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Expression of the EspB Protein of Enteropathogenic *Escherichia coli* within HeLa Cells Affects Stress Fibers and Cellular Morphology

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The EspB protein of enteropathogenic *Escherichia coli* (EPEC) is essential for the signaling events that lead to the accumulation of actin beneath intimately attached bacteria, a process that is known as the attaching and effacing effect. EspB is targeted to the host cell cytoplasm by a type III secretion apparatus. To determine the effect of intracellular EspB on the host cell cytoskeleton, we transfected HeLa cells with a plasmid containing the *espB* gene under the control of an inducible eukaryotic promoter. A HeLa cell clone that expressed *espB* mRNA and EspB protein after induction was selected for further study. The expression of EspB in these cells caused a dramatic change in cell morphology and a marked reduction in actin stress fibers. Cells expressing EspB were significantly impaired in their ability to support invasion by EPEC and *Salmonella typhimurium*. However, the expression of EspB within host cells could not compensate for the lack of EspB expression by an *espB* mutant strain of EPEC to restore attaching and effacing activity. These studies suggest that EspB is a cytoskeletal toxin that is translocated to the host cell cytoplasm, where it causes a redistribution of actin.

Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of infantile diarrhea in the developing world. EPEC binds epithelial cells as compact microcolonies in a pattern that has been referred to as localized adherence. The epithelial cell responds to the presence of the bacteria by reorganizing its cytoskeleton such that microvilli are replaced by cup-like pedestals upon which the bacteria rest. The resulting lesion, known as the attaching and effacing effect, is considered to be the hallmark of EPEC infection (4).

EPEC induces a host cell signal transduction cascade during infection that leads to the reorganization of filamentous actin (18) and a number of other cytoskeletal elements (9). Signals transduced by EPEC to the epithelial cell result in tyrosine phosphorylation of substrates that colocalize with the accumulated cytoskeletal elements beneath adherent bacteria (25). The major phosphorylation substrate detected in EPEC-infected cells is a bacterial protein known as Tir that is targeted to the host cell membrane, where it becomes the receptor for the EPEC adhesin intimin (14). EPEC also induces other signaling cascades, such as the activation of phospholipase-Cγ (16), protein kinase C (2), and NF-κB (28); fluxes of inositol phosphates (11); and changes in membrane potential (29). Precisely how bacterial effectors or specific cellular targets are involved in these processes is not yet clear.

All of the factors necessary for formation of the attaching and effacing lesion by EPEC are encoded by a 35.6-kb chromosomal locus referred to as the LEE (locus of enterocyte effacement) (21). The LEE can be roughly divided into thirds, with one end consisting largely of a type III secretion apparatus that directs the secretion of proteins encoded by the *esp* genes located at the other end (8). At least three secreted proteins, EspB, EspA, and EspD, are involved in the induction of signal transduction events within the epithelial cell that lead to cytoskeletal rearrangements, tyrosine phosphorylation, and second messenger cascades (10, 17, 20). At the nexus of the right- and left-hand regions of the LEE is the *eae* gene, which encodes the adhesin intimin. Mutants with disruptions in *eae* are unable to attach intimately to epithelial cells, yet they retain the ability to transduce signals that result in the translocation and tyrosine phosphorylation of Tir, indicating that intimin is not necessary for these signal transduction events (3, 25). Although *eae* mutants are capable of causing some actin rearrangement, these cytoskeletal structures are not sharply focused under adherent bacteria and are not organized into pedestals (3). An *eae* mutant has reduced, but residual, virulence in volunteers (6). Upstream of *eae* is the gene encoding Tir, which serves as the receptor for intimin upon insertion into the host cell membrane (14). Tir requires the Esp proteins for association with the host cell membrane, although the function of the Esp proteins in this process remains to be defined.

Since *esp* mutants are each deficient in the secretion of a single protein and are unable to induce host signal transduction cascades, these polypeptides are likely candidates for effectors that interact with host cells. Kenny and Finlay (15) demonstrated that EspB, but not EspA, remains associated with cells following protease treatment of infected monolayers. Several studies have recently confirmed that EspB is targeted to the host cytoplasm (19, 30, 31). Another recent study has implicated EspA as a component of a surface appendage involved in the delivery of EspB to the cytoplasm (19). Very little is known of the interactions between EspD and the host cell. Since EspB is the only protein secreted by EPEC that is known to be targeted to the host cell cytoplasm, this protein is currently the prime candidate for an effector molecule that usurps signaling mechanisms to disrupt the cytoskeleton. To test this hypothesis, we determined the effect of EspB expression within epithelial cells on cytoskeletal organization.

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MATERIALS AND METHODS

Bacterial strains, plasmids, tissue culture, and media. *E. coli* E2348/69 is the prototypic 0127:H6 wild-type strain of EPEC shown to be virulent in volunteers

(6). *E. coli* UMD864, containing an in-frame deletion of the *espB* gene, is isogenic to E2348/69 and has been described previously (7). *Salmonella typhimurium* 14028, also previously described (22), was obtained from the American Type Culture Collection. Bacteria were maintained at −80°C in 50% (vol/vol) Luria-Bertani (LB) broth–50% glycerol and grown either on LB agar or in LB broth. HeLa cells (ATCC CCL2) were grown at 37°C in an atmosphere of 95% air–5% CO_2 in Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL, Gaithersburg, Md.) supplemented with 10% (vol/vol) fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and, when appropriate, G418 (750 μg/ml) and dexamethasone (10^{-7} M).

Construction of an *espB* expression vector and transfection of HeLa cells. All PCRs were performed in 50-μl samples in a minicycler (MJ Research, Watertown, Mass.) with DeepVent polymerase (New England Biolabs, Beverly, Mass.). The *espB* gene was amplified from pMSD3 (7) by PCR with primer Donne-158 (GCG GCT AGC ATG AAT ACT ATC GAT AAT AAC AAT GCG GCA), consisting of nucleotides 112 to 141 of the *espB* gene (EMBL database accession no. Z21555) and incorporating an *Nhe*I site (underlined), and primer Donne-149 (GCG CTC GAG TTA CCC AGC TAA GCG AGC CGC T), consisting of nucleotides 1077 to 1056 of the *espB* gene and incorporating an *Xho*I restriction site (underlined). The PCR product was digested with *Nhe*I and *Xho*I and cloned into the corresponding sites of pMAM*neo* (Clonetech, Palo Alto, Calif.) directly downstream of the dexamethasone-inducible mouse mammary tumor virus long terminal repeat promoter. The resulting plasmid, pKT46, was purified for transfection of HeLa cells with the Transfectam reagent (Promega, Madison, Wis.) according to the manufacturer's instructions. Cells transfected with pKT46 or with pMAM*neo* alone were selected with G418 and cloned by limiting dilution.

Expression of EspB in HeLa cells. Transfected cells expressing *espB* mRNA were identified by reverse transcriptase (RT) PCR. Total RNA was isolated with RNAzol (TelTest, Friendswood, Tex.), according to the manufacturer's directions, from HeLa cells transfected with the pMAM*neo* vector alone and from eight individual HeLa cell clones transfected with pKT46 following induction with 10^{-7} M dexamethasone for 3 to 5 days. Two micrograms of total RNA from each sample was reverse transcribed with Superscript II RT (Gibco BRL), and the resulting cDNA was amplified by PCR with Donne-149 and Donne-3 (7), an internal *espB* forward primer representing nucleotides 508 to 543. A single transfected clone expressing EspB mRNA was selected for further study.

Fluorescence microscopy. HeLa cells were seeded in eight-well chamber slides, infected for 3 h, and stained with fluorescein isothiocyanate (FITC)-phalloidin to detect actin, as described previously (5, 18). To detect EspB, HeLa cells were seeded on coverslips that were placed in 24-well plates in DMEM and incubated in an atmosphere of 95% air–5% CO_2 until 85% confluence was reached. Overnight static cultures of EPEC strains grown in LB broth at 37°C were diluted 1:100 in DMEM without additives and incubated with aeration for 3 h at 37°C to preinduce the secretion of the Esp proteins. Following the preinduction, the HeLa cell cultures were washed three times with phosphate-buffered saline (PBS) and the medium was replaced with DMEM without additives. One-milliliter volumes of EPEC cultures were added to 1 ml of base medium overlying the HeLa cell monolayers and centrifuged at 800 × *g* for 10 min. Infected HeLa monolayers were incubated in an atmosphere of 95% air–5% CO_2 at 37°C for 3 h. Following infection, the cell monolayers were washed extensively in PBS, fixed with 2% formaldehyde, and permeabilized with 0.1% Triton X-100. The monolayers were then blocked overnight at 4°C in 3% bovine serum albumin (BSA)–0.2% sodium azide. All subsequent antibody treatments were performed at room temperature for 3 h. An affinity-purified anti-EspB antibody (30) was used at a dilution of 1:10 in PBS containing 0.3% BSA and detected with an anti-rabbit immunoglobulin G antibody conjugated to lissamine rhodamine B (Molecular Probes, Eugene, Oreg.) at a dilution of 1:200 in 0.3% BSA-PBS. Filamentous actin was detected with FITC-phalloidin (5 μg/ml) in PBS. The samples were examined with a Zeiss Axioskop epifluorescence microscope.

Confocal microscopy. Samples prepared as described above were also examined with a Zeiss LSM410 confocal laser scanning microscope with a 63×, NA 1.4 objective. Fluorescein and lissamine rhodamine signals were excited with the 488- and 568-nm lines of a 50-mW KrAr laser and detected through 515- to 540-nm band-pass and 590-nm long-pass filters, respectively. The diameter of the detector pinhole corresponded to one Airy unit at 590 nm, which corresponds to an optical thickness of 1 μm along the *z* axis. The conditions for laser attenuation and detector black level and gain were established by using HeLa cells expressing EspB, and these settings were maintained for the other samples.

Analysis of tyrosine kinase substrates. To analyze tyrosine-phosphorylated proteins, six-well tissue culture plates were seeded overnight with 10^6 HeLa cells per well, and 30 min prior to infection, the cells were washed with PBS and incubated with Eagle's minimal essential medium without additives. EPEC strains were incubated with the monolayers in tissue culture medium for 4 h following overnight incubation in LB broth. The infected monolayers were lysed in 1% Triton X-100 in the presence of protease inhibitors as described previously (25). For the addition of epidermal growth factor (EGF), the cell monolayers were treated with 0.125 μg of EGF/ml for 30 min prior to the preparation of cell lysates. The Triton-soluble fractions were isolated, resolved by sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis (PAGE), and analyzed by Western blotting with the anti-phosphotyrosine antibody PY20 (Pierce, Rockford, Ill.), as described previously (23).

Adherence and invasion assays. Adherence assays were performed as described previously (5). For each sample, 100 infected HeLa cells were analyzed. For individual cells, the bacterial clusters were counted as well as the bacteria in each cluster. The gentamicin protection assay was performed as described previously (23) with the following modifications to enhance invasion for each species. Overnight LB cultures of EPEC were diluted 1:100 in DMEM and then grown with aeration at 37°C to an optical density of 0.6 at 600 nm prior to infection. Overnight cultures of *S. typhimurium* were diluted 1:100 in LB broth and incubated without aeration at 37°C to an optical density of 0.6 at 600 nm prior to infection.

RESULTS

Expression of *espB* mRNA and EspB protein in HeLa cells. To determine the effect of intracellular expression of EspB on host cells, we cloned the *espB* gene under the control of a dexamethasone-inducible promoter in the eukaryotic expression vector pMAM*neo*. Following the transfection of HeLa cells, G418-resistant clones were isolated, induced with dexamethasone, and analyzed for expression of EspB. EspB mRNA expression in transfected clones was demonstrated by reverse transcription of total RNA followed by PCR amplification with primers specific for the *espB* gene. EspB message was detected only in cells transfected with *espB* following dexamethasone induction. In contrast, EspB message was not detected in *espB*-transfected cells that were not treated with dexamethasone nor in cells transfected with vector alone either in the absence or the presence of dexamethasone. No product was detected in the absence of RT, demonstrating that contaminating DNA was not the template for the PCR. A single clone, denoted B3, that expressed *espB* mRNA upon induction was selected for further study.

EspB protein expression in clone B3 was detected by confocal microscopy with an affinity-purified EspB antibody (Fig. 1). EspB protein was detected in transfected cells after dexamethasone induction but not in cells transfected with *espB* and cultured in the absence of dexamethasone induction nor in cells transfected with the vector alone, irrespective of dexamethasone treatment. EspB was distributed throughout the cytoplasm of cells expressing the protein. The apparent levels of EspB expression in transfected cells increased from days 3 to 5 following dexamethasone induction (not shown).

Effect of intracellular expression of EspB on attaching and effacing lesion formation by EPEC. EspB is targeted to the cytoplasm of cells infected with EPEC (19, 30, 31). To determine whether the expression of EspB within the host cell cytoplasm can bypass the need for EPEC to produce and translocate the protein, we tested the ability of an *espB* deletion mutant to generate attaching and effacing lesions in host cells expressing EspB. HeLa cells transfected with *espB* were treated with dexamethasone for 4 days and then infected with the *espB* deletion mutant (UMD864) or the wild-type EPEC strain. Infected cells were stained with FITC-phalloidin, which labels filamentous actin, and examined by fluorescence microscopy. In this assay, the accumulation of filamentous actin beneath adherent bacteria indicates the presence of an attaching and effacing lesion (18). Whereas wild-type EPEC bacteria were fully capable of forming attaching and effacing lesions in cells expressing EspB, the *espB* mutant was unable to induce the accumulation of cytoskeletal actin beneath adherent bacteria upon infection of these cells (data not shown). Similar results were obtained at earlier time points. Thus, the intracellular expression of EspB is not sufficient to complement the *espB* mutant for the attaching and effacing effect, nor does EspB expression within host cells interfere with attaching and effacing activity by wild-type EPEC. Therefore, it appears that attaching and effacing requires an aspect of EspB function that

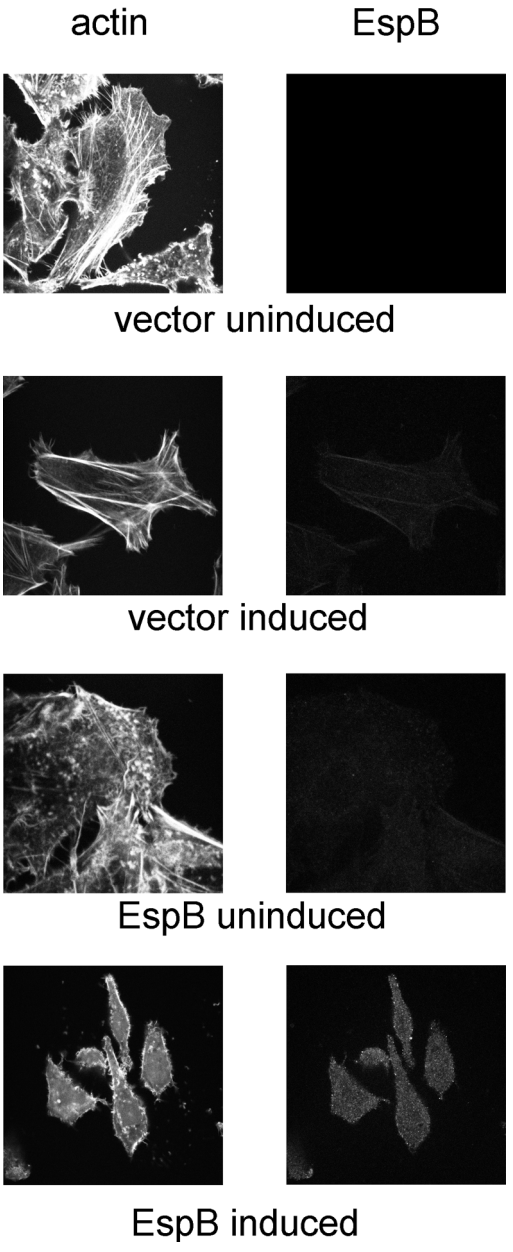

FIG. 1. Expression of EspB by HeLa cell clones. HeLa cells that had been transfected with vector alone or with *espB* were left untreated or induced for 5 days with dexamethasone as indicated. The cells were fixed, permeabilized, and stained with FITC-phalloidin to label filamentous actin (left) and with an affinity-purified antibody against EspB followed by a secondary antibody conjugated to lissamine rhodamine (right). The stained cells were examined with a laser scanning confocal microscope.

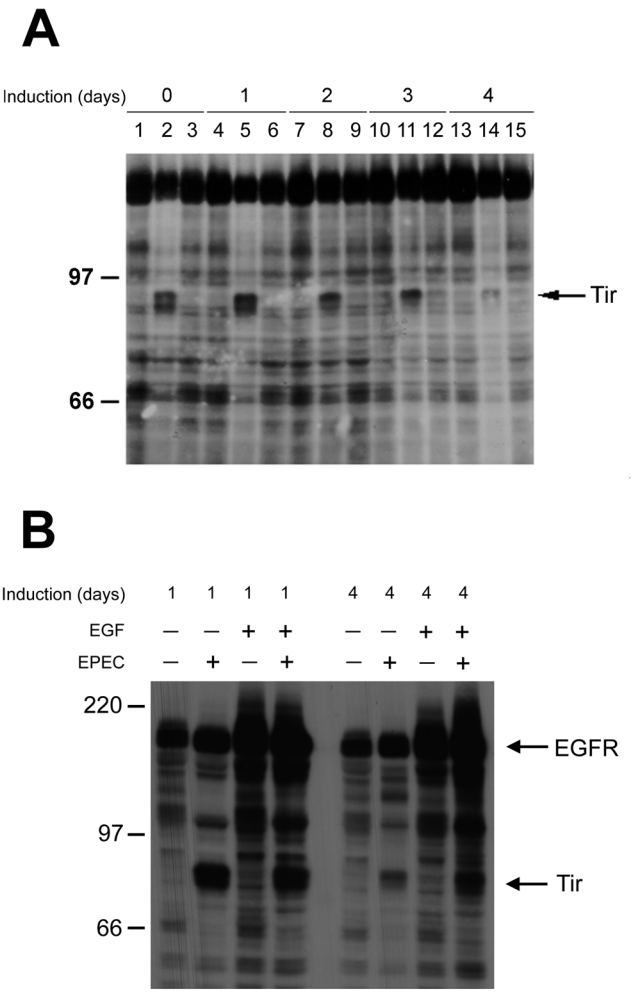

FIG. 2. Effect of EspB expression by HeLa cells on protein tyrosine phosphorylation patterns. Triton X-100-soluble proteins from HeLa cells were separated by SDS-PAGE and transferred to nylon membranes. Tyrosine-phosphorylated proteins were detected with an anti-phosphotyrosine monoclonal antibody and enhanced chemiluminescence reagents. (A) HeLa cells that had been transfected with the *espB* gene were induced with dexamethasone for the number of days indicated and were left uninfected (lanes 1, 4, 7, 10, 13), infected with the wild-type EPEC strain, E2348/69 (lanes 2, 5, 8, 11, 14), or infected with the *espB* mutant strain UMD864 (lanes 3, 6, 9, 12, 15). (B) HeLa cells that had been transfected with the *espB* gene were induced for 1 or 4 days with dexamethasone, either treated with EGF (+) or not (−), and infected with wild-type EPEC or not, as indicated. The positions of molecular weight standards are indicated on the left of each panel, and bands corresponding to the EGF receptor (EGFR) and Tir are indicated on the right.

is not recapitulated when the protein is synthesized in the cytoplasm of HeLa cells in our system.

A recent study has shown that EspB is required for Tir to become associated with the host cell membrane, where it is phosphorylated and is thought to participate in the nucleation of actin and in pedestal formation (14). Thus, we investigated whether intracellular expression of EspB affects the induction by EPEC of signaling pathways that lead to the translocation and phosphorylation of Tir in the development of the attaching and effacing lesion. To this end, we treated the cells with dexamethasone for up to 4 days and then infected them with wild-type or *espB* mutant EPEC. Protein samples were resolved by SDS-PAGE and subjected to Western blotting analysis with anti-phosphotyrosine antibodies. The results in Fig. 2A show that at no time point was the *espB* mutant able to induce translocation and phosphorylation of Tir in cells expressing EspB. Thus, while EspB is required for Tir translocation and phosphorylation (14), expression of EspB within host cells cannot compensate for a lack of EspB expression by the bacteria. As expected, phosphorylated Tir was not detected in uninfected cells that had been transfected with *espB*. Interestingly, at early time points (days 1 and 2) wild-type EPEC was able to induce the translocation and phosphorylation of abundant levels of Tir in cells expressing EspB. However, there was a progressive reduction in the amount of phosphorylated Tir at

later time points (days 3 and 4). This decrease in phosphorylated Tir during infection by wild-type EPEC contrasts with the observation that at comparable time points, wild-type EPEC remains capable of inducing actin accumulation.

To determine whether intracellular expression of EspB leads to a global decrease in tyrosine kinase activity, we tested the ability of cells transfected with *espB* to phosphorylate the EGF receptor. Following induction with dexamethasone for 1 or 4 days, cells transfected with *espB* were infected with EPEC and stimulated with EGF for 30 min. Lysates prepared from each sample were subjected to Western blot analysis with anti-phosphotyrosine antibodies. Figure 2B shows that while intracellular expression of EspB for 4 days greatly reduced the ability of wild-type EPEC to induce the translocation and phosphorylation of Tir, there was no change in the basal level of phosphorylation of the EGF receptor. Furthermore, stimulation of samples with EGF led to similar increases in EGF receptor phosphorylation regardless of the duration of EspB expression or whether the cells were infected with EPEC. These results suggest that the intracellular expression of EspB causes a specific decrease in the ability of EPEC to induce the translocation and/or the phosphorylation of Tir.

Effect of EspB expression on HeLa cell morphology. In the course of our studies of the interaction of EPEC with *espB*-transfected cells, we observed a remarkable change in the morphology of these cells, concurrent with the induction of EspB expression. Rather than demonstrating the typical polygonal epithelial phenotype, these cells were frequently spindle or sickle shaped (Fig. 1). Similar morphological changes were rarely seen among cells transfected with the pMAM*neo* vector alone, regardless of dexamethasone treatment, or among *espB*-transfected cells in the absence of dexamethasone induction. The changes in cellular morphology that were observed in *espB*-transfected cell lines were not associated with cell death, as the plating efficiencies for all samples were similar (data not shown). In several experiments, quadruplicate samples of *espB*-transfected clones and controls containing vector alone were seeded, with or without dexamethasone, in 24-well plates and incubated for 4 days to allow for optimal induction of EspB protein. The observer was blind to the identity of the samples. One hundred random cells were counted for each sample and scored for normal (polygonal) or altered (spindle or sickle) morphology. The proportion of *espB*-transfected cells that displayed an altered morphology was 83% ± 11% (mean ± standard deviation) after induction with dexamethasone compared to 3.8% ± 1.7%, 2.3% ± 1.5%, and 1.0% ± 1.4% in uninduced *espB*-transfected cells and cells transfected with vector alone in the presence and absence of dexamethasone, respectively ($P < 0.001$). The dramatic change in cellular morphology observed in transfected cell lines expressing EspB indicates that the intracellular expression of EspB alone is sufficient to profoundly alter cell shape.

Effect of intracellular expression of EspB on the actin cytoskeletons of HeLa cells. The results of confocal microscopy suggested that HeLa cells expressing EspB had reduced numbers of stress fibers (Fig. 1). This finding is consistent with the changes observed in cellular morphology. To confirm an effect of intracellular EspB expression on the distribution of filamentous actin, we performed additional experiments with fluorescence microscopy. HeLa cells transfected with *espB* or with vector alone, in the presence or in the absence of dexamethasone, were seeded on coverslips and incubated until 85% confluence was reached. Samples were then fixed, permeabilized, and stained with phalloidin conjugated to FITC. These experiments confirmed that stress fibers were markedly reduced in cells expressing EspB. In contrast, the uninduced cells transfected with *espB* and cells transfected with vector alone in the absence or presence of dexamethasone contained abundant stress fibers (Fig. 1; additional data not shown). These results confirm the observation made by confocal microscopy and indicate that the intracellular expression of EspB leads to a reorganization of filamentous actin in HeLa cells.

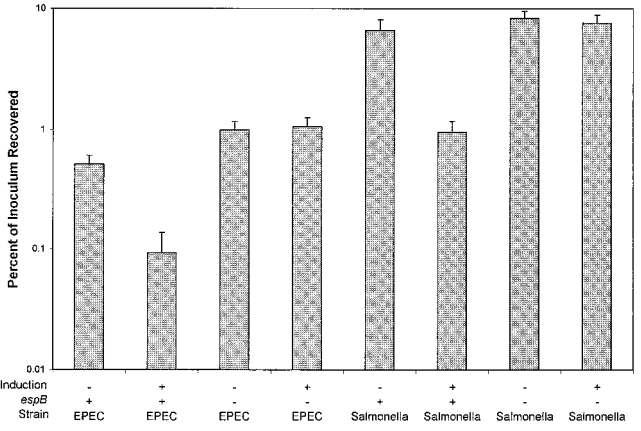

FIG. 3. Effect of EspB expression by HeLa cells on invasion by EPEC and *S. typhimurium*. HeLa cells that had been transfected with *espB* (+) or with vector alone (−) as indicated were left uninduced (−) or were induced for 4 days with dexamethasone (+), as indicated. These cells were infected with EPEC or *S. typhimurium* as indicated, and the percent of the inoculum surviving gentamicin treatment was calculated. The data shown are the geometric means of four experiments, each performed in triplicate. The error bars indicate the geometric mean plus one standard error of the geometric mean.

Effect of intracellular EspB expression on adherence and invasion by bacteria. Since expression of EspB within HeLa cells significantly alters cell shape and may interfere with the processing of signals leading to the translocation and phosphorylation of Tir, we determined whether there was a quantitative difference in the ability of EPEC to adhere to these cells. We observed no significant difference between the ability of EPEC to adhere to cells expressing EspB and its ability to adhere to uninduced cells or cells transfected with vector alone (data not shown).

Since the intracellular expression of EspB alters the actin cytoskeleton, we also tested the ability of these cells to support invasion of EPEC as measured by the gentamicin protection assay. Invasion of cells transfected with *espB* by *S. typhimurium* was also measured to determine whether any effect observed was specific for EPEC. Cells transfected with vector or *espB*, either uninduced or induced for 4 days with dexamethasone, were infected with EPEC or *S. typhimurium*. Following gentamicin treatment, the cell monolayers were lysed and the percentage of the inoculum that was recovered was determined by the plate dilution method. As shown in Fig. 3, there was a significant decrease in the ability of both EPEC and *S. typhimurium* to invade cells expressing EspB compared to their ability to invade uninduced cells transfected with *espB* (Student's *t* test; $P < 0.001$). In contrast, there was no difference in the ability of either EPEC or *S. typhimurium* to invade control cells transfected with vector alone based on whether or not the cells were induced with dexamethasone ($P = 0.43$ for EPEC; $P = 0.44$ for *S. typhimurium*). These results suggest that the intracellular expression of EspB leads to a disruption of the host actin cytoskeletal function required for efficient invasion. Interestingly, there was no significant difference in invasion of HeLa cells by *S. typhimurium* in the absence of induction, whether or not the cells were transfected with *espB*. However,

EPEC invasion of HeLa cells transfected with *espB* was significantly impaired in comparison to its invasion of cells transfected with vector alone even in the absence of induction ($P = 0.04$). This result suggests that EspB is expressed in transfected cells in the absence of dexamethasone induction and that this low level of EspB expression specifically interferes with EPEC invasion.

DISCUSSION

In this report we have shown that it is possible to select stable HeLa cell clones transfected with the *espB* gene and that, upon induction, such cells express *espB* mRNA and EspB protein. Moreover, the expression of *espB* in transfected cells causes profound changes in morphology and in filamentous actin distribution and function, but the cells remain viable. Intracellular expression of EspB also greatly diminishes the ability of wild-type EPEC to induce the translocation and/or tyrosine phosphorylation of Tir and to invade HeLa cells. Furthermore, we have demonstrated that intracellular expression of EspB is not sufficient to complement an *espB* mutant for the translocation and phosphorylation of Tir or for the production of mature attaching and effacing lesions. Thus, it appears that production of EspB in the cytoplasm of host cells does not duplicate the effects seen when EspB is delivered to the host cell cytoplasm by EPEC. Nevertheless, the effects of EspB on host cells when expressed in the absence of other bacterial factors may provide insights into EspB function.

Cells expressing the EspB protein display a morphology that is quite different from the typical polygonal shape of epithelial cells. After several days of EspB induction, such cells become elongated or sickle shaped. Although EspB is detected throughout the cytoplasm of most transfected cells, there is no apparent colocalization of this protein with actin structures (data not shown). However, in these cells the distribution of stress fibers is greatly reduced. The effect of intracellular EspB expression on the actin cytoskeleton is reminiscent of the function of the YopE and ExoS proteins of *Yersinia* spp. and *Pseudomonas* spp., respectively (12, 26). Like EspB, these effector molecules are secreted and translocated by a type III secretion system to the host cytoplasm, where they disrupt the actin microfilament network (12, 26, 27). Our results suggest that EspB also functions to reorganize actin structures in infected cells. Despite the similar effects of YopE, ExoS, and EspB on the host cytoskeleton, EspB does not share sequence similarities with these proteins. While the cytotoxic effect of YopE and ExoS in phagocytes serves to inhibit bacterial uptake by impairing the formation of microfilament structures, EspB may function in epithelial cells to release monomeric actin for localized reorganization of filamentous actin during pedestal formation. This concept is supported by the fact that cells transfected with *espB* remain capable of focusing high concentrations of actin beneath wild-type EPEC despite their relative lack of stress fibers. Thus, EspB may be thought of as a cytoskeletal toxin, delivered to the cytoplasm by the EPEC type III secretion system to subvert host cell actin.

It has been shown that EspB is required for the delivery of the translocated intimin receptor (Tir) to the host cell membrane (14), and we have recently demonstrated that EspB also requires EspA and EspD to be translocated into host cells (30). Taken together, these data suggest that EPEC uses a system similar to the Yop virulon of *Yersinia* spp., where numerous secreted Yops are required to form a complex of translocator proteins, which collaborate to inject other Yops into the host cell (1). EspA serves as a component of a surface structure that appears to bridge the bacteria to the host cell (19). It is tempting to speculate that EspD, the least-characterized Esp protein, may function in a manner analogous to YopB, which is essential for translocation of Yop effector proteins and has a membrane-disrupting activity (13). EspD shares with YopB a modest degree of sequence similarity. It is possible that the failure of EspB to complement an *espB* mutant when expressed within epithelial cells is due to a requirement for EspB to enter the host cell as part of a complex with another protein, for example, Tir. Interestingly, EspB is required for the translocation of an EspB-adenylate cyclase fusion protein (31). Alternatively, the quantity or the temporal or spatial distribution of EspB in transfected cells may not mimic those of EspB delivered by EPEC. Thus, future experiments investigating the quantity of EspB expressed in host cells or the route by which it is delivered may indicate that it is possible to bypass the delivery of EspB by the type III secretion system. In any case, it is clear that the Esp proteins and Tir act in concert to form a mature attaching and effacing lesion.

We demonstrated a significant decrease in the ability of either EPEC or *Salmonella* to invade cells expressing EspB. This decrease was not due to dexamethasone treatment or transfection alone, as illustrated by the level of invasion observed in control samples. EPEC and *Salmonella* experienced similar decreases in their ability to invade *espB*-transfected cells, suggesting that it is the disruption of the actin cytoskeleton, rather than a specific EspB effect on EPEC, that inhibited the ability of these bacteria to invade.

We observed a substantial reduction in the amount of phosphorylated Tir in transfected HeLa cells at time points optimal for expression of EspB. However, without appropriate reagents, we are unable to ascertain whether there is a decrease in the translocation of Tir into the cell, in its subsequent phosphorylation, or both. Cells expressing EspB are able to support both localized adherence and the ability of EPEC to focus actin under sites of bacterial attachment, yet these cells display a dramatic decrease in the amount of phosphorylated Tir and in the ability to support invasion by either EPEC or *S. typhimurium*. These observations are compatible with other studies, which show that attaching and effacing is possible in the absence of detectable levels of phosphorylated Tir (24), and they reinforce the concept that adherence and actin accumulation are separable from phosphorylation of Tir and cellular invasion by EPEC. If these events require different or sequential signals, then the results presented here may reflect these differences. Moreover, while EspB may play a role in the generation of both signals, it is clear that other effectors must be involved in order to reconstitute the attaching and effacing lesion. Certainly, further research is required to dissect the complex and dynamic interactions between EspB and host cells.

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