenylhydrazone (CCCP) to uncouple the proton motive force and then incubated with sRBC in the presence of CCCP for 2 h. To observe the reversibility of CCCP in hemolysis, the pellet containing *M. marinum* and red blood cells was resuspended in PBS and then repelleted and further incubated for 2 h before measurement of hemolysis.

In the polycryl alcohol (PEG) osmoprotection experiment, PEG1000, PEG3350, PEG6000, and PEG8000 were resuspended in PBS and added to red blood cells with or without *M. marinum* to a final concentration of 30 mM. Hemolysis was measured after 2 h of incubation at 32°C. To determine if protection from hemolysis by PEG8000 is reversible, the pellet containing *M. marinum* and red blood cells was resuspended in PBS and then repeltleted and further incubated for 2 h before measurement of hemolysis.

To determine the protection from hemolysis was determined and plotted against the Einstein-Stokes molecular diffusion radius, *Rₑₐ₉₀* (48).

**Expression and purification of recombinant ESX-1 proteins.** The *resAT*-6-His₉₀, *CFP*-10-His₉₀, and *Mh3881c*-His₉₀ proteins were affinity purified and had endotoxins removed. The *resAT*-6-His₉₀ and *CFP*-10-His₉₀ proteins were obtained from Colorado State University under the NIH TB Research Materials Contract. The inclusion bodies containing *resAT*-6-His₉₀ were solubilized with 6 M urea, and the protein was purified with the His-_bind resin (Novagen). Endotoxins were removed by washing the column with 10 mM Tris-HCl, followed by 0.5 M ASB-14. The eluted protein was eluted with 10 mM Tris-HCl containing 1 M imidazole. The eluted protein was dialyzed against 10 mM ammonium bicarbonate. The residual concentration of endotoxins was ≤0.24 ng/mg protein.

Two lots were obtained, one produced in 2001 and the other in 2007. Both lots were used for the analysis of membrane pore formation, and similar results were observed. We expressed and purified the rMh3881c-His₉₀ protein by using procedures similar to those described above.

**Detection of pore formation by purified ESX-1 proteins.** To determine the ability of ESX-1-secreted proteins to induce membrane pore formation, the above-described purified rESAT-6-His₉₀, rCFP-10-His₉₀, or rMh3881c-His₉₀, in a 50-μl volume was mixed with 100 μl of sRBC (containing 1 × 10⁷ cells) in a microcentrifuge tube. The tubes were incubated at 32°C for designated periods of time. The cells were then resuspended and centrifuged at 4,000 rpm for 7 min. The supernatants were transferred to 1 ml microcentrifuge tubes grown to mid-log phase and homologous recombinants were obtained.

The Δesat-6 mutant was resuspended in PBS and added to the red blood cell-protein mixture at a final concentration of 30 mM. Hemolysis was measured after 2 h of incubation at 32°C.

**Detection of pore formation in macrophage cell membranes.** *M. marinum* strains grown to mid-log phase were washed twice with DMEM, added to macrophage monolayers at an MOI of 50, centrifuged for 10 min at 1,500 × g to allow immediate bacterium-cell contacts, and incubated at 32°C in 5% CO₂ for designated periods of time. The release of lactate dehydrogenase (LDH) by the infected and noninfected cells was measured with a CytoTox-One Homogeneous Membrane Integrity Assay kit (Promega) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Pore formation in macrophage cell membranes was determined by an osmoprotection assay with various PEGs at a final concentration of 30 mM. To examine the induction of pore formation in macrophage cell membranes by *resAT*-6-His₉₀, *CFP*-10-His₉₀, or *Mh3881c*-His₉₀, the protein was dissolved in DMEM and added individually to Raw264.7 cells in a 96-well plate at designated concentrations. After 2 h of incubation at 32°C in 5% CO₂, the release of LDH was determined.

To detect membrane pore formation by a microscopic method, infected or noninfected macrophages were incubated with ethidium homodimer-1 (Molec- ures) and penetrated by the cells by using a protocol for the detection of internalized membranes was detected by fluorescence microscopy. In brief, macrophages were incubated for 40 min in phenol red-free culture medium containing ethidium homodimer-1 (4 μM) and calcine AM (2 μM), followed by imaging.

Ethidium homodimer-1 only penetrates permeabilized cell membranes and...
stains the nuclei red. Calcein AM permeates every cell membrane and is only metabolized by live cells to produce green fluorescence.

Preparation of *M. marinum* short-term culture filtrate and cell lysate. Preparation of *M. marinum* short-term culture filtrate and cell lysate was carried out as previously described (50). In brief, WT and mutant *M. marinum* cells were first grown in TH9 medium to mid-log phase. The bacteria were then washed and diluted 10 times in Sauton’s medium and cultured for 2 days to reach mid-log phase. The bacteria were washed again and diluted 10 times in Sauton medium and cultured for another 2 days. The culture supernatant was collected, filtered through a 0.2-μm filter, and concentrated 100 times with a Centricon centrifugal filter with a molecular weight cutoff of 3,000. The cell lysate was obtained by bead beating the bacterial pellet.

Western blotting. Proteins were separated by 4 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking with 2% bovine serum albumin, the membrane was incubated with a primary antibody diluted in 2% bovine serum albumin overnight, followed by incubation for 1 h with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad). The membrane was developed by enhanced chemiluminescence (Pierce) and exposed to films. The following primary antibodies were used at the dilutions indicated: anti-ESAT-6 (monoclonal antibody HYV 76-8; Abcam), 1:3,000; anti-CFP-10 (Colorado State University, NIH contract NOI-AI-75320), 1:2,000; anti-Mh3881c (Michael Lodes, Corixa Corporation, Seattle, WA), 1:5,000.

RESULTS

ESX-1 is essential for *M. marinum* to escape from the vacuole. *M. marinum* is able to escape from the MCV into the host cell cytosol, polymerize actin, and spread from cell to cell (17, 42, 43). Because mutations in various ESX-1 genes abolish *M. marinum* spreading (17), we hypothesized that the ESX-1 secretion system may play a role in either the escape of *M. marinum* from the MCV or initiation of actin polymerization. We first determined if ESX-1 is involved in *M. marinum* escape from the vacuole. We examined the association of WT or ESX-1 mutant bacteria with the vacuole membranes in live macrophages by using a fluorescent membrane dye, DiI. DiI is frequently used to label the live cell membranes and has worked well in previous studies labeling MCV membranes (42). As shown in Fig. 1A to C and Table 1, at 72 h postinfection only a small fraction (18%) of the WT bacteria colocalized with DiI, suggesting that the majority of the bacteria entered the cytosol. In sharp contrast, for all of the nine ESX-1 mutants examined, the majority of the bacteria (>80%) colocalized with DiI (Fig. 1D to F and Table 1), indicating that they reside predominantly within MCV membranes. To confirm the above observations, we used transmission electron microscopy to examine in greater detail the association of *M. marinum* with vacuole membranes. This study shows that only a small fraction of the WT bacteria are bound with MCV membranes (36%), while most are free of the membrane (64%) (Fig. 2A and Table 1). On the other hand, for the four ESX-1 mutants examined, more than 98% of the bacteria are surrounded by MCV membranes (Fig. 2B and Table 1). The mutants used in the above assays contain mutations in either the ESX-1 secretion apparatus (such as mh3877::tn and mh3871::tn) or the secreted substrates (such as Δsat-6, Δsfp-10+esat-6, and mh3881c::tn). Because the two groups of mutants showed similar phenotypes (Table 1), these results indicate that the ESX-1 secretion system plays a critical role in the escape of *M. marinum* from the MCV.

*M. marinum* escape from the vacuole is required for the polymerization of actin. Next, we determined if ESX-1 is required for *M. marinum* to initiate actin polymerization. At 72 h postinfection, 34% of the WT bacteria showed actin polymerization at one pole of the bacterium, forming the “actin comet tail” (Fig. 3A to C and Table 1), similar to previous observations (42, 43). In contrast, none of the nine ESX-1 mutants were able to polymerize actin (Fig. 3D to F and Table 1).

One possible explanation for the above results is that an ESX-1-secreted protein is directly involved in the recruitment and polymerization of actin. Alternatively, an ESX-1-secreted protein could be involved in compromising the integrity of MCV membranes to facilitate *M. marinum* escape into the host cell cytosol. To distinguish between these two possibilities, we treated the infected macrophages with a hypotonic solution to artificially deliver the ESX-1 mutant bacteria into the cytosol and then reexamined actin polymerization. Okada and Rechsteiner (31) showed elegantly that hypotonic shock treatment causes lysis of pinocytic/endocytic vesicle membranes without disrupting the plasma membrane. This finding supports that the hypotonic shock treatment could facilitate entry of the ESX-1 mutant bacteria into the host cell cytosol. Indeed, as shown in Fig. 4, after the treatment, mh3868::tn gained the ability to polymerize actin. Similar results were observed with mh3881c::tn (data not shown). These results indicate that the defect in the ability of the ESX-1 mutants to polymerize actin is due to their inability to escape from the vacuole rather than a deficiency in initiation of actin polymerization. They also provide direct evidence that *M. marinum* initiates actin polymerization only after it enters the host cell cytosol. Moreover, they suggest that ESX-1 is involved in secreting a pore-forming...
protein that may compromise the integrity of the MCV membranes to facilitate the escape of *M. marinum*.

**Evidence for membrane pore formation by *M. marinum***

*Listeria monocytogenes* represents a group of bacteria that are able to disrupt the vacuole membranes to enter the host cell cytosol by the secretion of pore-forming proteins (41). We hypothesized that the ESX-1 secretion system may play a similar role to facilitate *M. marinum* escape into the host cell cytosol. A direct test of this hypothesis would require the analysis of pore formation in the MCV membranes of infected cells, which is technically challenging. In an attempt to address this hypothesis, we took an alternative approach. We incubated *M. marinum* with host cells at a relatively high MOI and ex-

![FIG. 2. Transmission electron microscopy confirming the role of ESX-1 in *M. marinum* escape from the vacuole. BMDMs were infected with WT *M. marinum* or the ESX-1 mutants at an MOI of 2. At 72 h postinfection, the cells were processed for electron microscopy. For the ESX-1 mutants, only the results for mh3868::tn are shown; the results for the rest of the mutants are shown in Table 1. Panel A shows a section of a representative macrophage infected by WT *M. marinum*. The upper left insert shows an enlarged area of the cell. No host cell membranes are visible surrounding the bacteria. Note that a bacterium on the right shows an actin tail (indicated by arrows). Panel B shows a section of a representative macrophage infected by mh3868::tn. Almost all of the mutant bacteria are surrounded by vacuole membranes (indicated by arrowheads). The insert on the mid-left shows an enlarged area of the cell. Information on duplication of experiments, the number of cells examined, and statistical analyses is shown in Table 1.](http://iai.asm.org/)

![FIG. 3. ESX-1 secretion plays an essential role in polymerization of actin by *M. marinum* in macrophages. BMDMs were infected with WT *M. marinum* or the ESX-1 mutants at an MOI of 2. At 72 h postinfection, the cells were stained with Alexa Fluor phalloidin to detect F-actin. For the ESX-1 mutants, only the results for mh3868::tn are shown; the results for the rest of the mutants are shown in Table 1. The top panels show the images of phase (A), F-actin (B), and merge (C) from a representative macrophage infected by WT *M. marinum*. The bottom panels show the images of phase (D), F-actin (E), and merge (F) from a representative macrophage infected by mh3868::tn. The insert in the lower left corner is an enlarged section of the area indicated in each panel. Arrowheads indicate actin tails. Information on duplication of experiments, the number of cells examined, and statistical analyses is shown in Table 1.](http://iai.asm.org/)
amined pore formation in the cell plasma membranes. Membrane pore formation was determined by an osmoprotection assay (40) which has been used in a number of studies to demonstrate membrane pore formation and estimate pore size. This assay has been used to detect membrane pores and demonstrate membrane pore formation in the cell plasma membranes. Membrane pore formation was determined by an osmoprotection assay with different-sized PEGs to prevent hemolysis. PEG8000-R indicates recovery of hemolysis after the removal of PEG8000. The bacterium-to-red blood cell ratio is 25:1. Mm indicates M. marinum. (B) Estimation of the size of the pores induced by M. marinum. The osmolarity of each of the PEGs (PEG1000, PEG3350, PEG6000, and PEG8000) required to provide 50% protection from hemolysis is plotted as a function of its Einstein-Stokes molecular diffusion radius, $R_{\text{ES}}$ (40). The $R_{\text{ES}}$ value for each PEG is as follows: PEG1000, 1.0 nM; PEG3350, 1.9 nM; PEG6000, 2.5 nM; PEG8000, 3.2 nM. The graphs are the summation of two independent experiments, each performed in duplicate, and error bars indicate standard deviations.

To estimate the size of the membrane pores produced by M. marinum, PEGs of different sizes were used at various concentrations to determine the osmolarity required for each PEG to provide 50% protection from hemolysis (26, 40). Figure 5B shows the osmolarity of each PEG that provides 50% protection as a function of its Einstein-Stokes molecular diffusion radius, $R_{\text{ES}}$ (40). The response curve is hyperbolic and approaches a membrane stabilization limit asymptotically. By using a similar method developed by Scherrer and Gerhardt (40), we estimated pore size by extrapolating to the zero abscissa the linear regression between PEG1000 and PEG3350, and the intercept at $R_{\text{ES}}$ of 2.25 is believed to represent the radius of the membrane pore.

We then determined if ESX-1 plays a role in membrane pore formation. As shown in Fig. 6A and B, mutations in either the ESX-1 secretion apparatus (mh3877::tn and mh3871::tn) or the secreted substrate ($\Delta$sat-6) completely abolished the ability of M. marinum to induce membrane pore formation, demonstrating that ESX-1 secretion plays an essential role in this process. To determine if continuous ESX-1 secretion or pre-deposition of ESX-1-secreted proteins on the bacterial surface is necessary to cause pore formation, we treated WT M. marinum with CCCP, a membrane deenergizer that blocks energy-dependent pathways including ESX-1. Figure 6C shows
that the CCCP treatment abolished hemolysis completely and in a reversible manner, indicating that continuous energy-dependent ESX-1 secretion is required for \textit{M. marinum} to induce membrane pore formation.

**Evidence for membrane pore formation by ESX-1-secreted ESAT-6.** Three known ESX-1-secreted proteins, ESAT-6, CFP-10, and Mh3881c, are co-dependent for secretion (50). To determine the relative role of each individual protein in pore formation, we compared the hemolysis levels induced by different \textit{M. marinum} strains producing various amounts of these proteins. We have shown previously that Mh3881c is cleaved during secretion to produce an N-terminal 50-kDa and a C-terminal 11-kDa fragment (apparent molecular masses) (50; Fig. 7, lane 1). The 50-kDa fragment is relatively stable and present at abundant levels in the culture supernatant, while the majority of the 11-kDa fragment is degraded proteolytically (50; data not shown). As shown in Fig. 7, mh3881c::tn fails to secrete these three proteins and is defective in pore formation (lane 2). Both the secretion and pore formation defects of this mutant are almost fully restored by expression of the WT mh3881c gene (lane 3). On the other hand, when only the N-terminal 50-kDa fragment of Mh3881c was expressed in this mutant, it was secreted by the mutant at levels comparable to...
those produced by WT bacteria, but ESAT-6 secretion was not detected and CFP-10 secretion was minimal (lane 4). Because this strain shows a complete defect in pore formation, the data suggest that Mh3881c, or at least the 50-kDa fragment, does not contribute directly to pore formation. To determine if the secretion of ESAT-6 or CFP-10 plays a role, we examined pore formation by Δesat-6 and the complementation strain. Δesat-6 fails to secrete these three proteins and is defective in pore formation (lane 5). Expressing esat-6-His\(_{6}\) in Δesat-6 substantially restored the secretion of ESAT-6 and Mh3881c, although CFP-10 secretion was only recovered by a marginal level (lane 6). The reason that we used esat-6-His\(_{6}\) instead of esat-6 was to express this protein in a form that is exactly the same as the recombinant protein used in other assays (see below). Because this strain shows a substantial increase in hemolysis, which correlates with the much increased ESAT-6 secretion, the results suggest that ESAT-6 secretion plays a more important role in pore formation.

To determine if ESAT-6 has a direct role in membrane pore formation, we examined the pore-forming activity of recombinant ESAT-6 [rESAT-6-His\(_{6}\)] purified from Escherichia coli and compared it to that of rCFP-10-His\(_{6}\) or rMh3881c-His\(_{6}\). These recombinant proteins were affinity purified and free of detergents and had endotoxins removed (see Materials and Methods for details). As shown in Fig. 8A, rESAT-6-His\(_{6}\) from M. tuberculosis produced dose-dependent hemolysis after a 2-h incubation, i.e., partial hemolysis at 15 µg/ml and complete hemolysis at 30 µg/ml, almost equivalent to lysis with \(\text{H}_2\text{O}\). In contrast, neither rCFP-10-His\(_{6}\) nor rMh3881c-His\(_{6}\) from M. marinum caused hemolysis, even at a higher concentration of 60 µg/ml (Fig. 8B) or 120 µg/ml (data not shown). The combination of rESAT-6-His\(_{6}\) with rCFP-10-His\(_{6}\) or with rMh3881c-His\(_{6}\) produced hemolysis at levels similar to those produced by rESAT-6-His\(_{6}\) alone (data not shown). Hemolysis induced by rESAT-6-His\(_{6}\) was not due to the residual endotoxins present in the recombinant protein preparations (≤0.24 ng/mg protein), since lipopolysaccharide at a concentration of 0.007 ng/ml [equivalent to the level of endotoxins present in rESAT-6-His\(_{6}\)] at a concentration of 30 µg/ml] failed to induce a detectable level of hemolysis (data not shown). Consistent with the hemolysis results, rESAT-6-His\(_{6}\) at 60 µg/ml, but not rCFP-10-His\(_{6}\) or rMh3881c-His\(_{6}\) (even at 120 µg/ml), caused release of LDH from macrophages (Fig. 8C). Permeation of macrophage cell membranes was similarly observed by microscopic detection of penetration of ethidium homodimer-1 across the plasma membrane into the cytosol to stain the nuclei red (Fig. 8D). These results together suggest that ESAT-6 may play a direct role in membrane pore formation.

To provide a more direct demonstration that ESAT-6 by itself can induce pore formation in cell membranes, we performed an osmoprotection assay similar to that described above. As shown in Fig. 9A, the hemolysis induced by rESAT-6-His\(_{6}\) was blocked completely by 30 mM PEG8000 and PEG6000, ~40% by PEG3350, and to a small extent by PEG1000. The facts that hemolysis is induced by rESAT-6-His\(_{6}\) and that it can be blocked by PEGs of increasing sizes indicate that ESAT-6 indeed plays a direct role in membrane pore formation.
H2O, which caused 95% and complete hemolysis after 5 and 20 min of incubation, which increased to 93% after 10 min of incubation, respectively.

The kinetics of hemolysis produced by rESAT-6-His(6). Figure 9A characterized the ESAT-6-induced membrane pores by determining hemolysis after a 5-min incubation, which was completely blocked by 30 mM PEG6000 (data not shown). (B) The kinetics of membrane pore formation induced by ESAT-6. The concentration of rESAT-6-His(6) was 30 μg/ml for both panels. Error bars indicate standard deviations of data from two or three independent experiments, with each performed in duplicate.

Pore formation. It was noticed that the membrane pores induced by rESAT-6-His(6) are somewhat smaller than those produced by the bacteria (see Discussion). We further characterized the ESAT-6-induced membrane pores by determining the kinetics of hemolysis produced by rESAT-6-His(6). Figure 9B shows that rESAT-6-His(6) at 30 μg/ml caused 75% hemolysis after a 5-min incubation, which increased to 93% after 10 min. This is almost equivalent to the hemolysis produced by H2O, which caused 95% and complete hemolysis after 5 and 20 min of incubation, respectively.

**DISCUSSION**

The ESX-1 (type VII) secretion system plays an important role in the virulence of *M. tuberculosis* and *M. marinum*, but the precise molecular and cellular mechanisms by which it enhances virulence are not clearly defined. This paper describes a comprehensive study of these mechanisms which has led to several important observations. Firstly, by examining nine *M. marinum* ESX-1 mutants and the WT by fluorescence and electron microscopy detecting MCV membranes, this study demonstrates conclusively that ESX-1 plays an essential role in the escape of *M. marinum* from the MCV. The role of vacuole escape in mycobacterial pathogenesis is not clearly understood. The observations that *M. marinum* can polymerize actin inside host cell cytosol and spread from cell to cell (17, 42, 43) suggest that vacuole escape may play a role in mycobacterial spreading.

Secondly, we show that the ESX-1 mutant bacteria are able to polymerize actin after being delivered into the host cell cytosol by hypotonic shock treatment. This result suggests that the defect in the ability of the ESX-1 mutants to polymerize actin during normal cell infection is due to their inability to escape from the MCV rather than a deficiency in initiation of actin polymerization. This conclusion helps to redirect future research efforts aimed at identifying the mycobacterial molecules directly responsible for initiating actin polymerization. In addition, since this assay clearly shows that *M. marinum* induces actin polymerization only after it enters the cytosol, it suggests that actin tails can be used as a readout of cytosolic localization for *M. marinum*.

Thirdly, this study demonstrates for the first time that *M. marinum* utilizes ESX-1 to produce membrane pores ~4.5 nm in diameter in red blood cells and macrophages. Importantly, purified ESAT-6 by itself at a concentration of 30 μg/ml is sufficient to cause pore formation in cell membranes. These observations are a significant advancement of the previously published works. For example, *M. tuberculosis* (24) and *M. marinum* (17) have been shown to induce the permeation of cell membranes, and here we demonstrate that the cause of this permeation is pore formation. In addition, earlier studies show that purified ESAT-6 can cause permeation of liposome membranes (9, 24), and here we show that it causes pore formation in cell membranes. Our study suggests that ESAT-6 secreted by *M. marinum* ESX-1 could play a direct role in causing pore formation in MCV membranes to facilitate mycobacterial escape from the vacuoles.

We have noticed that purified ESAT-6 induces membrane pores that are somewhat smaller than those produced by bacteria. We hypothesize that the membrane pores could be formed by the insertion of multimers of ESAT-6, exposing their hydrophobic surface to the lipid bilayer and their hydrophilic surface to the center of the pore. This model suggests that the number of ESAT-6 molecules inserted to form a pore could determine the size of the pore. It is possible that ESAT-6 secreted by *M. marinum* ESX-1 could play a direct role in causing pore formation in MCV membranes to facilitate mycobacterial escape from the vacuoles.

A topic related to the above is whether ESAT-6 alone or ESAT-6 in complex with CFP-10 or the other ESX-1-secreted proteins forms the membrane pores during mycobacterial infection. Thus far, the published studies have assigned the membrane destabilization activity to ESAT-6 alone, exposing their hydrophobic surface to the lipid bilayer and their hydrophilic surface to the center of the pore. This model suggests that certain bacterial surface structures or molecules or some other secreted molecules could enhance membrane pore formation. For example, the secretion of phospholipases C by *Listeria* plays a role in enhancing membrane pore formation by the pore-forming toxin listeriolysin O (21). *M. marinum* and *M. tuberculosis* contain multiple copies of phospholipase C (6, 45), and *M. tuberculosis* has been shown to secrete phospholipase C (35). The involvement of phospholipase C in membrane pore formation is worthy of further investigation.

FIG. 9. Purified ESAT-6 induces pore formation in red blood cell membranes. (A) Membrane pores induced by rESAT-6-His(6) were blocked by PEGs of appropriate sizes in an osmoprotection assay. Hemolysis was also completely blocked by 30 mM PEG6000 (data not shown). (B) The kinetics of membrane pore formation induced by ESAT-6. The concentration of rESAT-6-His(6) was 30 μg/ml for both panels. Error bars indicate standard deviations of data from two or three independent experiments, with each performed in duplicate.
natant. Both of our studies suggest that during *M. marinum* infection at least some of the ESAT-6 molecules could be targeted to the host cell membranes not in a complex with MCV membranes. The reproducibility of observations and ease of manipulation make *M. marinum* an ideal system for studying these cellular processes important for the pathogenesis of mycobacteria.

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