Disruption of Cell Polarity by Enteropathogenic *Escherichia coli* Enables Basolateral Membrane Proteins To Migrate Apically and To Potentiate Physiological Consequences

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Enteropathogenic *Escherichia coli* (EPEC) disrupts the structure and barrier function of host intestinal epithelial tight junctions (TJs). The impact of EPEC on TJ “fence function,” i.e., maintenance of cell polarity, has not been investigated. In polarized cells, proteins such as β1-integrin and Na⁺/K⁺ ATPase are restricted to basolateral (BL) membranes. The outer membrane EPEC protein intimin possesses binding sites for the EPEC translocated intimin receptor (Tir) and β1-integrin. Restriction of β1-integrin to BL domains, however, precludes opportunity for interaction. We hypothesize that EPEC perturbs TJ fence function and frees BL proteins such as β1-integrin to migrate to apical (AP) membranes of host cells, thus allowing interactions with bacterial adhesins such as intimin. The aim of this study was to determine whether EPEC alters the polar distribution of BL proteins, in particular β1-integrin, and if such redistribution contributes to pathogenesis. Human intestinal epithelial T84 cells and EPEC strain E2348/69 were used. Selective biotinylation of AP or BL membrane proteins and confocal microscopy showed the presence of β1-integrin and Na⁺/K⁺ ATPase on the AP membrane following infection. β1-Integrin antibody afforded no protection against the initial EPEC-induced decrease in transepithelial electrical resistance (TER) but halted the progressive decrease at later time points. While the effects of EPEC on TJ barrier and fence function were Tir dependent, disruption of cell polarity by calcium chelation allowed a tir mutant to be nearly as effective as wild-type EPEC. In contrast, deletion of *espD*, which renders the type III secretory system ineffective, had no effect on TER even after calcium chelation, suggesting that the putative β1-integrin–intimin interaction serves to provide intimate contact, like that of Tir and intimin, making translocation of effector molecules more efficient. We conclude that the initial alterations of TJ barrier and fence function by EPEC are Tir dependent but that later disruption of cell polarity and accessibility of EPEC to BL membrane proteins, such as β1-integrin, potentiates the physiological perturbations.

The intercellular tight junction (TJ) serves two crucial roles for the intestinal epithelium. First, the apical intercellular contacts form a regulatable barrier to the paracellular passage of ions, water, and immune cells (32). Second, TJs are key for maintaining the polar distribution of some membrane proteins. TJs control cell polarity by limiting the diffusion of lipids and integral membrane proteins between apical and basolateral membranes (11, 28). This “fence function” of TJs creates an asymmetrical and limited distribution of proteins in the two distinct membrane regions of the cell. Proteins such as Na⁺/K⁺ ATPase, a transport protein essential for creating the electrochemical gradient for vectorial transport, and β1-integrin, a cellular adhesion molecule that participates in anchoring of polarized epithelial cells to the basement membrane, are examples of proteins that are limited to the basolateral compartment. Their restricted distribution plays a critical role in their function.

Enteropathogenic *Escherichia coli* (EPEC) causes diarrheal disease and is a major contributor to the high rate of infant mortality in developing countries (22, 33). Intimate adherence between EPEC and host intestinal epithelial cells results in the formation of attaching-and-effacing (A/E) lesions on the surface of epithelial cells (14). The formation of A/E lesions has been shown to be an intricate, multistep process that requires type III secretion machinery that is encoded on a pathogenicity island known as the locus of enterocyte effacement (19). The type III secretory apparatus provides an avenue through which bacterial proteins and effector molecules are translocated into the host cell cytoplasm. A hollow filamentous structure composed of EPEC secreted protein A (EspA) serves as the conduit for protein shuttling from the pathogen to the host cell (15, 27). Pores are formed in the host cell membrane by EspB and -D, thus allowing the bacteria to deliver effector molecules directly to the host cell cytoplasm (34). One of these molecules is the translocated intimin receptor (Tir). Tir is injected into the host cell (5, 12), phosphorylated, and inserted into the cell membrane, where it serves as a receptor for intimin, an outer membrane adhesin of EPEC. As a result, intimate adherence is facilitated. Interestingly, intimin has been shown to interact with cells independent of Tir and to bind β1-integrin in vitro (8). The physiological ramifications of these interactions have yet to be determined.

It has been shown previously that another enteric pathogen, *Yersinia pseudotuberculosis*, utilizes β1-integrin as a receptor for the outer membrane protein invasin, thus driving cell in-
vasion (10). Although β1-integrin is normally limited to the basolateral domains of nonspecialized intestinal epithelial cells, Y. pseudotuberculosis-induced neutrophil transmigration across intestinal epithelial monolayers was shown to disrupt TJs, allowing β1-integrin to redistribute to the apical membrane, where it could serve as a ligand for invasion and increase the invasion of this organism (18). The potential role of β1-integrin as a receptor for EPEC intimin has not been explored in host-pathogen model systems.

In intact epithelia, β1-integrin is restricted to the basolateral membrane and thus is not available for interaction with luminally positioned microbial pathogens. EPEC infection has been shown to induce alterations in host intestinal epithelial functions, including stimulation of the inflammatory responses (25, 26), changes in ion transport (4, 9), and disruption of the TJ barrier (2, 23, 30, 35). Disruption of the TJ barrier is accompanied by structural changes in the arrangement of TJ-associated proteins (29). While EPEC-induced TJ alterations have a profound effect on barrier function, the impact on fence function (maintenance of cell polarity) has not been examined. In this study, we show that EPEC infection of intestinal epithelial cells also perturbs the TJ fence, facilitating redistribution of basolateral membrane proteins. The relocalization of basolaterally restricted proteins, such as β1-integrin, to the apical cell surface provides the opportunity for novel interactions with EPEC. Using a well-defined intestinal epithelial model system, we demonstrate here a role for β1-integrin in the pathogenesis of EPEC infection.

**MATERIALS AND METHODS**

**Cell culture.** Cultured human intestinal epithelial cells (T84 cells) were grown on permeable filters in a 1:1 (vol/vol) mixture of Dulbecco’s modified Eagle medium and Ham’s F-12 medium supplemented with 6% newborn calf serum. Cells were passaged and plated on collagen-coated filters as described previously (9, 35). Prior to infection, the cells were placed in antibiotic-free medium with 0.5% newborn calf serum and 0.5% mannose overnight.

**Bacterial strains and infection of monolayers.** EPEC strain E2348/69, a wild-type strain initially characterized by Knutson et al. (13), and the tir mutant CVD463 (previously published as SE8969) (7) were generous gifts from James Kaper, University of Maryland. The espD mutant UMD870 was kindly provided by Michael Donnenberg, University of Maryland. Bacterial cultures were grown overnight in Luria-Bertani broth and then diluted (1:33) in antibiotic-free cell culture medium and Ham’s F-12 medium and then incubated for specific time periods. EPEC and equivalent amounts of antibiotic-free Dulbecco’s modified Eagle medium were added to the apical surfaces of T84 monolayers grown on collagen-coated permeable supports at a multiplicity of infection of 100. Bacteria and monolayers were then incubated at 37°C for 2 h. Nonadherent organisms were removed by gentle washing with warm medium and then incubated for specified times. This well-characterized model has been used to explore the impact of EPEC infection on various intestinal epithelial functions (9, 25, 26, 29).

**Biotinylation and immunoprecipitation.** Surface biotinylation of T84 membranes was performed as described by McCormick et al. (18). Briefly, T84 monolayers grown on 5-cm² permeable supports (Transwells; Costar, Cambridge, Mass.) were washed with cold Hanks balanced salt solution (HBSS) and cooled to 4°C. Apical or basolateral surfaces of control or EPEC-infected monolayers were selectively biotinylated by application of biotin sulfo-NHS-LC-pipecolinic acid (EZ-link sulfo-NHS-biotin; Pierce Biochemical, Rockford, Ill.), dissolved in HBSS at 0.5 mg/ml, for 20 min at 4°C. The reaction was then quenched by treatment with 50 mM NH₄Cl in HBSS for 20 min at 4°C. The monolayers were washed, and cells were scraped into 10 ml HEPES (pH 7.4)–3.5 mM MgCl₂–150 mM NaCl–1 mM phenylmethylsulfonyl fluoride–10 mM sodium fluoride–1 mM leupeptin–1 mM pepstatin–1 mM EDTA–10 mM NaCl–2 mM EDTA–0.4 M NaCl–0.4 M NaCl. The extracts were then centrifuged at 4,000 × g for 5 min, and the pellets were solubilized in the same buffer with the addition of 2% Triton X-100. A 500-μg aliquot of extracted protein, as quantified by the Bradford assay (1), was incubated overnight with 3.0 μg of monoclonal β1-integrin antibody (BD Transduction Laboratories, San Diego, Calif.) or 3.0 μg of Na⁺/K⁺ ATPase antibody (Sigma, St. Louis, Mo.), followed by incubation with 0.4 ml of 50% protein A-Sepharose for 1 h. Immunoprecipitates were washed with 10 ml NaCl–H₃PO₄, (pH 7.4)–1% Nonidet P-40–0.4 M NaCl–0.4 M EDTA–0.1 M NaCl–1 mM benzamidine–10 mM chymostatin–10 mM leupeptin–1 mM pepstatin, denatured in sample buffer, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting as previously described (25). Nitrocellulose membranes were blocked with Zymed blocking solution (Zymed Laboratories). Membranes were scanned and densitometric analysis was performed with the Alpha Imager 1220 System.

**Immunofluorescence confocal microscopy.** The localization of specific proteins was determined by immunofluorescence confocal microscopy experiments. Monolayers were rinsed after infection with ice-cold phosphate-buffered saline (PBS) and either fixed for 10 min in 1% formaldehyde and permeabilized for 10 min in 0.2% Triton X-100 or fixed and permeabilized for 20 min in 100% methanol at −20°C. Cells were then blocked for 20 min in 1% bovine serum albumin in PBS. Monolayers were incubated with antibody specific for Na⁺/K⁺ ATPase (Sigma) and/or β1-integrin (BD Transduction Laboratories) or phalloidin–fluorescein isothiocyanate (Molecular Probes, Eugene, Ore.) and assessed by confocal microscopy. For Fig. 2A, monolayers that had been used for electrophysiology experiments in the presence of β1-integrin antibody were washed thoroughly with PBS and incubated with 5 μg of anti-rabbit immunoglobulin G (IGG) conjugated to Alexa568 (Molecular Probes) per ml. The filters were then rinsed thrice with PBS, excised from the support, and mounted with the Prolong antifade kit (Molecular Probes). Confocal analysis was performed with an LSM510 laser scanning confocal microscope (Zeiss, Thornwood, N.Y.).

**Electrophysiological measurement of TER.** Confluent T84 monolayers, grown on 0.33-cm² collagen-coated permeable supports (Transwells; Costar) were used for electrophysiological assessment. Transepithelial electrical resistance (TER) was determined by passing 25 μA of current across the cell monolayers by using a simplified Ussing chamber apparatus as described by Madara et al. (16). The resulting voltage deflection was measured, and Ohm’s law (V = IR) was applied in order to calculate resistance.

**Disruption of TJ fence function prior to infection by using “calcium switch.”** Following the measurement of baseline TER, monolayers were exposed to medium containing 5 mM EDTA for 10 min in order to disrupt the cell polarity and allow basolateral membrane proteins to redistribute to the apical membrane. TER was measured to ensure that TJs were disrupted (17). Monolayers were then switched to medium containing a normal concentration of calcium and no EDTA in order to allow the reestablishment of the TJ barrier (3). TER was measured at regular intervals to ensure the recovery of TER; monolayers were then infected with either wild type EPEC, tir mutant strain CVD463, or espD mutant strain UMD870 in the presence or absence of β1-integrin antibody, and TER was measured at the indicated times.

**Statistical analysis.** Statistical analysis was performed by using a paired t test. All data represent the mean ± standard error of the mean (SEM). Significance was determined as P < 0.05.

**RESULTS**

EPEC infection allows for redistribution of basolateral membrane proteins. The distribution of specific basolateral membrane proteins following EPEC infection (29, 30) was determined by selective membrane labeling with biotin followed by immunoprecipitation and streptavidin detection. This approach revealed significant alterations in the distribution of two basolaterally restricted proteins, Na⁺/K⁺ ATPase (Fig. 1A) and β1-integrin (Fig. 1B), following EPEC infection. In uninfected cells, these proteins were primarily restricted to the basolateral membrane as expected, with the apical-to-basolateral ratios of both of these proteins, with the apical-to-basolateral ratios of both of these proteins being 21%/79% (± 15%) and 23%/77% (± 3%), respectively. EPEC infection led to a progressive redistribution of both of these proteins, with the apical-to-basolateral ratios for Na⁺/K⁺ ATPase and β1-integrin after 6 h of infection...
what expected, given that the effects of chelation are uniform across the monolayer while the impact of EPEC infection may be limited to cells harboring attached microcolonies. To determine whether distinct membrane regions were altered after infection, BODIPY-FL C₁₂-sphingomyelin (Molecular Probes) was incorporated into apical membranes prior to EPEC infection. After 6 h, nonpermeabilized uninfected and EPEC-infected monolayers were analyzed by confocal microscopy. In uninfected monolayers, the BODIPY-FL label was limited to the apical membrane, whereas BODIPY-FL was distributed throughout the apical and basolateral membranes of infected monolayers (data not shown), thus confirming the selective biotinylation results.

Confocal immunofluorescence analysis confirms redistribution of β₁-integrin. The results of the selective biotinylation studies were also supported by confocal immunofluorescence analysis. In uninfected control monolayers (Fig. 2A), β₁-integrin staining was restricted to basolateral membrane regions. After 6 h of infection with EPEC (Fig. 2B), however, the β₁-integrin staining was apparent on both the basolateral and apical membranes. Interestingly, the appearance of apically positioned β₁-integrin was not uniform across the monolayer but rather tended to cluster at focal areas. We therefore explored whether β₁-integrin associated with EPEC microcolonies at this time point.

Uninfected control and EPEC-infected monolayers were dual labeled for actin and β₁-integrin. In this way, A/E lesion formation could be identified as apical aggregates of actin. In uninfected monolayers, actin localized primarily to the region of the actomyosin ring at the apical part of the cell as well as in stress fibers along the basal surface (Fig. 2B). Little overlap in the staining of these two molecules was seen. In contrast, after 6 h of EPEC infection, focal aggregates of actin appeared at the apical membrane, consistent with A/E lesion formation.

In addition, bright areas of staining for β₁-integrin at the apical membrane, consistent with A/E lesion formation could be identified as apical aggregates of actin. In uninfected monolayers, actin localized primarily to the region of the actomyosin ring at the apical part of the cell as well as in stress fibers along the basal surface (Fig. 2B). Little overlap in the staining of these two molecules was seen. In contrast, after 6 h of EPEC infection, focal aggregates of actin appeared at the apical membrane, consistent with A/E lesion formation. In addition, bright areas of staining for β₁-integrin colocalized at these focal actin plaques. In order to confirm that these focal areas of apical actin and β₁-integrin staining were in fact situated under adherent EPEC microcolonies, fluorescent images were overlaid onto differential interference contrast images of the same field in which bacterial microcolonies are visualized. Figure 2C shows that, in fact, the focal areas of colocalized apical actin and β₁-integrin staining correspond to EPEC attachment sites.

Antibody to β₁-integrin attenuates the decrease in TER in response to EPEC infection. TJ barrier function can be assessed by measuring the TER across a confluent cell monolayer. One well-described physiological consequence of EPEC infection on model intestinal epithelia is a significant time- and dose-dependent decrease in TER. A possible role for apically positioned β₁-integrin on this functional phenotype has not been explored. Therefore, the potential downstream effects of interactions between β₁-integrin and the bacterial surface on the TJ barrier were assessed by blocking the association of β₁-integrin with EPEC over the time course of infection (Fig. 3). Cell monolayers were infected in the presence or absence of monoclonal antibodies against β₁-integrin. The presence of β₁-integrin antibodies had no influence on the EPEC-induced decrease in TER at early time points (2 h) but significantly attenuated the subsequent decrease in TER (Fig. 3). In the absence of antibody or in the presence of isotype IgG, TER

FIG. 1. EPEC infection induces redistribution of basolateral membrane proteins. T84 monolayers that were either uninfected; infected with EPEC for 2, 4, or 6 h, or EDTA treated were selectively surface membrane labeled with activated biotin. Extracted proteins were immunoprecipitated with either Na⁺/K⁺ ATPase or β₁-integrin antibodies and quantified by alkaline phosphatase-streptavidin immunoblots. (A) Immunoprecipitation specific for Na⁺/K⁺ ATPase in uninfected monolayers, monolayers infected for 6 h, and monolayers exposed to 4 mM EDTA for 10 min revealed a redistribution of protein from the basolateral (BL) to the apical (AP) surface following EPEC infection (P = 0.05; n = 3) and EDTA treatment (P = 0.004; n = 3). (B) Immunoprecipitation of β₁-integrin was performed on uninfected monolayers; monolayers infected with EPEC for 2, 4, or 6 h; and EDTA-treated monolayers. A progressive and significant redistribution of β₁-integrin to the apical surface was observed by 6 h postinfection (P = 0.03; n = 4). Immunoblots are representative of those from four separate experiments. Densitometry values are expressed as mean percentages of the total (apical plus basolateral) ± SEM.
continued to progressively drop. Interestingly, β1-integrin antibody did not prevent the apical redistribution of this protein as determined by immunofluorescent staining (data not shown). These data suggest that redistributed β1-integrin is involved in the physiological perturbations in the later stages of infection.

Expression of Tir is required for disruption of TJ barrier and fence function. Early stages of EPEC pathogenesis are critically linked to the well-characterized type III secretion system. The formation of a “molecular syringe” and injection of Tir into host cells occurs following initial cell contact. Furthermore, phosphorylation and insertion of Tir into the host cell membrane mediates the intimate adherence of EPEC via intimin and facilitates the translocation of effector molecules and subsequent physiological perturbations. The ability of the tir deletion strain CVD463 to perturb the TJ barrier was explored. Infection of T84 cell monolayers with the tir deletion strain had no significant effect on TER (Fig. 4A) even after 6 h of infection (2% ± 4% change in TER from baseline). This is presumably due to the absence of Tir-intimin interactions, which are needed for intimate attachment and effective delivery of effector molecules into host cells. In addition to the lack of impact on barrier function, the absence of Tir also blocked the redistribution of β1-integrin as assessed by selective surface biotinylation and immunoprecipitation (Fig. 4B). These data indicate that both the fence and barrier functions of the TJ

FIG. 2. Confocal micrographs show that EPEC infection allows β1-integrin to migrate to the apical membrane. (A) Uninfected monolayers and those infected with EPEC for 6 h were immunostained with antibodies to β1-integrin. Note that in uninfected monolayers the staining for β1-integrin is basolateral and limited to the lateral membrane. EPEC-infected monolayers revealed a significant presence of β1-integrin at the apical pole of the cells, indicating free access and redistribution to the apical membrane after infection. Data are presented as yz single focal planes. (B) Confocal microscopy of uninfected and EPEC-infected monolayers dual labeled for actin (red) and β1-integrin (green). In uninfected monolayers the β1-integrin is primarily limited to the basal surface, with some lateral localization, but restricted to the region basolateral to the apical actin-myosin ring. (C) Following EPEC infection, apically localized regions of actin aggregation are seen and correspond with microcolony attachment as viewed by differential interference contrast microscopy. Colocalization of β1-integrin to the same A/E lesions is indicated by a yellow signal.
FIG. 3. β₁-Integrin antibody attenuates the EPEC-induced decrease in TER. T84 monolayers were infected with EPEC in the absence or presence of 1 μg of β₁-integrin monoclonal antibody per ml or 1 μg of isotypic IgG per ml and incubated for 6 h. TER was measured at 2, 4, and 6 h and expressed as the percent change from baseline values. There was no significant difference (N.S.) in the decrease in TER following EPEC infection with and without IgG. In contrast, antibody against β₁-integrin provided a significant level of protection at both 4 and 6 h postinfection. The data shown represent the mean ± SEM (n = 21 to 24; *P = 0.054, 0.005, and 0.001 for 2, 4, and 6 h postinfection, respectively) from seven experiments with triplicate or quadruplicate samples.

Confocal analysis of monolayers stained for actin and β₁-integrin substantiated the electrophysiological findings. Figure 5B shows representative images of control monolayers and monolayers that were treated with 5 mM EDTA for 10 min and allowed to recover in Ca²⁺-containing medium for 3 h. Control monolayers show β₁-integrin staining (green) limited to the basolateral domain, basal to the apical actin-myosin ring of cells. Following EDTA treatment and recovery, β₁-integrin staining was seen interdigitating into or rising above the actin ring. These results visually confirm the relocalization of β₁-integrin to the apical domain of monolayers following EDTA-induced disruption and recovery of TJs. Confocal microscopic analysis of monolayers that were EDTA treated and recovered revealed significant colocalization of actin and β₁-integrin after

complex remain intact following infection with the tir deletion EPEC strain, suggesting that Tir is required to generate the initial downstream signals that perturb TJ functions and allow for free diffusion of membrane proteins between apical and basolateral membrane compartments.

Trapping of basolateral membrane proteins on the apical aspect of intestinal monolayers obviates the requirement for Tir in EPEC-induced disruption of TJs. In order to directly test whether access of EPEC to β₁-integrin early in infection could eliminate the dependence on Tir in perturbing TJs, monolayers were briefly treated with EDTA to disrupt TJs so that polarized membrane proteins were allowed to redistribute prior to infection. Calcium was then restored so that TJs could reform, theoretically trapping basolateral membrane proteins on the apical surface. In order to ensure that β₁-integrin remained on the apical surface following recovery from EDTA-induced disruption of TJs, selective apical biotinylation was performed on control monolayers not subjected to EDTA treatment and on monolayers subjected to EDTA treatment and allowed to recover for 3 h. β₁-Integrin was immunoprecipitated from protein extracts of biotinylated monolayers and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and biotinylated proteins were detected with streptavidin. As demonstrated in Fig. 5A, following EDTA treatment and recovery, biotinylated β₁-integrin was recovered from extracts of monolayers subjected to selective apical labeling. As shown previously, little β₁-integrin was present in the apical membranes of untreated control samples.

Confocal analysis of monolayers stained for actin and β₁-integrin substantiated the electrophysiological findings. Figure 5B shows representative images of control monolayers and monolayers that were treated with 5 mM EDTA for 10 min and allowed to recover in Ca²⁺-containing medium for 3 h. Control monolayers show β₁-integrin staining (green) limited to the basolateral domain, basal to the apical actin-myosin ring of cells. Following EDTA treatment and recovery, β₁-integrin staining was seen interdigitating into or rising above the actin ring. These results visually confirm the relocalization of β₁-integrin to the apical domain of monolayers following EDTA-induced disruption and recovery of TJs. Confocal microscopic analysis of monolayers that were EDTA treated and recovered revealed significant colocalization of actin and β₁-integrin after

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FIG. 5. (A) β₁-integrin is trapped on the apical surface after EDTA treatment. T84 monolayers were treated with 5 mM EDTA for 10 min to disrupt TJs and allow for redistribution of basolateral proteins to the apical surface. EDTA was then removed, and calcium was restored to resel TJs for a 3-h recovery period. Apical surfaces of the nontreated and EDTA-treated monolayers were biotinylated, and β₁-integrin was immunoprecipitated and detected with alkaline phosphatase-streptavidin. A significant amount of β₁-integrin was found to be biotinylated after EDTA treatment and recovery. (B) Control monolayers and monolayers treated with EDTA were allowed to recover and were then fixed and stained for β₁-integrin (green) and actin (red). In control monolayers β₁-integrin is basolateral to the actin-myosin ring. However, in EDTA-treated monolayers, β₁-integrin stains at and above this ring, indicating that it has moved to the apical pole after EDTA disruption. (C) EDTA-treated and recovered monolayers were then infected with Δtir EPEC and stained for β₁-integrin (green) and actin (red). Coalescing of actin staining was seen following infection, and regions of colocalized actin and β₁-integrin can be seen. (D) Redistribution of basolateral proteins to the apical surface of cell monolayers prior to infection renders Δtir EPEC capable of decreasing TER. T84 monolayers were treated with EDTA for 10 min, and TER was measured to confirm disruption of TJs. EDTA was then removed, and calcium was restored to resel TJs and trap redistributed basolateral membrane proteins on the apical surface. TER was measured prior to and throughout the course of infection with wild-type EPEC or Δtir EPEC. EPEC and Δtir EPEC both decreased TER significantly compared to uninfected controls, with \( P = 4.1 \times 10^{-11} \) and \( P = 2.43 \times 10^{-5} \), respectively \((n = 6)\). The presence of β₁-integrin antibody throughout the course of infection blocked the Δtir EPEC-induced decrease in TER, whereas isotype IgG had no effect \((Δtir \text{ EPEC versus } Δtir \text{ EPEC plus } β₁\text{-integrin antibody, } P = 0.002 [n = 9]; Δtir \text{ EPEC versus } Δtir \text{ EPEC plus } \text{IgG, } P = 0.33 [n = 5])\). Error bars indicate SEMs.
infection with the tir mutant strain (Fig. 5C). Together, these data suggest that tir mutant EPEC is able to infect cells harboring β₁-integrin on the apical membrane. In order to determine whether access to β₁-integrin on the apical surface altered the effect of the tir mutant strain on TER, monolayers were treated with EDTA and allowed to recover as described in Materials and Methods. The immediate drop in TER following the addition of EDTA was measured, and the subsequent recovery following the restoration of calcium was verified by serial TER measurements (Fig. 5D). After recovery of the TJ barrier, monolayers were infected with either wild-type EPEC or the tir deletion strain. In contrast to the lack of effect of the tir mutant strain on the TER of standard monolayers, the tir mutant decreased the TER of monolayers in which membrane polarity had been previously perturbed by EDTA treatment (Fig. 5D). Uninfected control monolayers recovered fully and remained stable throughout the course of the experiment (TER at 10 h, 117% ± 6.7% [n = 12]). EDTA-treated and recovered monolayers infected with HB101, a commensal strain, also remained stable throughout the experiment (TER 10 h, 136% ± 12.4% [n = 12]). Thus, the decrease in resistance following infection of EDTA-treated monolayers was limited to pathogenic bacteria, i.e., EPEC and the tir mutant strain, as this response was not seen with nonpathogenic E. coli. Furthermore, the decrease in TER in EDTA-treated and recovered monolayers infected with the tir mutant in the presence of β₁-integrin antibody was significantly attenuated, as shown in Fig. 5D. These data suggest that β₁-integrin participates in the interaction and pathogenesis of EPEC in the absence of Tir, ultimately leading to perturbations in the physiological functions of host intestinal epithelia.

In an attempt to determine whether intimin contributes to this phenomenon, similar experiments were performed with the intimin deletion strain CVD206 (6). Infection of normal T84 monolayers with the intimin-negative strain did not significantly decrease monolayer resistance through a 6-h time course of infection (uninfected monolayers, 130.8% ± 0.7%; intimin-negative EPEC-infected monolayers, 115.1% ± 14.1% [6 h postinfection; P = 0.16; n = 3]). It should be noted that at the higher multiplicities of infection employed in previously published studies, CVD206 decreased TER by approximately half of that seen in response to wild-type EPEC (23). In order to assess whether expression of the putative β₁-integrin ligand intimin was required for the tir mutant to decrease TER following an EDTA switch, experiments were performed as described above. The rationale for this experiment was that if any bacterial outer membrane structures other than intimin were able to interact with apical β₁-integrin, then TER should decrease to the same extent as that seen with the tir mutant following an EDTA switch. Following an EDTA switch and recovery, the TERs of monolayers infected with CVD206 were not significantly different from those of control monolayers for up to 6 h postinfection (EDTA alone, 153.9% ± 8.4%; EDTA plus CVD206, 131.1% ± 17.1% [P = 0.11; n = 3]). These data imply that intimin is required for the β₁-integrin-mediated drop in resistance following EPEC infection.

Effective type III secretion is required for disruption of the TJ barrier via interactions with β₁-integrin. There are two possible mechanisms that could explain the contribution of β₁-integrin to EPEC-induced decrease in TER. First, interactions between intimin and β₁-integrin could trigger signaling cascades that affect TJs. Alternatively, β₁-integrin may substitute for Tir, securing intimate attachment and effective delivery of effector molecules through type III secretion. In order to distinguish between these two possibilities, studies employing the espD mutant strain were performed. To assess the necessity of an intact type III secretion system in the β₁-integrin-mediated decrease in TER, the calcium switch model was used. While the tir deletion mutant was capable of decreasing TER after EDTA treatment and recovery, the espD mutant EPEC had no significant effect on the TER of these monolayers (Fig. 6). These experiments indicate a requirement for intact type...
III secretion for disruption of the TJ barrier regardless of the distribution status of β1-integrin on the cell surface. The role of β1-integrin in this process therefore is not a result of signaling cascades activated by the interaction of intimin and β1-integrin but rather is intrinsically tied to a functioning type III secretion system.

**DISCUSSION**

Interactions between enteric microbial pathogens and the single layer of host intestinal epithelial cells that provides defense against the luminal contents define the outcome of the pathogenic process. TJs provide the protective barrier between the lumen and paracellular space and assist in the maintenance of polarity between apical and basolateral membrane compartments. While this apical “seal” at cell-cell contacts is critical to epithelial cell function, the delicate roles of TJs are directly exploited by several enteric pathogens. For example, EPEC has been shown to disrupt the TJ barrier in part by causing dephosphorylation and internalization of the transmembrane TJ protein occludin (29). The data presented here indicate that the EPEC-induced perturbation of TJs also affects cell polarity, which subsequently contributes to pathogenesis.

This finding is particularly relevant with regard to the polar distribution of the potential intimin binding partner β1-integrin. The interaction of intimin and β1-integrin has been clearly demonstrated in vitro (8). The use of cell culture models to study this interaction, however, has yielded controversial results. Frankel et al. (8) found that latex beads coated with the C-terminal portion of intimin fused to maltose-binding protein (MBP-Int280) or soluble MBP-Int280 bound to HEp-2 cells. Others were unable to reproduce these data but instead found that preinfection of eukaryotic cells with an intimin-negative strain of EPEC, but not an EspB-negative strain, rendered HeLa, HEp-2, and Caco-2 cells able to bind both MBP-Int280 and E. coli HB101 expressing EPEC intimin (24). The conclusion of that study was that a bacterium-induced signaling event was responsible for the binding phenotype. Although these studies predated the discovery of Tir, the findings are interesting given that CVD463, a tir deletion mutant, was able to decrease TER following calcium switch TJ disruption and recovery. Neither the degree of cell confluence nor the expression and distribution of polarized proteins, such as β1-integrin, were examined in these studies.

Interestingly, a recent report by Sinclair and O’Brien (29a) showed that intimin-γ expressed on the outer membrane of the related pathogen enterohemorrhagic E. coli (EHEC) 0157:H7 binds to nucleolin, a eukaryotic receptor for the extracellular matrix protein laminin, in addition to its cognate, bacterially derived receptor Tir. Not only did attached EHEC colocalize to sites of surface-expressed nucleolin, but the presence of nucleolin antibody interfered with EHEC binding to eukaryotic HEp-2 cells, suggesting that this host receptor contributes to EHEC pathogenesis. The polar expression of nucleolin on intestinal epithelial cells, however, has not been examined. It is interesting that several enteric pathogens, i.e., EHEC, EPEC, and Yersinia, exploit two different host cell receptors that share a common function, the binding of an extracellular matrix protein.

This study illustrates that although altered barrier function has been an area of intense interest, the secondary loss of cell polarity following TJ disruption may be yet another aspect of EPEC pathogenesis. Indeed, a similar paradigm has been demonstrated for Y. pseudotuberculosis. In this case, the outer membrane protein invasin, which confers the invasive phenotype of this pathogen, also binds to β1-integrin (10). While *Yersinia* is believed to initially exploit specialized intestinal cells, such as M cells or dendritic cells, that may harbor β1-integrin on their apical surface, McCormick et al. (18) addressed the question of whether *Yersinia* might be able to access this basolateral receptor on nonspecialized intestinal epithelial cells. Like that with all enteric pathogens, infection with *Yersinia* is associated with intestinal inflammation, defined as the transmigration of polymorphonuclear leukocytes (PMN) across the intestinal epithelial layer. PMN cross this protective layer by traversing TJs, resulting in temporary disruption of the barrier (21). Perturbation of TJs, either by inducing PMN transmigration with the chemoattractant formylated Met-Leu-Phe or by calcium chelation with EDTA, resulted in the localization of β1-integrin to the apical surface of model intestinal epithelia, thus rendering monolayers more susceptible to invasion by *Yersinia*. Infection by *Yersinia* has also been shown to directly perturb the TJ barrier by causing ZO-1 and occludin to dissociate from the TJ complex (31). These effects on TJs were found to be dependent upon YopE, a *Yersinia* protein delivered into host cells by type III secretion.

Our data indicate that over the course of EPEC infection, the disruption of TJs is followed by the redistribution of cell surface proteins. This physiological alteration leads to exciting new possibilities for the discovery of novel pathogen-host interactions. Previously published in vitro data have convincingly demonstrated that β1-integrin is capable of interacting with the EPEC outer membrane protein intimin, the cognate ligand for Tir (8). Evidence of physiological relevance for this interaction, however, has not been pursued. The coupling of our immunofluorescence and selective biotinylation data showing the appearance of β1-integrin on the apical pole of intestinal epithelial monolayers following EPEC infection with the attenuation of barrier disruption in the presence of β1-integrin antibody provides this evidence. Interestingly, the drop in TER was identical for EPEC alone at 2 h and EPEC plus β1-integrin antibody at 4 h, suggesting that the later decrease may be predominantly β1-integrin mediated. Furthermore, “trapping” of β1-integrin on the apical cell surface prior to infection by the tir mutant strain resulted in a drop in TER. In contrast, the tir mutant had no effect on the TER of monolayers whose polarity was intact. Particularly convincing were data demonstrating that antibody to β1-integrin, but not isotype IgG, prevented the decrease in TER by the tir deletion strain following EDTA treatment. Furthermore, an intimin deletion strain did not decrease TER following EDTA treatment, suggesting that interaction between intimin and β1-integrin interaction is needed. The mechanisms by which β1-integrin–intimin interactions could effect alterations in TJs include direct signaling events triggered by intimin–β1-integrin association or substitution of β1-integrin for Tir in securing intimate attachment and effective delivery of type III secretion system effectors into host cells. Our data showing that the type III secretion system-defective *espD* mutant strain had no effect on TER in either intact or polarity-disrupted monolayers support the latter
机制。EspD 形成裂缝在基底膜细胞，允许 EPEC 信使分子进入胞质；在缺乏这种情况下的任何影响，对 TER 未见。我们注意到强烈的相关性与减少 TER 和一种功能型 III 秘密系统过程（未发表数据）。

这些数据分析支持分时调节的模型 EPEC 感染，如图所示。EPEC 感染之前已经进行了彻底的调查和确定，作为初步的附着和信使分子注入通过一种型 III 的分泌系统（未发表数据）。在这些异常变化可能与 TJs 初始变化 (19, 33)。在其中，一个特定 EPEC 信使分子，EspF，是必要的，以完成 EPEC 在 TJ 阻止 (20)。数据呈现，然而，显示，这可能是一个后来的阶段，可以是后续的附着效应分子通过 TJ 转运的进程。这后期的阶段差异在 TJs 泰特尼尔的迁移 β1-整联素到上皮表面，并且后续的与 intimin 相互作用。这个生理学意义上的相互作用是被预防的，或者一般观察到的 TER 落后于后期的阶段，当 β1-整联素抗体存在时。

机制的范围，EPEC 与具有上皮细胞的炎性上皮细胞层，以及影响生理机制是令人惊讶的区域，调查和继续在于。因此，有多种机制被提出，然后参与到在感染的过程中。 interacti 来自和 β1-整联素是另外一种方式 EPEC 利用炎性上皮细胞生理学功能。


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