Enteropathogenic *Escherichia coli* effector EspF interacts with host protein Abcf2

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Summary

Enteropathogenic *Escherichia coli* (EPEC) is a major causative agent of infant diarrhoea in developing countries. The EspF effector protein is injected from EPEC into host cells via a type III secretion system and is involved in the disruption of host intestinal barrier function. In addition, EspF is sorted to mitochondria and has a role in initiating the mitochondrial death pathway. To clarify the manner in which EspF affects host cells, we sought to identify eukaryotic EspF-binding proteins using affinity purification. Abcf2, a protein of unknown function and member of the ABC-transporter family, bound EspF in this assay. An interaction between EspF and Abcf2 was confirmed in a yeast two-hybrid system, by colocalization and by co-immunoprecipitation from EPEC-infected cells. Levels of Abcf2 were decreased in cells infected with EPEC in an EspF dose-dependent manner. Knock-down of Abcf2 expression by RNA interference increased EspF-induced caspase 9 and caspase 3 cleavage. In addition, Abcf2-knocked down cells showed increased caspase 3 cleavage upon treatment with the apoptosis inducing agent staurosporine. These results indicate that EspF induces or facilitates host cell death by targeting and interfering with the putative protective function of Abcf2.

Introduction

Diarrhoea caused by intestinal pathogens remains a significant problem worldwide, resulting in 2.5 million deaths in children annually (Kosek *et al.*, 2003). Enteropathogenic *Escherichia coli* (EPEC) is a leading bacterial cause of paediatric diarrhoea, particularly in developing nations (Nataro and Kaper, 1998). EPEC strains are distinguished from other *E. coli* by virtue of their attaching and effacing (A/E) ability and their lack of Shiga-toxin production. The A/E effect is characterized by the intimate binding of the bacteria to the host epithelial cell and the accumulation of actin beneath the bacteria leading to the formation of a pedestal upon which the bacteria rest. In addition to pedestal formation, the surrounding brush border becomes remodelled, or effaced. EPEC utilizes a type III secretion system (T3SS) encoded by the locus of enterocyte effacement (LEE) pathogenicity island to mediate intimate attachment (McDaniel *et al.*, 1995; McDaniel and Kaper, 1997). The LEE also encodes the genes for several effector proteins including the translocated intimin receptor (Tir). Tir is injected by the T3SS into the host epithelial cell plasma membrane where it functions as a receptor for the bacterial outer membrane protein intimin, resulting in intimate attachment (Kenny *et al.*, 1997). Tir is then phosphorylated by a host kinase and subsequently recruits the host cell proteins Nck, N-WASP and Arp 2/3 leading to the polymerization of actin beneath the bacteria and pedestal formation (Gruenheid *et al.*, 2001; Campellone *et al.*, 2002).

In addition to the T3SS, Tir and intimin, the LEE also encodes chaperones as well as additional effector proteins such as Map, EspF, EspG, EspH and EspZ which are secreted into the host cell cytoplasm via the T3SS to ultimately modulate host cell function (Garmendia *et al.*, 2005; Kanack *et al.*, 2005). The effector EspF has been demonstrated to induce apoptosis in host cells (Crane *et al.*, 2001). This ~21 kDa protein contains proline-rich repeats, a eukaryotic SH3-like protein binding domain, and an N-terminal mitochondrial targeting sequence at the first 70-amino-acid residues (McNamara and Donnenberg, 1998; Nougayrède and Donnenberg, 2004; Nagai *et al.*, 2005). Following injection into the host cell, EspF is sorted to mitochondria, an event associated with the disruption of mitochondrial membrane potential, the release of cytochrome c into the cytoplasm, and cleavage of caspases 9 and 3 (Nougayrède and Donnenberg, 2004; Nagai *et al.*, 2005), all of which are characteristic features of the mitochondrial death pathway. This pathway is typically activated by cellular damaging agents and stress and is characterized by the release of cytochrome c from the mitochondria into the cytosol, which, in an ATP-dependent manner, complexes with Apaf-1 and...
were verified by Western blotting, Edman-degradation products (Fig. 1). The identities and mass of each form amino-terminus, and low-molecular-weight degradation poorly with Coomassie blue and was blocked at the doublet migrating at 28 kDa, a 21 kDa form which stained > sulfate precipitation, yielding protein estimated to be 99% pure. Multiple forms of EspF were identified: a triplet of proteins (Mr 70–75 000) that co-eluted with EspF is marked with a plain arrow. The thin arrow points to a bacterial contaminant that was identified by spectrometry as transformylase. The different forms of purified EspF are marked with asterisks. Masses of markers (in kDa) are indicated.

In addition to causing host cell death, EspF from EPEC also disrupts tight junctions and lowers trans-epithelial resistance both in infected epithelial cell monolayers in vitro, as well as in the C57Bl/6j infected mouse model in vivo (McNamara et al., 2001; Shifflett et al., 2005; Guttman et al., 2006). EspF has been demonstrated to interact with the host protein cytokeratin-18 in a complex with 14-3-3ζ and to activate ezrin in EPEC-infected cells, which may play a role in the disruption of the tight junctions in epithelial cells (Simonovic et al., 2001; Viswanathan et al., 2004). EspF was also shown to bind with the host protein sorting nexin-9 via its SH3 domain, but no function could be attributed to the formation of this complex (Marchès et al., 2006).

Recently, it was demonstrated that an espF deletion mutant failed to induce elongation of surrounding microvilli in an EPEC human intestinal organ culture model when compared with wild-type bacteria, suggesting that EspF also plays a role in cytoskeletal remodelling (Shaw et al., 2005). EspF also appears to have a role in the inhibition of EPEC internalization by J774 macrophages (Quitard et al., 2006).

In view of the multifunctional nature of EspF, we considered the possibility that additional EspF targets exist in host cells. We thus sought to identify EspF binding proteins using affinity purification.

**Results**

**Identification of host ligand(s) for EPEC EspF**

We sought to identify host cellular binding partner(s) of EspF using affinity purification with immobilized purified EspF and HeLa cell lysates. Purification of EspF was achieved by multiple rounds of Ni-affinity and ammonium sulfate precipitation, yielding protein estimated to be >99% pure. Multiple forms of EspF were identified: a doublet migrating at 28 kDa, a 21 kDa form which stained poorly with Coomassie blue and was blocked at the amino-terminus, and low-molecular-weight degradation products (Fig. 1). The identities and mass of each form were verified by Western blotting, Edman-degradation and matrix-associated laser desorption/ionization time of flight (data not shown). A HeLa cell extract was passed through a nickel column containing immobilized EspF. As controls, the HeLa cell extract was loaded on an empty column containing only Ni-charged resin, and another column was loaded with EspF but not with the HeLa cell extract. After washing, bound proteins were eluted, concentrated by precipitation, separated by SDS-PAGE and stained with Coomassie blue. Bands of about 75 kDa were detected in the EspF column-bound fraction but not in the control column-bound fraction (Fig. 1A). Similar proteins of about 75 kDa were also retained by purified EspF using a Caco-2 cells lysate as a source of eukaryotic proteins (Fig. 1B). We excised the bands specific to the EspF HeLa cell column and subjected them to mass spectroscopy. Four proteins were identified, all with molecular masses near 75 kDa (Table 1): (i) Bip, an essential endoplasmic reticulum chaperone that belongs to the heat shock 70 protein family and is believed to play a role in facilitating the assembly of protein complexes inside the endoplasmic...
reticulum (Kleizen and Braakman, 2004); (ii) Hsc70, a ubiquitous constitutively synthesized chaperone that is implicated in a variety of cellular processes, including the folding of nascent proteins and the transmembrane translocation of proteins (Hartl, 1996); (iii) the lysyl-tRNA synthetase (LTRS), an essential protein responsible for the loading of lysine to its corresponding tRNA molecule (Cavarelli and Moras, 1993); and (iv) Abcf2, a member of the ATP binding cassette (ABC) protein superfamily whose function is unknown. Abcf2 is unusual among members of this family because it lacks transmembrane domains.

The peptides identified and their positions in the matching protein are shown.
EspF binds host protein Abcf2 in vivo

To confirm the result of the pull-down assay, we used a yeast two-hybrid system to study the interaction between EspF and each of the four eukaryotic proteins that bound EspF in vitro. EspF was fused in frame with the GAL4 DNA binding domain (bait vector pGBK). Bip, Hsc70, LTRS and Abcf2 were each separately fused with the GAL4 activation domain (prey vector pGAD). As an EspF binding positive control, we fused its chaperone, CesF (Elliott et al., 2002), to the GAL4 activation domain. Saccharomyces cerevisiae reporter strain AH109 was co-transformed with combinations of these plasmids and grown on a medium that selects for the plasmid pair (yeast minimal medium lacking leucine and tryptophan; SD-LT). Then the transformants were analysed for the expression of reporter genes HIS3 and MEL1, which are under control of a GAL4-inducible promoter. The transformants were patched on yeast minimal medium lacking leucine, tryptophan and histidine (SD-LTH), which specifically selects for protein interaction.

Fig. 2. EspF interacts with Abcf2 in a yeast two-hybrid system. A. Plasmid pairs were co-transformed into yeast reporter strain AH109 and grown on minimal medium lacking leucine and tryptophan (SD-LT) to select for plasmid transformation. Transcriptional activation of reporter HIS3 was detected by plating onto minimal medium lacking leucine, tryptophan and histidine (SD-LTH), which specifically selects for protein interaction. B. The transcriptional activation of the non-selective reporter MEL1 was assessed in transformants by measuring the secreted α-galactosidase activity in SD-LT liquid medium. Error bars represent standard error from the mean of three experiments.

As a step towards investigating the function of Abcf2 and the biological significance of its interaction with EspF, we examined the subcellular localization of Abcf2. The distribution of Abcf2 in HeLa cells was observed by immunostaining and confocal microscopy. Optical sections revealed that Abcf2 is found in the cytoplasm and in punctate localization in the nucleus (Fig. 3A). Abcf2 staining was not homogeneously distributed throughout the cytoplasm: superimposed on cytoplasmic labelling, we observed concentrations of dot-like structures (Fig. 3A, arrow), in a pattern resembling organelles such as mitochondria. As EspF has been shown to be sorted to mitochondria following injection in the host cytoplasm (Nougayrède and Donnenberg, 2004; Nagai et al., 2005), we further tested whether Abcf2 is associated with mitochondria. Mitochondria were labelled by ectopic expression of DsRed2-MITO, a fluorescent protein bearing a mitochondrial targeting signal (Fig. 3B). Abcf2 and mitochondria labelling produced a partial coincident staining pattern, consistent with both cytoplasmic and mitochondrial localization (Fig. 3C). Abcf2 staining also colocalized partially with the mitochondrial marker MitoTracker (not shown). As a negative control, we expressed GFP within cells (Fig. 3D), which is known to localize in the cytoplasm and nucleus but is excluded from mitochondria (Fig. 3E): as expected, there was no colocalization of GFP with mitochondria (Fig. 3F). Cellular fractionation followed by Western blotting also indicated that Abcf2 is found not only in the cytoplasmic fraction but also associated with mitochondria (Fig. 3G). Together, these data indicate that Abcf2 is found in the cytoplasm, nucleus and associated with mitochondria.

To further confirm the interaction between EspF and Abcf2 in infected cultured cells, we performed co-immunoprecipitation studies. HeLa cells were infected...
with the espF mutant strain UMD874 (negative control) and with UMD874 hosting plasmid pJN61, which encodes functional FLAG-tagged EspF (Nougayrède and Donnenberg, 2004). Cell lysates were then immunoprecipitated with anti-FLAG antibodies. Pelleted immunocomplexes and corresponding supernatants were analysed by Western blotting using anti-EspF and anti-Abcf2 antibodies (Fig. 4, upper panels). Abcf2 and EspF were detected in the supernatant fraction of cells infected with UMD874 (pJN61), whereas Abcf2 but not EspF was observed in supernatants of UMD874-infected cells. Immunoprecipitated FLAG-tagged EspF was detected as two bands, consistent with previous results (Nougayrède and Donnenberg, 2004). Specific co-immunoprecipitation of Abcf2 with FLAG-tagged EspF was observed in pellets of cells infected with UMD874 (pJN61). In reciprocal experiments, anti-Abcf2 antibodies were used to immunoprecipitate infected cell lysates (Fig. 4, lower panels). We observed detectable amounts of low-molecular-weight EspF in the immunoprecipitated fraction of UMD874 (pJN61)-infected cells but not in that of UMD874-infected cells. Note that low-molecular-weight EspF correlates with intracellular EspF following mitochondrial targeting sequence cleavage upon mitochondrial import (Nougayrède and Donnenberg, 2004). Taken together, these results indicate that Abcf2 interacts with EspF in infected HeLa cells and suggest that this interaction might occur in the mitochondria.

In contrast to the results obtained with Abcf2, EspF did not co-immunoprecipitate with Bip or Hsc70 (using anti-FLAG, anti-Bip or anti-Hsc70 antibodies; results not shown), consistent with the yeast two-hybrid results.

Fig. 3. Abcf2 colocalizes partially with mitochondria in HeLa cells. Abcf2 was detected by indirect immunofluorescence with anti-Abcf2 affinity-purified antibodies (A) and mitochondria were labelled by prior transfection with a plasmid encoding DsRed2-MITO, a red fluorescent protein fused with a mitochondrial targeting signal (B). A single slice was acquired with a confocal microscope, the pinhole being set to achieve high Z-resolution (~0.5 μm). In the composite image (C), Abcf2 appears green, mitochondria appear red, coincident fluorescent sources appear orange-yellow. As a negative control, HeLa cells were co-transfected with a GFP vector (D) and DsRed2-MITO vector (E). GFP is excluded from mitochondria (composite image: panel F, compare red colour with orange seen in C). Scale bars correspond to 20 μm. (G) Cells were lysed and fractionated, Abcf2 and the mitochondrial marker cytochrome c (CYC) were detected by Western blotting. MH represents the 'heavy mitochondrial' fraction (3000 g pellet), ML represents the 'light mitochondrial' fraction (16 000 g pellet) and S represents the post-mitochondrial supernatant.

Fig. 4. EspF and Abcf2 can be co-immunoprecipitated from infected HeLa cells. HeLa cells were infected with espF mutant UMD874 or with UMD874 expressing EspF-FLAG from plasmid pJN61. The supernatants (S) and pellets (P) from immunoprecipitations carried out with antibodies to FLAG (first and second panels) or Abcf2 (third and fourth panels) and revealed with Abcf2 immunoblotting (first and fourth panels) or EspF immunoblotting (second and third panels) are shown. The positions of the relevant proteins are indicated. The exposure time for EspF was intentionally adjusted to reveal the faster migrating EspF band, which is a result of processing upon mitochondrial import (Nougayrède and Donnenberg, 2004).
Hence, these two putative EspF interacting partners were not investigated further.

EspF levels correlate with Abcf2 depletion and increased cleavage of caspases 9 and 3

We next sought to test the validity of Abcf2 as a target for EspF. According to our hypothesis, if Abcf2 is a relevant target of EspF in the mitochondrial cell death pathway, then Abcf2 might have anti-apoptotic or, conversely, pro-apoptotic effects, and EspF might inhibit or activate, respectively, the activity of Abcf2. To investigate the functional significance of EspF binding to Abcf2, we used RNA interference (RNAi) to knock down abcf2 gene expression in host cells. HeLa cells were transfected with Abcf2-specific or scrambled non-silencing RNA (small interfering RNA; siRNA) duplexes. Significant silencing of Abcf2 was readily achieved as monitored by immunofluorescence (Fig. 5) and confirmed by Western blotting (not shown). As assessed by microscopy and flow cytometry, knocked-down cells appeared healthy: they showed normal morphology, cell size and granulometry, and cell cycle distribution (Fig. 5 and data not shown). Knocked-down cells did not display an increase in the background level of caspase 9 or caspase 3 cleavage as markers of apoptosis (results below), nor changes in light scatter (Fig. 5) [cell death, morphology and growth alterations are accompanied by changes in light scatter, and thus light scatter measurement is a simple and reliable assay of cell viability (Swat et al., 1991; Darzynkiewicz et al., 1992)].

Next, we transfected the cells with scrambled or abcf2-specific siRNAs and infected the cells with wild-type EPEC, the espF mutant strain UMD874 or UMD874 complemented with pJN61. Abcf2 content was demonstrated by Western blotting together with EspF, Hsc70 and actin as markers for protein loading, and cleaved caspase 9 and cleaved caspase 3 to monitor EspF-associated apoptosis (Nougayrède and Donnenberg, 2004). Interestingly, we detected reduced levels of Abcf2 in cells infected with wild-type EPEC or UMD874 (pJN61) as compared with those in cells infected with the espF mutant strain UMD874 (Fig. 6A), suggesting an association between expression of EspF and reduced levels of Abcf2 in host cells. Further reduction in Abcf2 levels was seen in cells transfected with specific siRNAs and infected with EPEC. Cleaved caspases 9 and 3 were observed in HeLa cells infected with UMD874 (Fig. 6A), indicating that EspF is not the sole contributor to the induction of apoptosis. Interestingly, reduced levels of Abcf2 correlated with increased levels of cleaved caspases 9 and 3 (Fig. 6A).
Densitometry quantification of protein content indicated that levels of cleaved caspase 9 were elevated about fourfold in infected cells following transfection with specific siRNAs as compared with those of infected cells transfected with scrambled control siRNA (Fig. 6B). These data suggest that further reduction of Abcf2 levels by RNAi sensitized HeLa cells to EspF-induced apoptosis, prompting the hypothesis that Abcf2 has an anti-apoptotic function that is inhibited by EspF. We thus examined whether knock-down of Abcf2 could sensitize the cells to another apoptotic stimulus. HeLa cells were transfected with Abcf2-specific siRNAs and then infected with wild-type EPEC strain E2348/69, espF mutant strain UMD864 or UMD874 complemented with plasmid pJN61 that encodes FLAG-tagged EspF (espF+F) as shown. The cells were washed, lysed, proteins were separated by SDS-PAGE, blotted and probed with antibodies against Abcf2, cleaved caspase 9, cleaved caspase 3 (apoptosis markers), Hsc70 and actin (protein loading markers).

To further investigate the effects of EspF on Abcf2 and on the subsequent activation of apoptosis in epithelial cells, we infected HeLa cells with wild-type EPEC, the espF mutant UMD874, or the complemented mutant UMD874 (pJN61). HeLa cells were infected with a range of moi (multiplicity of infection, i.e. the number of bacteria per cells at the onset of infection) of each strain, and we monitored by Western blotting the levels of cleaved caspase 9, Abcf2 and EspF (Fig. 7). Densitometry quantification of protein content revealed that about 50% more cleaved caspase 3 was observed in staurosporine-treated cells following transfection with specific siRNAs as compared with infected cells transfected with scrambled control siRNA (Fig. 6D). This observation suggests that Abcf2 has an anti-apoptotic, protective role against diverse apoptotic stimuli.
required to induce similar levels of caspase 9 cleavage. Even so, at the highest moi cleaved caspase 9 was observed in UMD874-infected HeLa cells (Fig. 7B), confirming that EspF is not the sole contributor to the induction of apoptosis. Moreover, there was an inverse relationship between the moi and the levels of Abcf2 in cells infected with EPEC producing EspF (Fig. 7A and C). This relationship was more pronounced than in cells infected with UMD874 (compare Fig. 7A and C with 7B). Thus, there is an inverse relationship between EspF levels and levels of Abcf2, and an inverse relationship between Abcf2 levels and caspase 9 cleavage. As more EspF is delivered to cells, levels of Abcf2, to which it binds, decrease, and levels of cleaved-caspase 9 increase.

To further confirm the role of EspF and T3SS in the decrease of Abcf2 level and examine whether Abcf2 alteration occurs in intestinal epithelial cells, we infected HeLa and Caco-2 cells with wild-type EPEC, the espF mutant UMD874, the escN mutant CVD452 (T3SS deficient), or the mutants hosting pJN61 (encoding FLAG-tagged EspF). HeLa or Caco-2 cells were infected with each strain, and we monitored by Western blotting the levels of cleaved caspase 9, and Abcf2 (Fig. 8). We detected reduced levels of Abcf2 upon infection with wild-type EPEC or UMD874 (pJN61) as compared with infection with the espF mutant or the T3SS-deficient mutant strains. In contrast to the espF mutant, the escN mutant induced no detectable caspase 9 cleavage, suggesting that the low levels of caspase 9 cleavage seen in cells infected with the espF mutant are due to another T3SS effector. Once again, reduced levels of Abcf2 correlated with increased levels of cleaved caspase 9. Taken together these data indicate that Abcf2 is a relevant target for EPEC EspF and that EspF targets Abcf2 to promote, or facilitate, apoptosis.

**Discussion**

The T3SS-translocated EPEC effector protein EspF is involved in the disruption of host tight junctions and barrier function, and also in the promotion of cell death. The challenge now is to identify the host proteins that interact with EspF, to decipher the signalling pathways that are altered as a result and to provide molecular links to EspF-associated phenotypes. Using the yeast two-hybrid inter-
action trap method, other researchers identified the host proteins cytokeratin-18 and nixin-9 as EspF interacting partners (Viswanathan et al., 2004; Marchès et al., 2006). In the current study, we used an alternative approach to identify another host protein, Abcf2, as an interacting partner of EspF. This interaction was discovered using affinity purification followed by mass spectrometry identification; Abcf2 from a HeLa cell lysate was retained on a column of purified EspF. We further provide four lines of evidence indicating together that EspF targets Abcf2 in infected cells; (i) an interaction between EspF and Abcf2 was confirmed in yeast two-hybrid analyses. (ii) We observed that Abcf2 is primarily cytoplasmic, consistent with previously published observations (Huh et al., 2003; Tsuda et al., 2005), but also partially associated with mitochondria. As upon infection, EspF is injected by the EPEC T3SS into host cell cytoplasm, then sorted to mitochondria (Nougayrède and Donnenberg, 2004; Nagai et al., 2005), the binding of EspF to Abcf2 in host cells is compatible with the observed subcellular localization of Abcf2. (iii) EspF and Abcf2 were co-immunoprecipitated from EPEC-infected HeLa cells, indicating that the interaction occurs during EPEC infection. Furthermore, the form of EspF that was immunoprecipitated with Abcf2 corresponded to the low-molecular-weight form processed upon mitochondrial import (Nougayrède and Donnenberg, 2004). (iv) Levels of Abcf2 were decreased in HeLa and Caco-2 cells infected with EPEC in an EspF-dependent manner, and further intentional reduction of Abcf2 levels by RNAi increased EspF-induced apoptotic mitochondrial pathway recruitment (Nougayrède and Donnenberg, 2004; Nagai et al., 2005), as demonstrated by increased caspase 9 and caspase 3 cleavage. This last line of evidence indicates that the binding of EspF to Abcf2 is biologically relevant.

Prior to this report, the function of Abcf2 was unknown and only fragmentary information was available. Abcf2 was first characterized as the product of an iron-inhibited transcribed gene (Ye and Connor, 2000). Based on amino acid sequence similarities and phylogeny, Abcf2 belongs to the ABC protein superfamily (Kerr, 2004). ABC proteins, which are found in all organisms, are characterized by the ABC units that contain a nucleotide binding domain with Walker A and B motifs and an alpha-helical domain bearing a signature motif that distinguishes ABC proteins from other ATPases. Most proteins that belong to the ABC superfamily also contain transmembrane domains and function as membrane transporters, translocating solute molecules against a concentration gradient (McKeegan et al., 2003). There are 48 ABC proteins encoded within the human genome, which can be divided into seven subfamilies (ABCA to ABCG) (Dean et al., 2001). The ABCF group is unusual, because it contains proteins harbouring a pair of linked ABC units that are not fused to transmembrane transport complexes (drug efflux pumps), but that confer resistance to antibiotics (macrolides, lincosamides and streptogramin B family) that act at the ribosome and inhibit translation (Kerr, 2004). It is proposed that these twin-ABC proteins might confer resistance by a direct protection of the drug target site. In eukaryotes, mammalian Abcf1, closely related to Abcf2, was shown to interact with ribosomes and with the eukaryotic initiation factor 2 (eIF2),
which plays a key role in the process of translation initiation and in its control (Tyzack et al., 2000). The yeast orthologue twin-ABC protein GCN20 also associates with ribosomes, and activates the eIF2α kinase (Vazquez de Aldana et al., 1995). The phosphorylation of eIF2 subunit alpha by eIF2α kinase integrates signalling in stressful conditions, and reduces the rate of translation initiation (potentially preventing the synthesis of pro-apoptotic proteins) and at the same time activates the transcription of genes specific to the integrated stress response (ISR) program, both effects contributing to cytoprotection (Harding et al., 2003). Blocking eIF2α phosphorylation sensitizes cells to insults, resulting in enhanced cell death (Lu et al., 2004). Interestingly, abci2 appears to be a target gene of the ISR (hence it is a stress response gene) as it is significantly more transcribed upon eIF2α phosphorylation (Lu et al., 2004).

Could Abcf2 function as a cytoprotective, anti-apoptotic factor? We observed that as more EspF is delivered to cells, levels of Abcf2 decrease, and caspase 9 cleavage increases. Further experimental reduction of Abcf2 levels by RNAi sensitized HeLa cells to EspF-induced cleavage of caspases 9 and 3. Furthermore, we observed that knock-down of Abcf2 by RNAi also sensitized the cells to staurosporine-induced caspase 3 cleavage. As cells transfected with Abcf2-specific siRNAs showed normal growth and morphology in the absence of apoptotic stimuli, this increase of caspase cleavage is not likely due to general requirement for Abcf2 to maintain cell health. These findings are consistent with the hypothesis that EspF binding to Abcf2 inhibits its anti-apoptotic function. According to this hypothesis, overexpression of Abcf2 would be expected to protect cells from apoptosis induced by EspF or other stimuli. Unfortunately, transfection of neither HeLa cells nor COS-7 cells with a vector containing abci2 under the control of a strong promoter resulted in increased levels of Abcf2 (data not shown), possibly because high levels of this protein are already expressed in these cells. Recent observations that the abci2 gene is amplified and overexpressed in cancer cells are consistent with these results (Yasui et al., 2004; Tsuda et al., 2005). It is known that enforced expression of anti-apoptotic genes can render cells refractory to cytotoxic conditions (Yasui et al., 2004) (and references therein). In addition, amygdalin, a potential anticancer compound from Prunus sp. was shown to inhibit abci2 gene transcription in a colon cancer cell line (Park et al., 2005). Our observations together with these published data suggest that Abcf2 functions as a cell protective factor. These data support the hypothesis that EspF targets Abcf2 and reduces its level, thus inhibiting its anti-apoptotic function, resulting in induction or facilitation of host cell death. It will prove interesting to further decipher this pathway to determine whether the EspF interaction with Abcf2 results in the inhibition of eIF2α phosphorylation, thus inhibiting the ISR and sensitizing cells to insults. Additionally, it will be important to determine how EspF reduces levels of Abcf2 in EPEC-infected cells; for example whether binding to EspF induces the degradation of Abcf2 through ubiquitination or via another pathway.

Other data provide additional clues to the mode of action of EspF. In a large scale protein interaction mapping in Drosophila (Giot et al., 2003), it was found that the Abcf2 orthologue CG9281 (81% homology) might be engaged in a multiprotein interaction complex including CG18255, the fruit fly myosin light chain (MLC) kinase. MLC phosphorylation is a common intermediate in the pathophysiologic regulation of the barrier function. EPEC induces MLC phosphorylation, which might explain the alteration in intestinal epithelial permeability (Yuhan et al., 1997). If this interaction complex is confirmed conserved in mammals, this pathway might link EspF and Abcf2 to the opening of the tight junctions.

The identification of Abcf2 as a target of EspF linked to apoptosis has many implications and provides many avenues for further research. Present and future findings should advance our understanding of EPEC infection processes and basic cell biology.

**Experimental procedures**

*Enhanced purification of EspF, pull-down assay and identification of EspF-binding proteins*

C-terminal hexahistidine-tagged EspF was overexpressed in a BL21DE3 slyD mutant under T7 promoter control in an artificial operon with its chaperone CesF as described elsewhere (Nougayrède and Donnenberg, 2004). To remove the chaperone and achieve high purity, the native eluate was precipitated with ammonium sulfate, solubilized in 8 M urea buffer, affinity-purified on Ni-agarose (Qiagen) and eluted at low pH. For the capture assay, 1 mg of EspF was reapplied to Ni-agarose, then washed extensively in PBS 0.1% Tween 20 followed by PBS 10 mM imidazole. To prepare eukaryotic cell proteins, HeLa or Caco-2 cells grown to confluence were collected and approximately 8 × 10^5 cells were lysed in 5 ml of PBS with 1% Triton X-100 and anti-protease cocktail (Boehringer) and the lysate was cleared by centrifugation. To remove Ni-binding proteins, imidazole was added (10 mM final) to the supernatant, which was then incubated three times over 48 h at 4°C with 500 μl of Ni-agarose, then washed extensively in PBS 0.1% Tween 20 followed by PBS 10 mM imidazole.

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the peptides formed extracted with acetonitrile-formic acid and analysed with a mass spectrometer (LC-MC and MS-MC) acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence. The data were analysed by database searching using the Sequest algorithm.

**Plasmid constructions and yeast two-hybrid assay**

Plasmid pGBK-EspF was constructed by subcloning the Ndel/XhoI DNA fragment of pJN40 (Nougayrède and Donnenberg, 2004) into the Ndel/Sall sites of pGBKTT7 (Clontech) to create a GAL4 DNA-binding domain-EspF fusion protein. Plasmid pGAD-CesF was constructed by PCR amplification of cesF using EPEC E2348/69 genomic DNA as template and primers Donne-694 (5′-CTC GAG TCA AAG TGA TAG TTT TAT TAT TTC) and Donne-695 (5′-CAT ATG AAT GAA CAA TTT TGC AAA GAT C) followed by cloning (Ndel/Xhol) into pGADT7 (Clontech) to create a GAL4 activation domain-CesF fusion protein. Plasmid pGAD-Abcf2 was constructed by PCR amplification of IMAGE clone #2988125 (MGC-1425) using primers Donne-697 (5′-CAT ATG GCC AGC AGG ACC TGC AAA GAT C) and Donne-698 (5′-CAT ATG GGC ATC GAC CTG GGC CAA GAA G) using IMAGE clone #2988125 (MGC-1425) as template, and cloned (BamHII/EcoRI) into pGEX-4T (Amersham) to create a fusion protein with glutathione S-transferase (GST). One milligram of Abcf2[1–102]-GST fusion protein was affinity-purified over glutathione-agarose and used to immunize two rabbits. To enhance specificity, the final combined sera were depleted of anti-GST antibodies on a column of cross-linked GST-glutathione sepharose, then affinity purified on a column of cross-linked Abcf2[1–102]-GST-glutathione sepharose. Bound antibodies were eluted at pH 2 and dialysed. Western blot analysis for reactivity with lysates from HeLa or Caco-2 cells revealed that the purified antibodies recognized a major 75 kDa band.

**Cell culture, infection assay, immunoprecipitation and Western blotting analysis**

HeLa (ATCC CCL2) and Caco-2 (ATCC HTB-37) cells were maintained by regular serial passage in Iscove modified Dulbecco’s medium (IMDM with 25 mM Hepes, Biofluid) or in Dulbecco’s Modified Eagle medium (DMEM with glutamax, Gibco), supplemented with 10% fetal calf serum (FCS) for HeLa cells or 20% FCS for Caco-2 cells. Cells were split regularly to maintain exponential growth and a fresh culture was started from a liquid nitrogen stock every 15–20 passages.

Reference wild-type EPEC E2348/69, mutant strains UMD874 (EspF-), CVD452 (EscN-, type III secretion deficient) and plasmid pJN61, which encodes a C-terminal FLAG-tagged EspF, were described previously (Nougayrède and Donnenberg, 2004).

Infections were performed by diluting Luria–Bertani overnight bacterial cultures 1:10 in interaction medium [DMEM/F12 with 15 mM Hepes (Invitrogen), 2% heat-inactivated FCS] and incubating for 2 h at 37°C with agitation; the cultures were further diluted to an OD600 of 0.2 prior to performing dilutions and infecting epithelial cell cultures. The resulting moi were checked by plating and counting the bacterial colony-forming units present at the onset of infection. Cells were infected with a moi of 100:1 or with serial dilutions, as indicated in the text. Cells were processed after 3 h infection.

Immunoprecipitations and Western blotting analyses were performed as described previously (Nougayrède and Donnenberg, 2004). Antibodies that recognize cleaved caspase 9 (Asp330) and cleaved caspase 3 (Asp175) were purchased from Cell Signalling Technologies. Goat serum anti-Hsc70 (K-19) and goat serum anti-Bip (GRP 78 N-20) were purchased from Santa Cruz Biotechnology. monoclonal anti-cytotochrome c antibody (7H8, 2C12) from PharMingen, monoclonal anti-actin from ICN and monoclonal anti-FLAG (M2) from Sigma. Monoclonal antibodies against EspF are described elsewhere (Nougayrède and Donnenberg, 2004). When needed, the relative levels of antigens were quantified by densitometry using the Kodak 1D 3.6.1 system and ImageJ software. For siRNA experiments, Abcf2 content was normalized based on that of scrambled siRNA-transfected, uninfected cells. Cleaved caspase 9 content was normalized based on that in scrambled siRNA-transfected, wild type-infected cells. Cleaved caspase 3 content was normalized based on that in scrambled siRNA-transfected, stauroporine-treated cells. For experiments in which the moi was varied, the cleaved caspase 9-values were normalized
based on the signal ratio determined at the highest moi used for each group, and Abcf2 values were normalized by the signal ratio determined from the untreated control.

Cell fractionation, fluorescence microscopy and flow cytometry analysis

Separation of cells into cytoplasm, mitochondria and light organelle fractions was performed as described (Susin et al., 2000; Nougayrède and Donnenberg, 2004). For fluorescence microscopy visualization of mitochondria, plasmid pDsRed2-MITO (Clontech), encoding the red fluorescent protein DsRed2, fused to a mitochondrial targeting sequence and pEGFP-N2 (Clontech), encoding enhanced green fluorescent protein (EGFP), were transfected using FuGene6 (Boehringer) in cells 24 h before fluorescence study. Alternatively, the mitochondrial dye MitoTracker (Molecular Probes) was used following the manufacturer’s instructions. Indirect immunofluorescence was performed as described (Nougayrède and Donnenberg, 2004). F-actin was stained with rhodamine-phalloidin (Molecular Probes) and DNA with DAPI (Sigma). For confocal microscopy, the slides were observed with an Olympus BX50WI krypton/argon laser confocal microscope. Sections were scanned at 0.5 µm optical thickness and images were processed with FluoView software v2.1. For standard fluorescence microscopy, images were acquired using a Leica DMRB fluorescence microscope equipped with a DFC300FX digital camera. Conditions for black level and gain were established by using control microscope equipped with a DFC300FX digital camera. Condi-

Depletion of Abcf2 with siRNA

Small interfering RNA sequences for the knock-down of Abcf2 were designed according to Tuschl and collaborators’ recommendations (Elbashir et al., 2001). siRNA duplex kabf123 was designed to target the sequence AAG AAT GAG GCC AAT GGC AGA, kabf154 the sequence AAG TAG ATT TGC TGA CCA AGG and kabc243 the sequence AAC AGT ACT GAT GTT CAC ATC. A scrambled siRNA targeting the sequence AAT TCT CGG AAC GTG TCA CGT, conjugated with rhodamine, was used as a non-silencing control and to estimate the transfection efficiency by fluorescence microscopy. HPP-purified siRNA were pur-

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