INFECTION AND IMMUNITY, Dec. 1998, p. 6049–6053 Vol. 66, No. 12
0019-9567/98/$04.00+0

Enteropathogenic *Escherichia coli* Virulence Genes Encoding Secreted Signalling Proteins Are Essential for Modulation of Caco-2 Cell Electrolyte Transport

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Received 8 June 1998/Returned for modification 24 July 1998/Accepted 29 September 1998

The pathophysiology of enteropathogenic *Escherichia coli* (EPEC) diarrhea remains uncertain. In vitro, EPEC stimulates a rapid increase in short-circuit current (I_{sc}) across Caco-2 cell monolayers coincident with intimate attaching and effacing (A/E) bacterial adhesion. This study has examined the roles of specific EPEC virulence proteins in this I_{sc} response. EPEC genes encoding EspA, EspB, and EspD, essential for signal transduction in host cells and A/E activity, were also required for modulation of Caco-2 electrolyte transport.

Enteropathogenic *Escherichia coli* (EPEC) infections among infants and young children are common in many developing countries (24); however, the pathophysiology of resultant diarrhea remains uncertain. EPEC produces no recognized enterotoxins (22, 25, 28); rather, diarrhea results from direct interaction of bacteria with the small intestinal epithelium. EPEC adheres to enterocytes and transduces signals (13) which produce an attaching and effacing (A/E) lesion in the brush border membrane characterized by a loss of microvilli and intimate adhesion of bacteria to the apical cell membrane, beneath which host cell cytoskeletal elements accumulate (18, 19, 34).

To study the effects of EPEC interaction on host cell electrolyte transport, we developed an in vitro model of EPEC infection in which monolayers of the human intestinal cell line Caco-2 are rapidly infected with EPEC before being mounted into Ussing chambers (2). Using this technique, we demonstrated a rapid stimulation of short-circuit current (I_{sc}) across Caco-2 cell monolayers caused by EPEC which indicated modulation of transepithelial electrolyte transport (2). Moreover, the peak of this I_{sc} response coincided with development of A/E lesions and was partially (~35%) chloride dependent, consistent with EPEC-induced stimulation of chloride secretion.

The A/E phenotype is determined by a 35-kbp region of the EPEC chromosome designated the locus of enterocyte effacement (LEE) (7, 26). At the right end of this pathogenicity island are genes encoding three EPEC secreted proteins, EspA, EspD, and EspB, which are essential for signal transduction to host cells and A/E activity (4, 8, 14, 21); these proteins are exported via a type III secretion apparatus encoded by *esc* and *sep* genes at the left end of the LEE (7, 11). EPEC-induced signal transduction results in a number of intracellular changes, including a cascade of inositol phosphates, phosphorylation of host cell proteins, and most significantly, tyrosine phosphorylation of a large protein (formerly Hp90) in the host cell membrane (6, 9, 29). This protein, recently shown to be bacterial in origin and renamed Tir (15), acts as the receptor for intimin, a 94-kDa outer membrane protein product of the LEE *eae* gene (12). Intimin binding is essential for intimate adhesion of EPEC to host cells and promotes accretion of cytoskeletal elements beneath attached bacteria to produce the mature A/E lesion (30).

The present study was designed to investigate the roles of specific EPEC virulence proteins in the generation of I_{sc} in Caco-2 cell monolayers; we utilized isogenic EPEC mutants deficient in expression of EspA, EspB, or EspD, in functional type III secretion (*escN* mutant), or in the expression of intimin (*eae* mutant). The origins and characteristics of EPEC strains used in this study are listed in Table 1. Bacteria were grown in bicarbonate-buffered Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., Poole, United Kingdom) in an atmosphere of 5% CO_2 in air at 37°C for 4 to 5 h to stimulate expression of LEE-encoded virulence proteins (activated EPEC) (10, 17). As described elsewhere (2), bacteria (10^8 to ~10^9 CFU) were centrifuged onto Caco-2 cell monolayers, which had been grown on Transwell polycarbonate microporous cell culture inserts, to facilitate synchronous initial attachment. Infected monolayers were immediately mounted into a modified Ussing chamber apparatus and maintained at 37°C in DMEM (gassed with 5% CO_2-95% O_2) for 60 min; during this time I_{sc} and transepithelial electrical resistance (TEER) of monolayers were measured continuously (2). Uninfected Caco-2 cell monolayers, subject to all the procedures involved in the infection method except exposure to bacteria, maintained both a constant I_{sc} and a stable TEER throughout the 60-min study period.

To investigate the respective roles of Esp proteins, functional type III secretion, and intimin-mediated adhesion in EPEC-induced electrical responses, Caco-2 cell monolayers were infected with EPEC wild-type strain E2348/69; with *espA*, *espB*, *espD*, *escN*, or *eae* mutants of E2348/69; or with their complementary transformants in which the deficient gene was reintroduced on a plasmid. Light microscopy studies revealed that for all strains, initial infection consistently produced adhesion of discrete bacterial colonies to at least 75% of cells (data not shown).

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TABLE 1. Characteristics of EPEC strains used in this study

Strain	Description	Signal transduction	Intimin expression	A/E lesions	Reference(s)
E2348/69	EPEC (O127:H6); infant diarrhea outbreak (United Kingdom)	+	+	+	33
UMD872	E2348/69 Δ*espA1::aphA-3*	−	+	−	14
UMD872pMSD2	UMD872 containing plasmid bearing *espA*	+	+	+	14
UMD864	E2348/69 Δ*espB1*	−	+	−	4, 13
UMD864pMSD3	UMD864 containing plasmid bearing *espB*	+	+	+	4, 13
UMD870	E2348/69 Δ*espD1::aphA-3*	−	+	−	21
UMD870pLCL123	UMD870 containing plasmid bearing *espD*	+	+	+	21
CVD452	E2348/69 Δ*escN::aphA-3*	−	+	−	11
CVD206	E2348/69 Δ*eae*	+	−	−	3, 29
CVD206pCVD438	CVD206 containing plasmid bearing *eae*	+	+	+	3, 12

EPEC stimulates I_{sc} in Caco-2 cell monolayers. Consistent with our previous observations (2), infection of Caco-2 cells with the wild-type EPEC strain, E2348/69, stimulated a rapid increase in I_{sc}, which peaked 10 to 15 min after initial infection (mean change in I_{sc} ± standard error of the mean [SEM] at 10 min, 26.4 [2.6] μA/cm^2 above the basal I_{sc} of uninfected monolayers; $n = 8$; Student's t test, $P < 0.001$) before falling gradually (Fig. 1A). The peak of this I_{sc} response was not associated with any significant change in TEER (ΔTEER) of monolayers; however, between 10 and 60 min after initial infection, E2348/69 induced a 31% loss in TEER (mean ΔTEER [SEM] between 10 and 60 min postinfection, −81 [8] Ω · cm^2; $P < 0.001$; $n = 8$) similar to that described previously (2).

EspA, EspB, and EspD are required for stimulation of Caco-2 cell I_{sc}. Changes in I_{sc} following infection of Caco-2 cell monolayers with E2348/69 gene deletion mutants or their plasmid transformants are shown in Fig. 1. The most striking finding was an absence of the characteristic EPEC-induced I_{sc} response (Fig. 1A) when Caco-2 cell monolayers were infected with signal transduction-defective mutant UMD872 (Δ*espA*), UMD864 (Δ*espB*), UMD870 (Δ*espD*), or CVD452 (Δ*escN*) (Fig. 1B). Moreover, the I_{sc} response was qualitatively restored, albeit at a reduced peak magnitude, when cells were infected with complementary plasmid transformants UMD872 pMSD2 (EspA$^+$) or UMD864pMSD3 (EspB$^+$) (Fig. 1C). Infection with the EspD$^+$ transformant strain UMD870pLCL123 induced a more gradual increase in I_{sc} than that stimulated by the parental strain; this response peaked after ~30 min (Fig. 1C). Therefore, the EPEC genes required for expression and functional type III secretion of three separate signalling proteins (i.e., EspA, EspB, and EspD) all appear to be prerequisite for eliciting an increase in I_{sc} in Caco-2 cell monolayers. In contrast, infection with the intimin-deficient strain CVD206 (Δ*eae*) or its transformant CVD206pCVD438 (intimin positive) induced I_{sc} responses which were qualitatively similar to, although somewhat attenuated compared to, that seen with E2348/69 infection (Fig. 1D), thereby revealing that modulation of transepithelial electrolyte transport is independent of intimate EPEC adherence to host cells.

EPEC signal transduction and intimin expression are necessary for inducing loss of TEER. The reduction in TEER of Caco-2 cell monolayers, seen during infection with strain E2348/69, was not apparent during infection with signal transduction-deficient strain UMD872 (Δ*espA*), UMD864 (Δ*espB*), UMD870 (Δ*espD*), or CVD452 (Δ*escN*) (Fig. 2A). However, marked reductions in TEER were induced by transformant strains UMD864pMSD3 (EspB$^+$) and UMD870pLCL123 (EspD$^+$) (Fig. 2B); infection with strain UMD872pMSD2 (EspA$^+$) did not significantly reduce TEER. In contrast, infection with the intimin-deficient strain CVD206 (Δ*eae*) produced a significant increase in TEER over 60 min (Fig. 2A), while infection with its intimin-positive transformant CVD206 pCVD438 caused a reduction in TEER comparable to that induced by E2348/69 (Fig. 2B). A detrimental effect of EPEC infection on TEER has been described for several cultured epithelial cell lines, and although this effect was initially attributed to a transcellular defect (1), it is now considered to result primarily from the disruption of intercellular tight junctions, which reduces barrier function of the cell monolayer (27, 31). In Caco-2 cells, we found that both EPEC signal transduction and intimin expression were prerequisite for inducing loss of TEER. A similar observation was previously made by Canil et al. (1), who also noted an increase in TEER during infection with the intimin-deficient strain CVD206. Recently, specific signalling events induced in host cells by EPEC have been found to be dependent on intimin binding (16). Therefore, the detrimental effect of EPEC on the TEER of Caco-2 cell monolayers may occur after intimin binding as a result of late signalling events which alter the integrity of tight junctions. The increase in TEER observed during infection with the intimin-deficient strain CVD206 requires further investigation.

In the absence of conventional enterotoxin production, the A/E phenotype is central to EPEC pathogenicity; however, this interaction of bacteria with host cells is complex and the precise mechanism(s) of EPEC induced diarrhea remains to be determined. Although loss of functional brush border membrane caused by A/E lesion formation and increased paracellular permeability induced by EPEC (27, 31) may contribute to the generation of an osmotic diarrhea, in vitro evidence indicates that EPEC is also able to directly modulate electrolyte transport in host cells. In HeLa and Caco-2 epithelial cell lines, Stein et al. (32) found that EPEC infection caused a reduction in resting membrane potential, indicating an altered distribution of ions across cell membranes. Significantly, this effect was dependent on signal transduction to host cells (*sep* and *espB* mutants) but was independent of intimate attachment to host cells (*eae* mutant) (32). More recently, we have described an EPEC-induced stimulation of I_{sc} in Caco-2 cell monolayers

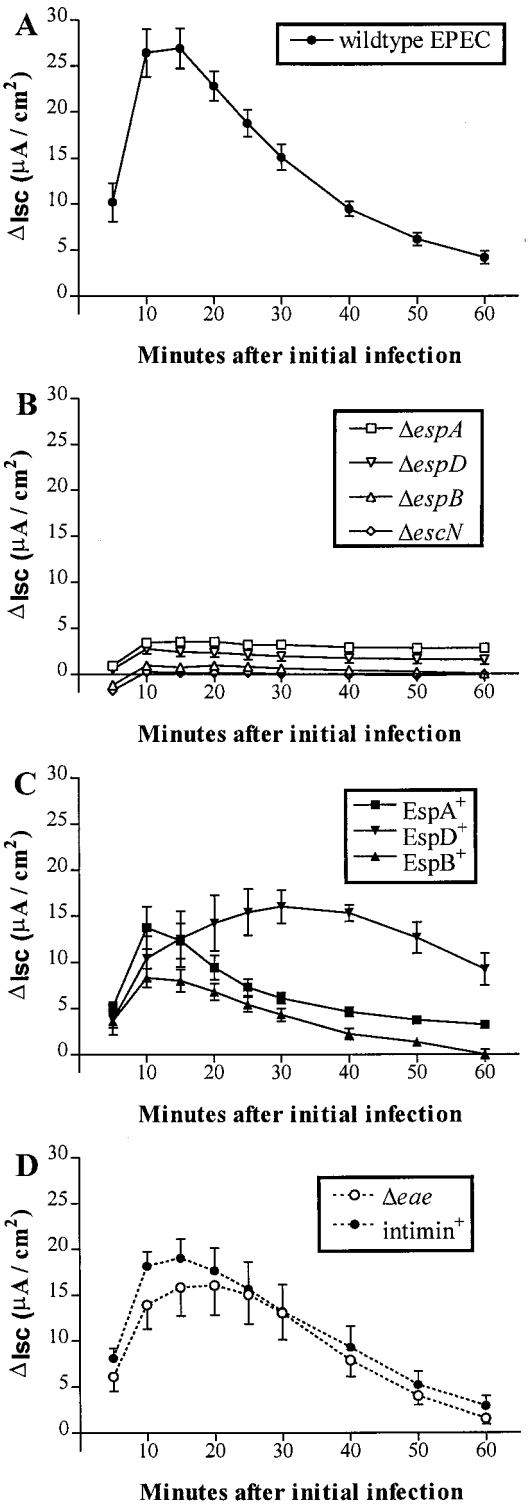

FIG. 1. I_{sc} change (ΔI_{sc}; $\mu A/cm^2$) in Caco-2 cell monolayers during infection with wild-type EPEC E2348/69 (A); signal transduction-defective mutant UMD872 (Δ*espA*), UMD864 (Δ*espB*), UMD870 (Δ*espD*), or CVD452 (Δ*escN*) (B); complementary plasmid transformant UMD872pMSD2 ($EspA^+$), UMD864pMSD3 ($EspB^+$), or UMD870pLCL123 ($EspD^+$) (C); and intimin-deficient mutant CVD206 (Δ*eae*) or its plasmid transformant CVD206pCVD438 (intimin positive) (D). Each point represent the mean change in I_{sc} ± SEM (error bars) of 5 to 10 monolayers compared with the basal I_{sc} of uninfected cells ($n = 25$).

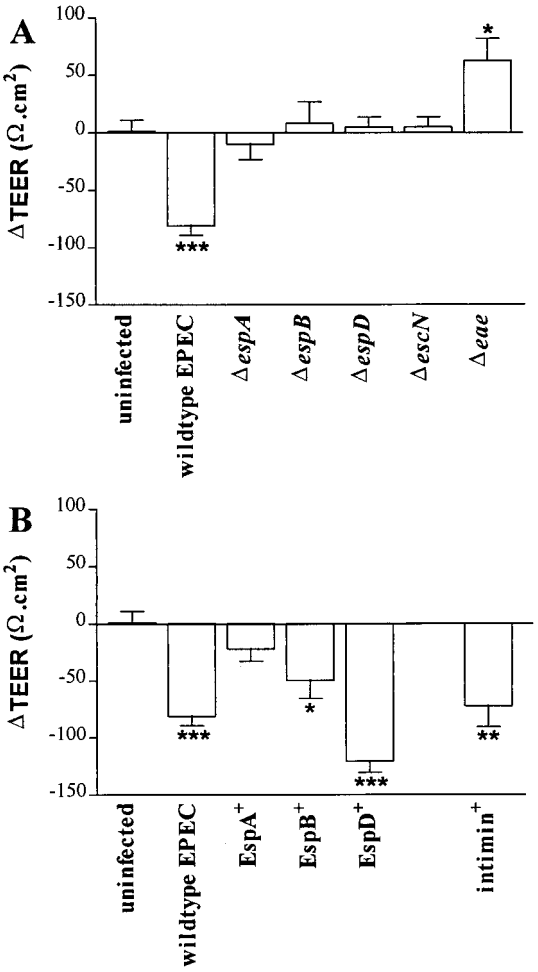

FIG. 2. Change in TEER (ΔTEER; $\Omega \cdot cm^2$) in uninfected Caco-2 cell monolayers between 10 and 60 min and after infection with wild-type EPEC E2348/69 or gene-deficient mutant UMD872 (Δ*espA*), UMD864 (Δ*espB*), UMD870 (Δ*espD*), CVD452 (Δ*escN*), or CVD206 (Δ*eae*) (A) or complementary plasmid transformants UMD872pMSD2 ($EspA^+$), UMD864pMSD3 ($EspB^+$), UMD870 pLCL123 ($EspD^+$), or CVD206pCVD438 (intimin positive) (B). Each bar represents the mean ± SEM (error bar) for 5 to 10 infected monolayers. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ versus uninfected monolayers ($n = 25$).

which provided direct evidence of altered transepithelial electrolyte transport (2); in the present study we examined the roles of various EPEC virulence proteins in the generation of this I_{sc} response. We have found that EPEC stimulation of I_{sc} requires functional secretion of all three Esp proteins (EspA, EspB, and EspD) needed for signal transduction and A/E activity. This observation accords well with recent reports which have sought to explain the dynamics of the EPEC-host cell interaction. Type III secretion mechanisms, akin to that found in EPEC (11), are a feature of many gram-negative pathogens and are typically responsible for the export of virulence proteins which interact directly with host cells (23). The EspA protein of EPEC was recently shown to be a constituent of an organelle on the bacterial surface which creates a bridge to host cells essential for translocation of EspB (20), and translocation of Tir also requires EspA (15). The role of EspD in this export pathway is as yet unclear; however, it is apparent that all three secreted proteins are required for signalling which leads to A/E lesion formation and the stimulation of I_{sc}. Previously, we demonstrated that the peak of the I_{sc} response

stimulated by wild-type EPEC coincided with development of A/E lesions, as assessed by transmission electron microscopy (2), suggesting that these EPEC phenotypes share similar kinetics. Moreover, in Caco-2 cell monolayers grown on glass coverslips, A/E lesions are identifiable by fluorescent staining of actin accumulated beneath adherent bacteria (19) 15 min after initial rapid infection with activated EPEC strain E2348/69 (data not shown); this lesion development coincides with peak I_{sc} stimulation seen in Ussing chamber studies. This finding is also true for those A/E phenotypic strains used in the present study which demonstrated I_{sc} kinetics typical of the wild type, irrespective of the magnitude of their peak I_{sc} increase. In contrast, this result is not the case for the $EspD^+$ transformant strain UMD870pLCL123. This strain, which stimulated a more gradual increase in I_{sc} than does the wild type, also induces lesion formation at a lower rate such that the A/E phenotype is undetectable at 15 min and only evident after 60 min of infection (data not shown). Significantly, it has been found that the extracellular production of individual Esp proteins may be adversely affected by the absence of other *esp* genes, and in gene-deficient mutants this effect may not be fully redressed by the presence of a plasmid bearing the missing gene (21). We believe that this phenomenon may account for the failure of the transformant strains to elicit an I_{sc} response equivalent to that caused by the wild-type EPEC strain and, in particular, is responsible for the uncharacteristic response seen for infection with strain UMD870pLCL123 ($EspD^+$). Interestingly, this strain produces abnormally long EspA filaments (20), a consequence of which may be abnormal protein translocation and signal transduction.

That EPEC-induced modulation of transepithelial electrolyte transport is a consequence either of the cascade of signalling events which are central to A/E activity (e.g., flux of inositol phosphates or activation of protein kinases) or of the cytoskeletal rearrangements which occur during lesion formation remains unclear. However, the ability of strain CVD206 (Δ*eae*) to cause a characteristic I_{sc} increase in Caco-2 cell monolayers indicates that this stimulation is independent of intimate adherence. Although it lacks intimin, strain CVD206 retains the ability to transduce signals to host cells and cause some cytoskeletal disorganization (3, 29). The residual virulence of this strain in vivo (5) suggests that these elements of EPEC pathogenesis alone are indeed sufficient to cause diarrhea in the absence of intimate adherence.

This work was supported by a grant from Action Research (to S.K.) and by Public Health Service awards AI32074 (to M.S.D) and AI21657 (to J.B.K.) from the National Institutes of Health.

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Editor: P. E. Orndorff