Targeting of Enteropathogenic Escherichia coli EspF to Host Mitochondria Is Essential for Bacterial Pathogenesis

CRITICAL ROLE OF THE 16TH LEUCINE RESIDUE IN EspF*

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The attachment of enteropathogenic Escherichia coli (EPEC) to host cells and the induction of attaching and effacing (A/E) lesions are prominent pathogenic features. EPEC infection also leads to host cell death and damage to the intestinal mucosa, which is partly dependent upon EspF, one of the effectors. In this study, we demonstrate that EspF is a mitochondrial import protein with a functional mitochondrial targeting signal (MTS), because EspF activity for importing into the mitochondria was abrogated by MTS deletion mutants. Substitution of the 16th leucine with glutamic acid (EspF(L16E)) completely abolished EspF activity. Infection of HeLa cells with wild type but not the espF mutant (ΔespF) decreased mitochondrial membrane potential (ΔΨm), leading to cell death. The ΔΨm decrease and cell death were restored in cells infected with ΔespF/pEspF but not ΔespF/pEspF(L16E), suggesting that the 16th leucine in the MTS is a critical amino acid for EspF function. To demonstrate the impact of EspF in vivo, we exploited Citrobacter rodentium by infecting C3H/HeJ mice with ΔespF>Ctr ΔespF>Ctr/pEspF>Ctr or ΔespF>Ctr/pEspF(L16E)>Ctr. These results indicate that EspF activity contributes to bacterial pathogenesis, as judged by murine lethality and intestinal histopathology, and promotion of bacterial colonization of the intestinal mucosa.

Enteropathogenic Escherichia coli (EPEC)† associated with severe infantile diarrhea represents a major health problem in developing countries and is also responsible for occasional out-

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1 The abbreviations used are: EPEC, enteropathogenic E. coli; LEE, locus of enterocyte effacement; ΔΨm, mitochondrial membrane potential; EGFP, enhanced green fluorescent protein; EH, ethidium homodimer; EspF, EPEC-secreted protein F; LDH, lactate dehydrogenase; mtHsp70, mitochondrial heat shock protein 70; MTS, mitochondrial targeting signal; Rho123, rhodamine 123; TTSS, type III secretion system; WT, wild type; MnSOD, manganese superoxide dismutase; TUNEL, TdT-mediated dUTP nick-end labeling; Map, mitochondrial-associated protein; moAb, monoclonal antibody; Km, kanamycin; PBS, phosphate-buffered saline; cuf, colony-forming unit; DEME, Dulbecco’s modified Eagle’s medium; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate.

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indicated that EspF can interact with cytookeratin 18, and Nougayrede and Donnenberg (26) have shown that EspF can migrate into host mitochondria and induce host cell death. Importantly, recent studies with C. rodentium (strain DBS100) revealed that C57BL/6, NIH Swiss, or C3H/HeJ mice, infected with the espF mutant, was partly attenuated as compared with that of wild-type C. rodentium (6, 8). Although these studies have clearly demonstrated that EspF acts as a bacterial effector, the biological relevance of each EspF activity in bacterial infection of the intestinal mucosa remains unclear.

In this context, by creating a series of single amino acid substitutions in the mitochondrial targeting signal (MTS) of EspF, we investigated the biological relevance of EspF activities to migration into host mitochondria, as it pertains to bacterially induced cell death, injury of the intestinal barrier, or bacterial colonization of the intestinal mucosa. One of the single amino acid substitutions at the 16th leucine in EspF abolished the ability to migrate into mitochondria, and this was also relevant to the EspF activity of initiating the mitochondrial death pathway. The single amino acid-substituted EspF mutant was created using C. rodentium, and the cloned plasmid was introduced into the espF mutant of C. rodentium in order to evaluate the impact on the pathogenesis of bacterial infection of the murine intestine. Our data provide for the first time convincing evidence that EspF activity contributes to injury of the intestinal mucosa including cell death and enhances of bacterial colonization of the intestinal mucosa.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—Bacterial strains and plasmids used in this study are listed in Table I. We created an EPEC (strain E2348/69) espF mutant by insertion of a kanamycin (Km)-resistant cassette gene. Two mutagenesis kits (Stratagene). pMnSOD-CR and pEspFCR were constructed by ligating the BamHI and XhoI fragment of the espF gene (from Sigma). Anti-intimin polyclonal antibody was used as described previously (30).

**Plasmid Constructions**—pEspF was constructed by ligation of the BamHI and XhoI fragment of the espF gene into the BamHI and SalI sites of pBR322 and pSU18 (chloramphenicol (CP)-resistant). pBREspFCR and pEspFCR were constructed by ligating the BamHI and XhoI fragment of the espF CR complementary gene (from +150 bp to −174 bp of C. rodentium espF CR into the BamHI and SalI sites of pBR322 and pSU18 (chloramphenicol (CP)-resistant) (31), respectively. The full-length or truncated pEGFP-EspF and pEspF-EGFPs were constructed as follows. The various espF gene fragments amplified by PCR were digested with EcoRI and BamHI and then ligated into the corresponding sites of pEGFP-N3 or pEGFP-C2. The point mutants on pEspF-EGFP, pEspF, and pEspFCR were created with a QuikChange site-directed mutagenesis kit (Stratagene), pMnSOD-EGFP and pTom20-EGFP were constructed, respectively, by ligation of MnSOD and ton20 fragments amplified by RT-PCR using total cDNA of HeLa cells with pEGFP-N3.

**Conditions of Eukaryotic Cell Culture and Bacterial Culture**—HeLa and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) with 10% fetal calf serum (Sigma) at 37 °C in the presence of 5% CO2. In the EPEC infection experiment, bacteria were grown overnight in LB broth containing 1% mannose at 37 °C with or without appropriate antibiotics (ABPC, 50 μg/ml or CP; 25 μg/ml). The bacteria were diluted 1:10 into modified EPEC adherence medium (13) consisting of DMEM supplemented with 40 mM HEPES (pH 7.4), 2% fetal calf serum, and 1% mannose with or without antibiotics and grown with shaking at 37 °C for 2 h. Cultured bacteria were added at a multiplicity of infection (moi) of 100 in the modified EPEC adherence medium.

**Infection of Cultured Cells with EPEC**—The state of infection was followed by the method of Crane et al. (13). HeLa cells (2 × 105 per well) were grown (on coverslips in the experiment with immunofluorescent staining) on 12-well cell culture plates in DMEM with 10% fetal calf serum for 16 h at 37 °C in the presence of 5% CO2. The culture medium was changed to modified EPEC adherence medium prior to bacterial infection. The precultured bacteria as described above were added to HeLa cells and incubated at 37 °C in the presence of 5% CO2. After 1 h of infection, the plate was washed.
with PBS three times and replaced with RPMI 1640 medium supplemented with 40 mM HEPES (at pH 7.4), 1% mannosse, and 0.1% bovine serum albumin with or without antibiotics.

Influential Staining and Immunoblotting—The cells on a coverslip were washed with PBS three times and immunostained with the appropriate antibodies as described previously (32). The coverslips were mounted on VectorShield (Vector Laboratory) for observing by a confocal laser-scanning microscope (MicroRadiance Plus, Bio-Rad). Immunoblotting analysis was carried out as described previously (33).

Intracellular Expression of EspF-EFGP Fusion Proteins—COS-7 cells (~50% confluent on 12-well plate) on coverslips were transfected with 1 μg/ml EFGP fusion protein expression vectors (pEspF-EFGP etc.) using FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol in the absence or presence of 10 μM valinomycin (uncoupler of mitochondrial inner membrane potential). After 16 h of incubation, the cells were treated with 100 μM Mitotracker (Molecular Probes) in DMEM (fetal calf serum-free) for 30 min in the absence or presence of 10 μM valinomycin and then washed three times with PBS. After fixation with 4% paraformaldehyde in PBS, coverslips were washed with PBS and then immunostained. They were observed by confocal laser-scanning microscopy. To determine the ratio of EspF-EFGPs localized in mitochondria, 100 cells expressing EspF-EFGPs were counted (a cell whose mitochondrial EFGF fluorescent signal was strongly overlapped with the cytosolic signal and the nuclear signal was being localized in mitochondria). The counts were performed three times, independently.

Fractionation of Mitochondria from HeLa Cells Infected with EPEC—The mitochondrial fractionation was based on a method described previously (34, 35). HeLa cells (2 × 10^7) were infected with ΔespFΔpEspF or ΔespFΔpEspF (L16E) for 3 h and washed with PBS four times. The following procedures were performed at 4 °C. Cells collected with a rubber-bell homogenizer were homogenized using a Microtube homogenizer (I.S.O) in buffer A (250 mM sucrose, 25 mM HEPES-KOH (at pH 7.4), 2.5 mM KCl, 5 mM MgCl2, 0.5 mM EGTA, and 5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF)). The homogenate was centrifuged at 600 × g for 10 min, and the pellet was used as the nuclei and unbroken cell fraction, while the supernatant was centrifuged at 7000 × g for 10 min. The resulting pellet was the cytosolic fraction including bacteria, while the supernatant was centrifuged at 100,000 × g for 1 h, yielding a pellet (mitochondrial fraction) and another supernatant (cytosolic fraction). To obtain highly purified mitochondria, the crude mitochondrial fraction was layered on a discontinuous sucrose gradient consisting of 4.5 ml of 1.6–1.0 M sucrose from the bottom and centrifuged at 82,000 × g for 1 h. After 200 min, the middle band was collected and was used as a mitochondrial fraction.

Mitochondrial Inner Membrane Potential (Δψm)—HeLa cells preincubated with the Δψm-sensitive dye rhodamine123 (Rh123) at 2 μg/ml (36) in DMEM (fetal calf serum-free) were washed with PBS three times and then infected with EPECs at an moi of 100 as described above. After 6 h of infection, both detached (supernatant) and attached cells were collected and the number of cfu per mouse was calculated. For the detection of released cytochrome c, the equal amounts of cytosolic proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-cytochrome c and anti-actin antibody as an internal standard control.

Detection of EspF Secreted by EPEC into the Culture Medium—The secretion of EspF was analyzed by immunoblot as described previously (29).

Infection of Mice with C. rodentium—6-week old, female C3H/HeJ mice (JC1, CLEA Japan) highly susceptible to C. rodentium (38), were housed for a week in the animal facility of the Institute of Medical Science, University of Tokyo in accordance with guidelines drafted by the University of Tokyo. Wild-type C. rodentium (WT_CR), ΔespFΔcr, and complementary strain ΔespFΔpEspFΔcr and ΔespFΔpEspFΔcr (L16E) were cultured overnight in LB broth with or without 25 μg/ml of CP at 37 °C. The respective cultures (2 ml) were centrifuged (1000 × g, 5 min) at room temperature, and after the supernatant had been discarded, bacteria were resuspended with 2 ml of LB broth. In order to examine the survival of EPEC, 200 μl of bacterial suspension (~2 × 10^8 cfu/ml) were inoculated into ten mice by oral gavages. Survival was assessed daily over the course of the infection for up to 20-days postinfection.

For determination of the number of adherent bacteria on the colon and colonic weight, five mice were inoculated in the same manner. At 9-days postinfection, these mice were sacrificed, and 5.5 cm of distal colon from the rectum were cut vertically along the colon. These samples were washed with PBS to remove fecal pellets. Weights were then determined, and the specimens were homogenized in 5 ml of ice-cold PBS with a Potter Elvehjem homogenizer (digital homogenizer, AS ONE). The homogenates were serially diluted with ice-cold PBS and plated on MacConkey agar plates with or without 25 μg/ml of CP. Colonies of C. rodentium, which were checked with PCR by amplifying the espF gene, were counted at least in five fields of view that included the proprial muscular layer through the luminal side at × 50 magnification and then converted to numbers per 1 mm^2.

RESULTS

Subcellular Localization of EspF Secreted from EPEC—After 3 h of infection of HeLa cells with WT EPEC or the espF mutant (ΔespF), the subcellular localization of EspF was examined using immunofluorescence microscopy with anti-EspF, anti-mitochondrial inner membrane heat shock protein 70 (mtHsp70) antibody and TO-PRO3. As shown in Fig. 1A, the EspF signal (green) in HeLa cells merged with the mitochondrial signal (red) (Fig. 1A, panel j or o), in which the intensity profiles of EspF and mtHsp70 signals as scanned along the X-X’ axis in panels l and m, were similar (Fig. 1B). Since the EspF signal mostly overlapped with the bacterial signal, we subsequently scanned the signals toward the Z-axis moving along to the X-X’ to ensure the special relationship among the three signals for bacteria, EspF, and mitochondria. As shown in Fig. 1C, the major EspF signal was merged with that of the mitochondria. A similar subcellular localization was observed in other mammalian cell lines such as HEp-2, Caco-2, T84, and COS-7 infected with EPEC, supporting the notion that EspF secreted by EPEC is imported into host mitochondria.

EspF MTS Functions in Mitochondrial Import—To investigate whether or not the MTS exists in EspF, we constructed various truncated versions of EspF, and each was cloned into either pEGFP-N3 (for pEGFP-EFP derivatives) or pEGFP-C2 (for pEGFP-EFP derivatives) (Fig. 2A). The resulting plasmids introduced into COS-7 cells were analyzed for cellular distributions of the signals for EspF-EFGP (EGFP, green) and Mitotracker, a mitochondrial specific marker (red), using immunofluorescence microscopy. As shown in Fig. 2, A and B, the full-length EspF-EFGP, EspF(1–72)-EFGP, EspF(1–43)-EGFP, and EspF(1–24)-EGFP were colocalized with mitochondria.

T. Nagai and C. Sasakiwara, unpublished data.
dria, whereas EspF-(24–206)-EGPF, EspF-(134–206)-EGPF, EGPF-EspF-(134–206), and EGPF-EspF were dispersed in the cytoplasm, indicating that the N-terminal EspF sequence functions as the MTS.

EspF Migration into Mitochondria Is Dependent on Mitochondrial Inner Membrane Potential—Because mitochondrial inner membrane potential ($\Delta \Psi_m$) has been shown to be required for mitochondrial proteins (other than mitochondrial outer membrane proteins) to be imported into mitochondria (39), we examined whether or not EspF migration into mitochondria would depend on $\Delta \Psi_m$. COS-7 cell transfectants carrying pEspF-EGFP, pMn-SOD-EGFP, or pTom20-EGFP were investigated in the presence or absence of valinomycin (10 $\mu$M) for subcellular localization using immunofluorescence microscopy. As can be seen in Fig. 3 (panels e, m, and u), because MitoTracker is incorporated into mitochondria in a manner dependent on $\Delta \Psi_m$, in valinomycin-treated cells the MitoTracker signal was not confined to the mitochondria instead being distributed within the cytoplasm, confirming that the $\Delta \Psi_m$ had disappeared. Tom20 could be imported into mitochondria regardless of valinomycin treatment (Fig. 3, panel v). Under these conditions, EspF, as well as Mn-SOD, which localizes in the matrix, was unable to migrate into mitochondria of cells treated with valinomycin (Fig. 3, panels f and n), strongly indicating that migration of EspF into mitochondria requires $\Delta \Psi_m$.

The 16th Leucine Residue of EspF Is Critical for Its Ability to Migrate into Host Mitochondria—N-terminal presequence of the mitochondrial import protein is involved in recognition by mitochondrial import machinery called TOM (translocase of outer membrane), which has the potential to form positively charged amphiphilic helices (40). According to the helical wheel...
structure of the 6–23 amino acids of EspF, one-half is rich in hydrophobic amino acids (see Fig. 3A, white circle), whereas the other side is rich in hydrophilic amino acids including two positively charged amino acids such as Arg (Fig. 3A, gray and black circles), thus forming a positively charged amphiphilic secondary structure. Previous studies have indicated that Leu and Arg in the MTS are particularly important for import into mitochondria, and that point mutants at either of these amino acids occasionally result in loss of the capacity to be imported (41, 42). Thus, we created a series of single amino acid substituted mutants, along with the MTS based on EspF-EGFP (Fig. 3B). These point mutants, designated EspF(L2E), EspF(L12E), EspF(G13E), EspF(R14Q), EspF(L16E), EspF(V17E), and EspF(R22Q) were investigated for their capacity to be transported into mitochondria. We created EGFP-fused EspF mutants in pEGFP-N3, which were introduced into COS-7 cells by transfection and investigated their localization in mitochondria. The results showed capacity of EspF(L16E) to be almost completely abolished, whereas other point mutants such as EspF(R14Q), EspF(V17E), and EspF(R22Q) retained this ability and others such as EspF(L2E), EspF(L12E), and EspF(G13E) had slightly less activity (Fig. 4, B and C). Because it has been suggested that the hydrophobic amino acids in MTS of the import proteins are important for interacting with the mitochondrial receptor, and that the positively charged residues involved in protein uptake into mitochondria depend on $\Delta \Psi_m$ (43), we substituted the 14th and 22nd Arg residues with Gln. The localization in mitochondria was investigated by the same methods as those used for the point EspF mutants. Interestingly, the import capacity of the resulting EspF(R14Q-R22Q)-EGFP was almost completely abolished, while EspF(R14Q) and EspF(R22Q) retained this ability. Thus, the 16th Leu in the MTS of EspF appears to be critical for interaction with some putative mitochondrial receptor, whereas the
Targeting of EspF Secreted by EPEC to Mitochondria

EspF Induces Cell Death and Can Reduce Mitochondrial Membrane Potential—$\Delta \Psi_m$ is necessary for producing ATP, a major source of bioenergy, via oxidative phosphorylation, and plays a regulatory role in cell fate, “survival versus death” (44). Previous studies found that EPEC infection of cultured epithelial cells elicited apoptosis and necrosis, in which the role of EspF is important (13). Therefore, we investigated HeLa cell responses to EPEC infection by focusing on the relationship between cell death and $\Delta \Psi_m$. HeLa cells pretreated with rhodamine123 (Rho123), a fluorescent dye sequestered by active mitochondria, were infected with EPEC for 30 min in the presence of EH (1 $\mu$g/ml), a cell-impermeable fluorescent dye, allowing it to flow into the host cytosol and nucleus upon disruption of the cytoplasmic membrane (13). The time course imaging of cell responses was analyzed using phase contrast and fluorescence microscopy up to 5.5-h postinfection. As seen in the series of photographs, the Rho123 signal (green: active mitochondria) within the cells gradually decreased up to 3.5-h postinfection, after which the infected cell lapsed into a rapid cell burst without cell shrinkage (Fig. 6A, white arrowheads at 2.5–3.5 h and yellow arrowheads at 3.5–5 h). Following the cell burst, an influx of EH into the nucleus resulted from the loss of cytoplasmic membrane integrity (Fig. 6A, white arrowhead at 3.5 h and yellow arrowhead at 4.5 h). These results thus suggested that the collapse of $\Delta \Psi_m$ direct ed by EPEC infection leads to cell burst. (The videos of these phase (S1), fluorescent (S2), and merged (S3) images are published on the Journal of Biological Chemistry website as Supplemental Data.) These cellular responses were not detectable when HeLa cells were infected with the ΔespF.2

Hence, we investigated whether or not a $\Delta \Psi_m$ reduction would occur upon the import of EspF into mitochondria. To test this, HeLa cells pretreated with Rho123 were infected with WT, ΔespF, or the espF complement strains. (The amount of EspF secreted by these strains were almost the same (Fig. 6B).) HeLa cells at 6-h postinfection were collected and subjected to FACS flow cytometry in order to analyze the Rho123 intensity profile (see “Experimental Procedures”). In this assay, a low intensity Rho123 peak appeared in 99% of HeLa cells upon treatment with 0.05% Triton X-100 representing loss of $\Delta \Psi_m$, whereas a high intensity Rho123 peak appeared in 99% of untreated HeLa cells, representing a high $\Delta \Psi_m$ level (Fig. 6C, top panel). As shown in Fig. 6C, upon infection with WT or ΔespF, a low intensity Rho123 peak was detected in 78.7 or 81.8% of the cell population, respectively. Furthermore, when HeLa cells were infected with ΔespF/pEspF or ΔespF/pEspF(L16E), low intensity Rho123 peaks were seen in 81.8 or 23.3% of the cell population, respectively, indicating that the dissipation of $\Delta \Psi_m$ induced by EPEC depends upon the import of EspF into mitochondria. Kenny and Jepson (21) previously indicated that Map, one of the EPEC TTSS-mediated effectors secreted, targets mitochondria and disrupts $\Delta \Psi_m$. Therefore, we investigated Δmap and ΔespF-Δmap for their effects on $\Delta \Psi_m$. As shown in Fig. 6C (bottom panels), infection of HeLa cells with Δmap and ΔespF-Δmap resulted in low intensity Rho123 peaks in 58.5 and 8.9% of cells, respectively, suggesting that, though the presence of Map affects $\Delta \Psi_m$ to some extent, the major factor leading to dissipation of $\Delta \Psi_m$ in this assay is EspF.

To further characterize the cell death induced by EspF during EPEC infection, we carried out an LDH assay to quantify...
the amount of LDH released from the damaged cell cytoplasm into the medium. The amounts of LDH released from HeLa cells into the medium at 2-, 4-, and 6-h postinfection were measured as described under “Experimental Procedures.” As shown in Fig. 6D, upon WT infection of HeLa cells, cytotoxicity increased markedly in a time course manner as compared with that shown by untreated cells. However, the cytotoxicity was significantly reduced in HeLa cells infected with ΔespF. Similarly, upon infection of HeLa cells with ΔespF/pEspF, cytotoxicity was high as compared with that of ΔespF/pEspF(L16E) infection, suggesting that EspF import into the mitochondria is involved in cell death. Since ΔespF and ΔespF/pEspF(L16E) infection of HeLa cells still elicited cell death to some extent, it is likely that some additional factor(s) associated with EspF participate in the induction of cell death. A similar cytotoxic effect on HeLa cells by C. rodentium-borne EspF (EspFCR) was observed when we introduced pBREspF CR but not pBREspF CR(L16E) into the EPEC ΔespF (Fig. 6D). Further-
more, we examined the \( \Delta \text{map} \) and \( \Delta \text{espF} \)-\( \Delta \text{map} \) for their capacities to induce cell death under the same conditions. As shown in Fig. 6D, the absence of Map from EPEC but not EspF had no appreciable effect on the EPEC-induced cytotoxicity in HeLa cells. When the LDH assay was conducted using another cell line such as a colonic T84 cell monolayer, no significant differences in the cytotoxicity induced by WT and \( \Delta \text{espF} \) infection were observed. However, when cytotoxicity on the T84 cell monolayer was measured by counting the cells taking up EEH, the cytotoxic pattern was similar to that of the LDH assay using HeLa cells and was dependent on the ability of EspF to migrate into mitochondria.\(^2\) To investigate whether or not the import of EspF into mitochondria affects bacterial pathogenesis, \(-2 \times 10^5\) of wild-type \( C. \text{rodentium} \) (WT\(_{CR}\)), \( \Delta \text{espF}_{CR}, \Delta \text{espF}_{CR}^{p \text{EspFCR}}, \) and \( \Delta \text{espF}_{CR}^{p \text{EspFL16E}_{CR}} \) were orally administered via the stomach to 10 C3H/HeJ mice, and survival of the mice was monitored up to 20 days after inoculation (Fig. 7B). Mice infected with WT\(_{CR}\) showed 100% mortality up to day 12, whereas mice infected by \( \Delta \text{espF}_{CR} \) survived through day 12 with 10% mortality after day 14. Similarly, mice infected with \( \Delta \text{espF}_{CR}^{p \text{EspFCR}} \) showed 80% mortality up to day 14, whereas those infected with \( \Delta \text{espF}_{CR}^{p \text{EspF(L16E)}_{CR}} \) exhibited 20% mortality by day 16 (Fig. 7B). Furthermore, C3H/HeJ mice (n = 5) infected with \( C. \text{rodentium} \) strains were sacrificed on day 9 and typical pathological features of the large intestine were macroscopically observed (Fig. 7C). Although intestines from the mice inoculated with LB were healthy with solidified feces, those from mice infected with WT\(_{CR}\), those from mice infected with WT\(_{CR}\), and typical findings of bacterial-induced intestinal colitis. The intestines from mice inoculated with \( \Delta \text{espF}_{CR} \) remained healthy with solidified feces, albeit some portions of the intestine were slightly swollen. The intestines from mice inoculated with \( \Delta \text{espF}_{CR}^{p \text{EspF}_{CR}} \) showed similar to those of specimens with WT\(_{CR}\). However, the intestines from mice inoculated with \( \Delta \text{espF}_{CR}^{p \text{EspF(L16E)}_{CR}} \) barely showed such findings, though the content of solidified feces was slightly less than with \( \Delta \text{espF}_{CR} \). The mice infected with these strains were sacrificed on day 9 and were also investigated for colon weight, numbers of bacteria colonizing the colon, and intestinal mucosal layer thickness as described under “Exper-

**Fig. 5.** Intracellular distributions of \( \text{EspF} \) or \( \text{EspF(L16E)} \) secreted by EPEC. A, HeLa cells were infected with \( \text{espF} \) complement strains, \( \Delta \text{espF}_{CR}^{p \text{EspF}} \) (panels a–d) or \( \Delta \text{espF}_{CR}^{p \text{EspF(L16E)}} \) (panels e–h), and the cells were immunostained by the same method as described in the legend to Fig. 1. Shown are the EspF signals (panels a and c), mtHsp70 signals (panels b and f), TO-PRO3 (panels c and g) and the merged images (panels d and h). B, subcellular localization of \( \text{EspF} \) and \( \text{EspF(L16E)} \) using cell fractionation. HeLa cells infected with \( \Delta \text{espF}_{CR}^{p \text{EspF}} \) or \( \Delta \text{espF}_{CR}^{p \text{EspF(L16E)}} \) were fractionated into nuclei-unbroken cells, mitochondria, microsomes, and cytosol by the fractional centrifugation method (see under “Experimental Procedures.”) These fractions were immunblotted with anti-EspF, anti-cytochrome oxidase subunit II (COX-II, mitochondrial marker), or anti-intimin (EPEC marker), or anti-aldolase (cytosol marker) antibodies.
Alteration of mitochondrial inner membrane potential and induction of cell death by infecting of HeLa cells with EPEC. A, time course imaging of HeLa cells infected with EPEC. HeLa cells pretreated with 2 μg/ml Rho123 were infected with WT at an moi of 100 in the presence of 1 μg/ml of EH. At 1-h postinfection, the phase contrast, Rho123 signal (green) and EH signal (red) were examined using fluorescence...
immunoblotting with anti-cytochrome c. Cells were obtained by a digitonin-based subcellular fractionation technique. These fractions were separated by SDS-PAGE and detected by anti-C. rodentium antiserum and then visualized using immunofluorescence microscopy. Phalloidin (actin) staining was used to counterstain the tissue. High numbers of bacteria were visible over the epithelial surfaces including intestinal crypts with WT\textsubscript{CR} or ΔespF\textsubscript{CR} pEspF\textsubscript{CR} infection but not ΔespF\textsubscript{CR} or ΔespF\textsubscript{CR} pEspF\textsubscript{L16E}\textsubscript{CR} infection (Fig. 7G). The bacteria were frequently visible in proximity to the lamina muscularis mucosae in colonic sections (Fig. 7G, blue arrowheads), whereas large amounts of exfoliative epithelial tissue inside the lumen were stained with anti-C. rodentium antiserum (Fig. 7G, white arrowheads). The intestinal tissue infected with WT\textsubscript{CR} or ΔespF\textsubscript{CR} pEspF\textsubscript{CR} showed marked hyperplasia, whereas hyperplasia was significantly milder with ΔespF\textsubscript{CR} or ΔespF\textsubscript{CR} pEspF\textsubscript{L16E}\textsubscript{CR} infection. Thus, these results from this series of experiments further support the notion that the ability of EspF to migrate into host mitochondria is critical for bacterial colonization of the intestinal epithelium and the initiation of disease processes.

**Discussion**

EspF is a cytotoxic factor secreted by EPEC that is responsible for the release of LDH from cells into the culture supernatant at 2, 4, and 6 h post-infection, and cytotoxicity was calculated as described under "Experimental Procedures." These indices were significantly reduced by infection with ΔespF\textsubscript{CR} as compared with WT\textsubscript{CR}. The same was true for infection with ΔespF\textsubscript{CR}/pEspF\textsubscript{L16E}\textsubscript{CR} or ΔespF\textsubscript{CR}/pEspF\textsubscript{CR} (Fig. 7, D–F). Bacteria present in the frozen distal colon sections on day 9 postinfection were also stained using anti-C. rodentium antiserum and then visualized using immunofluorescence microscopy. Phalloidin (actin) staining was used to counterstain the tissue. High numbers of bacteria were visible over the epithelial surfaces including intestinal crypts with WT\textsubscript{CR} or ΔespF\textsubscript{CR} pEspF\textsubscript{CR} infection but not ΔespF\textsubscript{CR} or ΔespF\textsubscript{CR} pEspF\textsubscript{L16E}\textsubscript{CR} infection (Fig. 7G). The bacteria were frequently visible in proximity to the lamina muscularis mucosae in colonic sections (Fig. 7G, blue arrowheads), whereas large amounts of exfoliative epithelial tissue inside the lumen were stained with anti-C. rodentium antiserum (Fig. 7G, white arrowheads). The intestinal tissue infected with WT\textsubscript{CR} or ΔespF\textsubscript{CR} pEspF\textsubscript{CR} showed marked hyperplasia, whereas hyperplasia was significantly milder with ΔespF\textsubscript{CR} or ΔespF\textsubscript{CR} pEspF\textsubscript{L16E}\textsubscript{CR} infection. Thus, these results from this series of experiments further support the notion that the ability of EspF to migrate into host mitochondria is critical for bacterial colonization of the intestinal epithelium and the initiation of disease processes.

**Discussion**

EspF is a cytotoxic factor secreted by EPEC that is responsible for the release of LDH from cells into the culture supernatant at 2, 4, and 6 h post-infection, and cytotoxicity was calculated as described under "Experimental Procedures." These indices were significantly reduced by infection with ΔespF\textsubscript{CR} as compared with WT\textsubscript{CR}. The same was true for infection with ΔespF\textsubscript{CR}/pEspF\textsubscript{L16E}\textsubscript{CR} or ΔespF\textsubscript{CR}/pEspF\textsubscript{CR} (Fig. 7, D–F). Bacteria present in the frozen distal colon sections on day 9 postinfection were also stained using anti-C. rodentium antiserum and then visualized using immunofluorescence microscopy. Phalloidin (actin) staining was used to counterstain the tissue. High numbers of bacteria were visible over the epithelial surfaces including intestinal crypts with WT\textsubscript{CR} or ΔespF\textsubscript{CR} pEspF\textsubscript{CR} infection but not ΔespF\textsubscript{CR} or ΔespF\textsubscript{CR} pEspF\textsubscript{L16E}\textsubscript{CR} infection (Fig. 7G). The bacteria were frequently visible in proximity to the lamina muscularis mucosae in colonic sections (Fig. 7G, blue arrowheads), whereas large amounts of exfoliative epithelial tissue inside the lumen were stained with anti-C. rodentium antiserum (Fig. 7G, white arrowheads). The intestinal tissue infected with WT\textsubscript{CR} or ΔespF\textsubscript{CR} pEspF\textsubscript{CR} showed marked hyperplasia, whereas hyperplasia was significantly milder with ΔespF\textsubscript{CR} or ΔespF\textsubscript{CR} pEspF\textsubscript{L16E}\textsubscript{CR} infection. Thus, these results from this series of experiments further support the notion that the ability of EspF to migrate into host mitochondria is critical for bacterial colonization of the intestinal epithelium and the initiation of disease processes.

**TUNEL Staining in Vivo**—The TUNEL assay involves labeling of the 3′-hydroxyl DNA ends generated by DNA fragmentation during apoptosis by means of terminal deoxynucleotidyl transferase (TdT) and labeled dUTP. However, several reports suggest that non-apoptotic DNA fragmentation is labeled also by the TUNEL assay (46). Since EspF can initiate the mitochondrial death pathway, we visualized intestinal cell death using TUNEL staining of the murine intestine infected with WT\textsubscript{CR}, ΔespF\textsubscript{CR}, ΔespF\textsubscript{CR}/pEspF\textsubscript{CR}, or ΔespF\textsubscript{CR}/pEspF\textsubscript{L16E}\textsubscript{CR} (Fig. 8). Frozen distal colonic sections on day-9 post-infection were stained for dead nuclei by the TUNEL method and counterstained with rhodamine-phalloidin, anti-C. rodentium serum, and DAPI—TUNEL-positive cells were counted. The numbers of TUNEL-positive cells per mm\textsuperscript{2} of a section showed cell death caused by infection with WT\textsubscript{CR} or ΔespF\textsubscript{CR}/pEspF\textsubscript{CR} to be decreased to one-fifth of that with ΔespF\textsubscript{CR} or ΔespF\textsubscript{CR}/pEspF\textsubscript{L16E}\textsubscript{CR} (Fig. 8A). Fig. 8B shows the representative data from the TUNEL-positive cells in the infected intestinal section. Based on the results of this series of experiments, we concluded that the ability of EspF to migrate into mitochondria is biologically relevant to intestinal injury including the cell death that results from bacterial infection.

**Discussion**

In the present study, we have investigated the impact of EspF as an EPEC effector in infection and obtained molecular evidences to support the concept that the capacity of EspF to migrate into the host cell mitochondria is important for bacterial pathogenesis. To this end, we used mice orally infected with a C. rodentium espF\textsuperscript{mutant}.

Mitochondria are an important target for many pathogens determining the fate of infected host cells (47). For example, human T-lymphotrophic virus type I protein p13\textsuperscript{(III)} has an amphilic MTS and decreases mitochondrial membrane potential (48). PorB is the porin protein of Neisseria gonorrhoeae and Neisseria meningitidis. Though N. gonorrhoeae PorB has no typical MTS, it interacts with the mitochondrial outer membrane via binding to VDAC (voltage-dependent anion channel) protein, leading to apoptotic cell death (49). In contrast, the PorB of N. meningitidis protects cells from apoptosis (50). The N-terminal cleavage product of VacA (p34) secreted from Helicobacter pylori has no MTS, but can be imported into mitochondria and trigger apoptotic cell death (51). SipB secreted from Salmonella enterica serovar Typhimurium via TTSS can also be imported into mitochondria and lead to cell death via induction of mitochondrial autophagy (52). Interestingly, although no genetic evidence has yet been obtained, Map secreted from EPEC via the TTSS has a typical MTS in the N-terminal 42 amino acids involved in import into mitochondria (22). Furthermore, Tir, the intimin receptor, has also been indicated to migrate into mitochondria and thereby lead to cell death (53). Together with those of a recent study indicating that EspF has the capacity to migrate into mitochondria (26), these findings indicate that the delivery of various effectors into host mitochondria from EPEC is likely to play some important roles in bacterial infection.

We demonstrated here that EspF can be imported into mitochondria, and that this protein import is crucial for EPEC infection. MTS involved in mitochondrial import of proteins reportedly takes part in recognition by several mitochondrial outer membrane proteins such as Tom20, Tom22, and Tom70, followed by translocation into the matrix through a general import pore (40). Since the MTS of EspF is located at the N terminus, EspF might initially be recognized by the Tom20-Tom22 complex instead of Tom70. A genetic and functional study of Tom20 indicated that the MTS recognized by Tom20 frequently possesses a motif composed of ΦXXΦΦ (Φ represents a hydrophobic amino acid, whereas Υ represents an arbitrary amino acid with a hydrophilic plus long side chain) (54). Thus, we looked for a putative consensus motif in the MTS of EspF, and found 5-ISNAA-9 and 13-GRQLV-17 to fit the motif. The fact that EspF(L16E) (13-GRQEV-17) was poorly transported into mitochondria raised the possibility that the EspF MTS might also be imported into mitochondria via an interaction with Tom20.

As mentioned above, many pathogenic bacteria can kill host cells by inducing cell death, although the type of cell death varies among target host cells, depending on cellular physiological or experimental conditions (55). Crane et al. (13) reported that EPEC infection of epithelial cells caused necrosis-like cell death, whereas ectopic expression of EspF in epithelial cells triggered apoptotic cell death (13). In this study, we demonstrated that import of EspF into mitochondria resulted in a decrease in ∆Ψ\textsubscript{m}, accompanied by necrotic cell (see Fig. 6). Recent studies have shown Salmonella-induced macrophage necrotic cell death to be caspase-1-dependent (56), while Bordetella bronchiseptica-induced necrotic cell death in epithelial cells triggered caspase-1-dependent cell death via the intrinsic mitochondrial pathway (56).

**Experimental Procedures**

Results are representative of three independent experiments. Data are presented as means ± S.D. E, release of cytochrome c into the cytosol. HeLa cells were infected with EPECs as in D. After 2, 4, and 6 h of infection, the cytosolic proteins of infected HeLa cells were obtained by a digitonin-based subcellular fractionation technique. These fractions were separated by SDS-PAGE and detected by immunoblotting with anti-cytochrome c. The same blots were analyzed with anti-actin to control for protein loading. As a control for apoptosis, HeLa cells were incubated with 2 μM staurosporine (STS) for 6 h.
FIG. 7. Studies of mice infected with *C. rodentium*. (*C. rodentium* EX33 strains were as follows, LB:a, WTc:b, ΔespF_c:c, ΔespF_c/pEspF(L16E)_c:d, and ΔespF_c/pEspF(E116L)_c:e). A, Western blot of EspF_c secreted by *C. rodentium* strains into DMEM. B, survival curves. C3H/HeJ mice (*n* = 10) were orally infected with 2 × 10^8 CFU of *C. rodentium* strains and monitored daily up to 20 days. Percentage of mice surviving from initial population is shown. C–G, effect of EspF_c on colonic tissue. C3H/HeJ mice (*n* = 5) were inoculated with *C. rodentium* strains, as described in methods for B and sacrificed 9-days postinfection. Colonies (from cecum to rectum) showing typical features of infection with the respective strains are shown in C. After weighing the colons (5.5 cm from rectum) without fecal pellets (shown in D), the specimens were homogenized and plated on MacConkey agar plates. Colonies were then counted to determine the number of adherent bacteria in the mouse colon (shown in E, the *y* axis values are presented on a log scale). Portions of these distal colons were fixed, frozen, cut into 7-μm sections, and immunostained with anti-*C. rodentium* antiserum (green) and counterstained for actin with rhodamine-phalloidin (red). Thicknesses of mucosal layers were measured (shown in F). Typical immunofluorescent images are shown in G. Data are presented as means ± S.D.
cells is caspase-1-independent (57). Nevertheless, various forms of necrotic cell death were prevented by adding glycine (inhibitor of necrosis caused by nonspecific ion fluxes through the cytoplasmic membrane). Furthermore, EspF-induced cell death was not inhibited by glycine but was inhibited by z-VAD-fmk (pancaspase inhibitor), although no cleavage of polyADP-
Targeting of EspF Secreted by EPEC to Mitochondria

ribose polymerase (PARP), a substrate for caspases, was observed. It has recently been shown that two different types of cell death occur in Jurkat cells, with the type being determined by the intracellular ATP concentration (58). The authors observed the concentration of intracellular ATP to act as a molecular switch controlling the type of cell death. Indeed, they reported that with a low concentration of intracellular ATP, necrotic cell death could be blocked by adding z-VAD-fmk without Lamin B, a substrate for caspases, being cleaved. These features of necrotic cell death are similar to those of cell death induced by EPEC infection. Intriguingly, Cranel et al. (13) reported that EspF has some capacity to reduce the intracellular ATP concentration (13). If true, this raises the possibility that necrotic death of epithelial cells in response to EPEC infection may be triggered by reducing the ATP concentration via the release of cytochrome c, which functions in respiration as a key molecule, from mitochondria resulting from the import of EspF into mitochondria.

Recent studies have indicated another biological activity of EspF, i.e., involvement in alteration of intestinal epithelial barrier function (24, 59). Dickman et al. (60) suggested that Rota virus infection decreased metabolism and cellular ATP concentrations, resulting in the destruction of cell-cell junctions. Thus, we are currently attempting to address whether EspF import into the mitochondria causes destruction of cell-cell junctions via a decreased cellular ATP concentration.

Infection of the murine intestine with C. rodentium has recently been established as the most reliable model of EPEC pathogenesis. The espF<sub>CR</sub> mutant created in C. rodentium (strain EX-33) in our study confirmed lower virulence, with oral administration to C3H/Hej mice, than with WT<sub>CR</sub>. Importantly, the ability of EspF<sub>CR</sub> to migrate into mitochondria as determined by EspF[L16E]<sub>CR</sub> was shown to be highly relevant to bacterial pathogenicity, as judged by the mouse mortality rate, colon weight, and intestinal mucosal layer thickness. Furthermore, EspF activity was also required for promotion of bacterial colonization. Though we have no other evidence as yet, we speculate that EspF activity might be needed for the pathogen to stimulate intestinal cell metabolism, thereby increasing the opportunity for bacteria to attach to the freshly renewed cell surface, possibly conferring some advantage over epithelial cells in a normal metabolic state. In fact, as shown in the histopathological study of murine intestine infected with WT<sub>CR</sub>, it seems likely that the hyperplasia caused by the pathogen increases opportunities for the bacteria to colonize the intestinal cryptae, as compared with the condition associated with the espF<sub>mut</sub> mutant. Since infection of mice with ΔespF<sub>CR</sub> or ΔespF<sub>CR</sub>PespF[L16E]<sub>CR</sub> still caused some intestinal hyperplasia as compared with the untreated intestine, bacterial effectors other than EspF, as described in the Introduction, must also be required for full bacterial virulence.

In summary, EspF secreted via the TTSS of EPEC targets host mitochondria. The N-terminal 24 amino acids serve as a mitochondrial targeting signal. In migration of EspF into host mitochondria, L64<sup>16</sup> and Arg<sup>14,22</sup> in the MTS are critical. Assessment of mitochondrial membrane potential (ΔΨ<sub>m</sub>) in infected epithelial cells indicated that EspF is required for loss of ΔΨ<sub>m</sub> to be triggered by EPEC infection. Furthermore, EspF is associated with the release of cytochrome c from mitochondria into the cytoplasm, which leads to host cell death. Finally, the significance of the ability of EspF to migrate into mitochondria during bacterial infection was established for the first time by creating the C. rodentium mutants, ΔespF<sub>CR</sub>, ΔespF<sub>CR</sub>Δesp<sub>P</sub> and ΔespF<sub>CR</sub>ΔespF[L16E]<sub>CR</sub>, in the murine infection model. Our findings thus provide clues to elucidating the role of EspF in initiation of the mitochondrial death pathway, which appears to be an important mechanism by which EPEC promotes colonization of the intestinal mucosa.

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Targeting of Enteropathogenic *Escherichia coli* EspF to Host Mitochondria Is Essential for Bacterial Pathogenesis: CRITICAL ROLE OF THE 16TH LEUCINE RESIDUE IN EspF

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